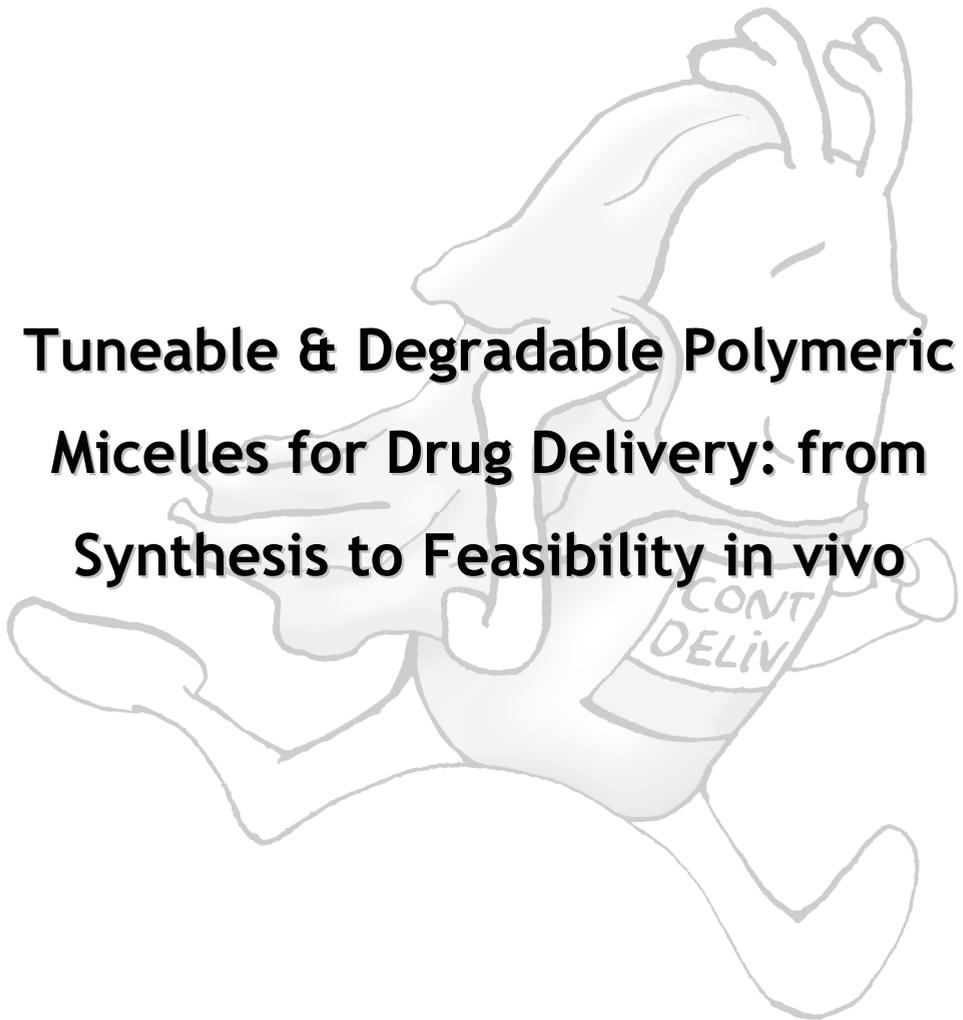


**Tuneable & Degradable Polymeric
Micelles for Drug Delivery: from
Synthesis to Feasibility in vivo**



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Netherlands Organisation for Scientific Research



Tuneable & degradable polymeric micelles for drug delivery: from synthesis to feasibility *in vivo*

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PhD thesis with summary in Dutch

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Tuneable & Degradable Polymeric Micelles for Drug Delivery: from Synthesis to Feasibility *in vivo*

Controleerbare & Degradeerbare Polymeren Micellen
voor Geneesmiddelafgifte: van Synthese
tot Haalbaarheid *in vivo*

(met een samenvatting in het Nederlands)

proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag
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door

Cristianne Johanna Ferdinand Rijcken

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'Niemand komt je iets brengen, je moet overal zelf voor knokken. Dus hard werken maar ook goed genieten' (ma)

Table of contents

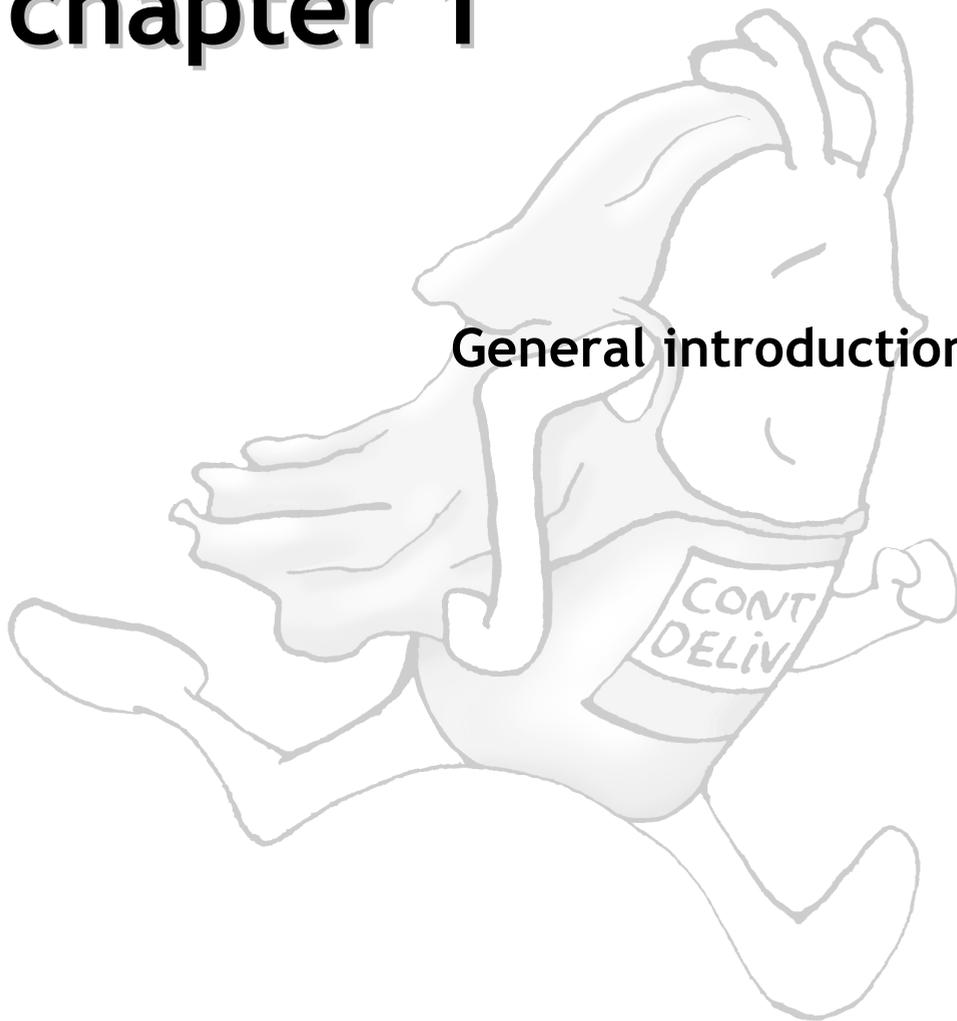
	1	General introduction	9
2	a)	Pharmaceutical micelles: combining longevity, stability & stimuli-sensitivity	28
	b)	Triggered destabilisation of polymeric micelles & vesicles by changing polymers polarity: an attractive tool for drug delivery	67
3		Step-by-step synthesis of monodisperse methacrylamidoalkyl-oligolactates	121
4		Novel fast degradable thermosensitive polymeric micelles based on mPEG- <i>block</i> -poly(<i>N</i> -(2-hydroxyethyl)-methacrylamide-oligolactates)	139
5		Photosensitiser-loaded biodegradable thermosensitive polymeric micelles: preparation, characterisation and <i>in vitro</i> PDT efficacy	165
6		<i>In vitro</i> cellular internalisation of rhodamine-labelled polymeric micelles	195
	7	Hydrolysable core crosslinked thermosensitive polymeric micelles	213
8		Biodistribution of (paclitaxel-loaded) degradable core crosslinked polymeric micelles after intravenous administration	239
9		Synthesis & characterisation of shell functionalised core crosslinked polymeric micelles with thermoresponsive & biodegradable properties	261
	10	Summary & perspectives	281

Appendices

	Nederlandse samenvatting	303
	List of abbreviations	309
	List of publications & abstracts	313
	Curriculum Vitae	317
	Dankwoord	319

chapter 1

General introduction



Drug delivery systems

New drug entities are nowadays discovered and developed via computational studies on structure - activity relationships (QSAR) to predict receptor-ligand binding, combinatorial chemistry and high-throughput screening, while therapeutic antibodies and proteins are produced by various sophisticated biotechnological approaches¹⁶. All these efforts lead to a broad variety of active compounds with a high specificity and potency. These drug candidates should be present at the right place (e.g. cell or tissue) in the body, in an adequate concentration for a certain time period to exert the desired therapeutic effect and meanwhile preferably not reside in non-targeted sites or at least be harmless to healthy cells. The research field that is dedicated to targeted drug delivery, which is defined as the selective transport of a drug to specific places in the body where its activities are required, aims to address these demands⁵². To improve an undesirable circulation and biodistribution profile of an active compound, it can be encapsulated in a controlled drug delivery system (*vide infra*). Ideally, the drug-loaded carrier accumulates in the diseased tissue but not in healthy tissue. Next, the encapsulated drug should be selectively released at its targeted site. Obviously, the potential building blocks for carriers should be safe, not causing inflammatory reactions or (severe) toxicity. Consequently, the main focus is on bioresorbable and biodegradable drug delivery systems.

Polymeric assemblies

Carriers are either of natural^{30, 34, 68, 76} or of synthetic origin, the latter being more appealing as their physicochemical properties are very much tuneable. Synthetic polymers are used in a great variety of drug delivery systems²¹, e.g. hydrogels^{84, 85}, microspheres/capsules⁸³, polymer coated liposomes^{65, 76}, (stent) coatings^{1, 82}, polymer-drug conjugates¹⁹, dendrimers^{20, 25} and various nanoparticles^{39, 45}. Amphiphilic block copolymers self-assemble into various nanoscale morphologies such as micelles^{28, 47}, nanoaggregates^{61, 72}, nanocapsules^{8, 22, 66}, nanogels⁴⁴ and polymersomes¹⁸. Among these, polymeric micelles are one of the most extensively studied assemblies as they display very favourable properties such as a large encapsulation volume for hydrophobic drugs, a hydrophilic shell that keeps the micelle in solution, a small size, multifunctionality, possibility for active targeting and tuneable disintegration behaviour. Furthermore, hybrid micellar systems such as peptide-amphiphiles³,

9, 31, 38, phospholipid-substituted polymers^{49, 67, 79, 89} and even DNA-containing block copolymers^{46, 59} bring together the best of the biomolecular and the synthetic worlds. Various excellent reviews about amphiphilic block copolymers and micelles have been published^{2, 4, 23, 33, 35, 40, 55, 58, 63, 64, 75, 77, 78}. The characteristics of an ideal polymeric micellar system for drug delivery purposes are summarised in Table 1. The drug-loaded polymeric micelles currently evaluated in clinical trials are given in Table 2.

Attractive properties of polymeric micelles are their small size (< 100 nm) and their propensity to evade scavenging by reticulo-endothelial system (RES) present in liver, spleen and bone marrow after intravenous (i.v.) administration (Figure 1). Besides, the size of the nanoparticles prohibits that they are excreted via renal clearance and consequently, in the ideal case, the micelles display a prolonged circulation. Selective accumulation of drug-loaded micelles at the targeted sites (e.g. tumour or inflamed tissue) is desired and achievable by taking advantage of the so-called enhanced permeation and retention effect (EPR) of colloidal particles. Contrary to healthy organs, tumour tissue comprises leaky vasculature and after extravasation of polymeric micelles, they will be retained there due to the impaired lymphatic drainage^{27, 50, 54}. Furthermore, the encapsulation of various hydrophobic drugs (including cytostatic agents and photosensitisers (PSs)) in micelles solves formulation difficulties attributed to their poor solubility.

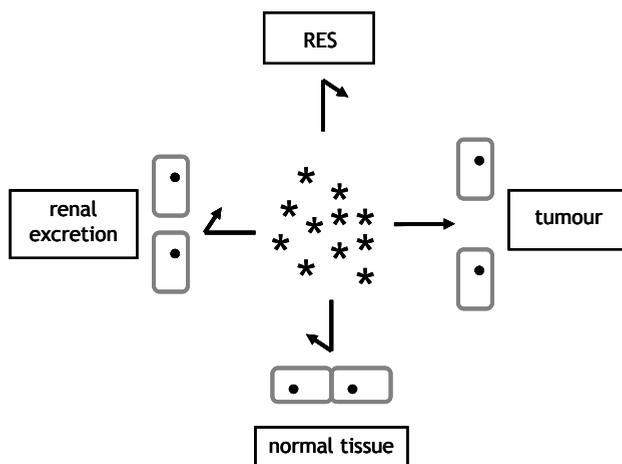


Figure 1 Concept of selective tumour accumulation of nanosized drug delivery systems after intravenous administration³⁵

aspect	properties	advantages
formulation	synthetic origin	minimal endotoxins
	ease of preparation	low costs
	self assembly in absence of (co)solvents	low levels of impurities
	low CMC	high stability upon dilution
	high drug-loading	applying small volumes
	stable drug-retention	no premature release
	high stability on storage	long shelf-life
administration	small size (10 - 150 nm)	sterilisation via filtration
	no side effects at site of administration	patient comfort
	small volume	“
disposition	no viscous solutions/dispersions	“
	inert to infusion lines/bags	no contamination
	stable in blood stream	no premature release
	masking the drug (against degradation)	reducing potential toxic effects due to high blood concentrations & allowing efficient use of drug
therapeutic efficacy	small size (< 150 nm) and hydrophilic shell with reduced a-specific protein/cell adherence ('Stealth')	minimise uptake RES - prolonged circulation - less administrations - increased patient compliance
	selective accumulation at target (EPR)	prevent effect on non-targeted cells
	locally or externally triggered release	maintain constant blood or tissue levels
toxicity	controlled release mechanism over prolonged period of time	levels
	renal clearance of unimers	no long-term accumulation
	intracellular degradation	“
	no muta- or teratogenicity	no long-term toxicity

Table 1 Characteristics of an ideal polymeric micellar drug delivery system

name - composition	dose (interval)	phase (patients)	benefits	side effects	comments / references
NK105 pactitaxel in hydrophobised mPEG-b-p(Asp)	3 weeks	I (17)	MTD to 180 mg/m ²	neutropenia	C _{max} and AUC of NK105 approximately 30-fold higher than that of Taxol [®] phase II ongoing against advanced pancreatic and gastric cancers
Genexol pactitaxel in mPEG-b-PLA	300 mg/m ² 1 x per 3 weeks refractory breast cancer	I (21) II (41)	MTD threefold increased up to 390 mg/m ² (free PTX 110 mg/m ²) 58 % response, 5 complete and 19 partial	no hypersensitivity reactions and thus no premedication 20 % hypersensitivity reactions	³⁶ ⁴³
Paxceed pactitaxel in mPEG-b-PDLLA	75 mg/m ² rheumatoid arthritis	I (15)	75 % of the patients had significant improvement	no serious adverse events	⁹⁰ further clinical development seemed not to have been pursued as from 2004 no data are published on Paxceed ⁶
NK911 doxorubicin in hydrophobised mPEG-b-p(Asp)	50 mg/m ² (equal to free DOX)	I	higher tumour values than free DOX	neutropenia, less toxicity than free DOX, no infusion related reactions ⁸	with respect to free DOX, Doxil increased AUC _{blood} 237 x and AUC _{tumour} 25 x, NK911 only AUC _{blood} 29 x and AUC _{tumour} 3.4 x ^{51, 80} phase II ongoing against pancreatic cancer ⁶⁰
SP1049 doxorubicin in Pluronic L61 + F127	75 mg/m ² 1 x per 3 weeks	I (26) I (21)	MTD from 50 for free DOX to 70 mg/m ² AUC _{blood} 200 x higher than free drug, in hypervascular mice full tumour ablation	antitumour activity against some advanced resistant solid tumours ¹⁷ neutropenia, anaemia, mucositis and alopecia	antitumour activity against some advanced resistant solid tumours ¹⁷ combination studies with other active agents warranted ⁷ , phase II ongoing against oesophagus cancer/ soft tissue sarcoma phase I ongoing against advanced solid tumors ³⁷
SN 38 = NK012 mPEG-b-(polyglutamate-g-camptothecin) NC 6004 = nanoplatin™ cisplatin complexed to mPEG-b-polyglutamate			AUC _{blood} 95 x higher than free cisplatin (in mice)	reduced the nephro- and neurotoxicity	phase I ongoing ⁸¹

Table 2 Overview of all drug-loaded polymeric micelles currently evaluated in clinical trials (MTD = maximum tolerated dose)

Photodynamic therapy

Photosensitisers, i.e. light-sensitive agents, are applied in photodynamic therapy (PDT), which is an emerging modality for the treatment of different types of cancer and other diseases. The principle of PDT consists of the uptake of a photosensitiser by a targeted tissue followed by illumination with light of a specific wavelength. The absorption of light by the PS results in oxygen-dependent photochemical reactions leading to damage of the illuminated tissue. The consequences are threefold, being direct cell death via apoptosis and/or necrosis, induction of vascular damage leading to thrombosis and haemorrhages, and finally acute inflammation reactions with resulting anti-tumour-immunity (Figure 2) ¹².

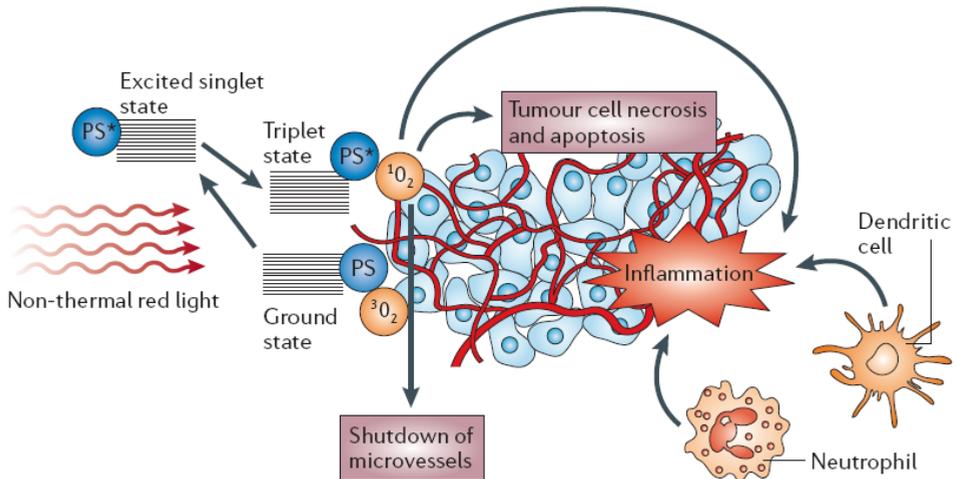


Figure 2 The threefold effect of photodynamic therapy on tumours ¹²

The ideal PS is easily administered with minimal direct effects (i.e. allergic effect or dark toxicity). Upon systemic circulation, sufficient and selective accumulation of the PS at the target is desired. Next, a reliable activation upon illumination with a specific wavelength is needed to prevent any accidental PDT effect. The different categories photosensitisers currently applied in clinical PDT are listed in Table 3 ⁵. The main (severe) drawbacks of these PSs are their low tumour accumulation after i.v. administration and relative high skin accumulation, thereby causing severe (sunlight-induced) skin burns. Hydrophobic PSs are desired because they generally result in a high phototoxicity, but they accumulate extensively in the skin, while a more

hydrophilic compound is rapidly excreted from the body (i.e. less skin penetration) but is less effective. To circumvent this paradox, polymeric micelles that sufficiently and selectively accumulate in their intact form at the targeted tumour tissue can be advantageous as a delivery system for hydrophobic PSs. After controlled release of the PSs, subsequent illumination is expected to generate a substantial phototoxic effect.

category	drug	substance	manufacturer
porphyrin	Photofrin [®]	HpD	Axcan Pharma Inc.
porphyrin	Levulan [®]	ALA	DUSA Pharmaceuticals Inc.
porphyrin	Metvix [®]	M-ALA	PhotoCure ASA
porphyrin	Visudyne [®]	vertiporfin	Novartis Pharmaceuticals
texaphyrin	Antrin [®]	lutexaphyrin	Pharmacylics
chlorin	Foscan [®]	temoporfin	Biolitec Pharma Ltd.
chlorin	LS11	talaporfin	Light Science
chlorin	Photochlor	HPPH	RPCI
phthalocyanine	Photosens [®]	sulphonated Al-phthalocyanine	General Physics Institute

Table 3 Overview of the photosensitisers currently applied in the clinic ⁵

Drug encapsulation & release

Besides in PDT, the concept of drug targeting is also applied in other cancer therapies, since either fast blood clearance, inefficient cell entry or detrimental side effects severely limit the use of many cytostatic agents. Examples of (mainly) hydrophobic anticancer drugs that have been encapsulated in polymeric micelles are paclitaxel ^{13, 29, 41, 48}, cisplatin ¹⁰, cyclosporin ⁵³, doxorubicin ^{26, 42}, camptothecin ⁸⁶ and methotrexate ⁹¹. Hydrophobic compounds are physically entrapped in or covalently bound to the micellar core ^{32, 88}. In case of covalent binding, drug release should occur through degradation of the linkage between the drug and the polymer, which can in principle be environmentally controlled. In physical entrapment, drug loading and retention depends on the interaction forces between the drug molecules and the carrier building blocks, whereas release generally takes place by passive diffusion. The use of stimuli-responsive polymers introduces a(n) (external) control of the release behaviour of loaded carriers ²⁴. Among the

several options available (see **chapter 2**), temperature responsiveness has been extensively investigated as a way to release drugs from polymeric micelles^{11, 14, 15, 62, 74}. However, since their clinical application requires hypo- or hyperthermia, other triggers (pH- or photosensitivity and biodegradability) are often implemented in thermosensitive micelles. A new type of biodegradable thermosensitive polymeric micelles, which have recently been developed in our laboratories, are based on block copolymers of ω -methoxy poly(ethylene glycol) (mPEG) and poly(alkyl-(meth)acrylamides) grafted with lactate side chains (Figure 3). Typically, micelles of these block copolymers are formed by heating an aqueous solution from 0 °C to above their critical micelle temperature (CMT)^{56, 57, 70, 71}. Subsequent ester hydrolysis of the lactate grafts increases the hydrophilicity of the polymer and finally results in micellar dissociation (Scheme 1); the corresponding time frame for disassembly is dependent on the type of polymeric backbone. For instance, polymeric micelles composed of a hydrophilic mPEG₅₀₀₀ block and a thermosensitive block based on (*N*-(2-hydroxypropyl)methacrylamide-dilactate) (mPEG-*b*-p(HPMAM-Lac₂, Figure 3) destabilised after one week at physiological conditions (pH 7.4, 37 °C)⁷¹.

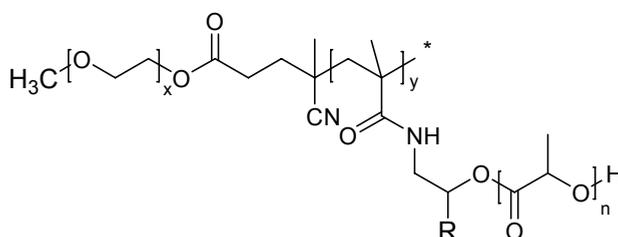
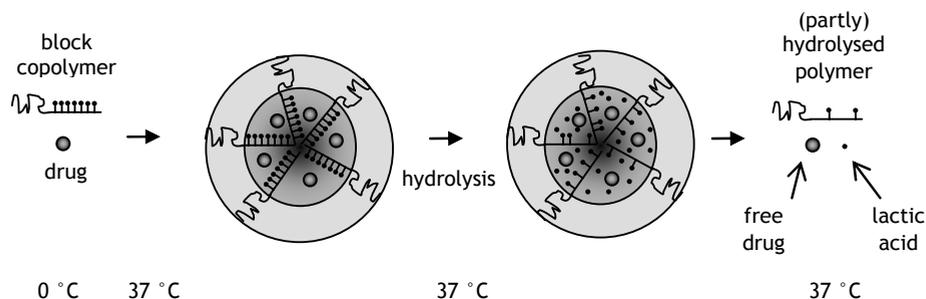


Figure 3 General chemical structure of mPEG-*b*-poly(methacrylamide-oligolactates) (for HPMAM-Lac₂: R is CH₃, n is 2)



Scheme 1 Concept of thermosensitive biodegradable polymeric micelles

Paclitaxel was almost quantitatively entrapped in these micelles up to a final concentration of 2 mg/mL (22 % (w/w)). Nevertheless, *in vitro* studies revealed that the encapsulated paclitaxel was rapidly released, i.e. more than 50 % after 5 hours at pH 7.4⁷⁰, which indicated diffusion instead of degradation controlled release. Moreover, a biodistribution and therapeutic study in tumour bearing mice demonstrated that the micellar formulation did not result in enhanced tumour uptake of the drug. In fact, an equal anti-tumour effect as the commercial formulation (Taxol) was observed⁶⁹. Interestingly, the micellar carriers themselves showed no toxicity whereas the cremophor used in the commercial formulation is known to cause vehicle-related toxicities^{53, 69}.

In literature, some micellar formulated drugs displayed an increased blood residence time (i.e. an elevated AUC compared to the free drug)^{13, 48, 53}, less drug-induced side effects^{26, 29} and in some cases a higher anti-tumour activity⁴¹. The differences in blood levels, tumour toxicity and side effects are however not very pronounced^{42, 69}, presumably as a result of rapid leakage, extraction of the drug and/or micellar dissociation due to serum interactions upon i.v. administration. Despite all the tremendous efforts made in the field, a drug-loaded micelle that fulfils all the requirements of an ideal micellar system is not yet on the market. Nevertheless, seven micellar formulations are (currently) evaluated in clinical trials (Table 2). The results of initial phase I trials demonstrated that drug-loaded micelles had an enhanced therapeutic effect relative to the free drug, but this was less pronounced than for liposomal formulations. Other paclitaxel carriers currently clinically applied are XyotaxTM (polyglutamate conjugated PTX) and AbraxaneTM (PTX albumin-bound nanoparticles). The most important common advantage of all these dosage forms with respect to Taxol[®] is the absence of cremophor which is correlated with serious allergic reactions.

In sum, the ideal micellar system comprising the main elements of high and stable drug-loading with enhanced tumour accumulation and controllable release mechanisms *in vivo* is yet to be developed.

Aims of this thesis

In the broad field of nanoparticulate drug carriers, biodegradable thermosensitive polymeric micelles based on methacrylamide-oligolactates display various attractive features as drug delivery vehicles:

- ❑ drug-loaded micelles are easily formed by simple heating an aqueous polymer-drug mixture
- ❑ encapsulation of relatively large quantities of various hydrophobic compounds is possible (up to 22 % (w/w) loading capacity was observed for paclitaxel)
- ❑ micelles can easily be sterilised via filtration through 0.22 µm filters
- ❑ the small micellar size (50 - 100 nm) is expected to result in a long circulation and tumour-infiltrating ability
- ❑ micellar dissociation is tuneable by modifying the chemical structure of the constituent block copolymers, which may provide a means to control drug release
- ❑ *in vitro* no direct cellular toxicity was measured at least up to 1 mg/mL
- ❑ *in vivo* (rats and mice) no direct toxicity symptoms were observed at concentrations up to 75 mg/kg (~ 15 mg/mL) as opposed to various toxic side effects of cremophor⁸⁷ or infusion-related side-reactions of liposomes⁷³
- ❑ after micellar dissociation, excretion of (hydrophilised) polymers will be possible via the renal pathway

However, before clinical application will become feasible, the topics of stable drug encapsulation during circulation, tumour accumulation and (intracellular) drug release at its aimed site of action *in vivo* need to be addressed in particular. Ultimately, the almost infinite freedom to design and modify synthetic block copolymers (large tuneability) should definitely lead to the ideal micellar system (Table 1).

The aims of the current study were:

- ❑ synthesis of polymeric micelles based on different methacrylamide-oligolactates with tailorable destabilisation profiles (Figure 3)
- ❑ stable encapsulation of hydrophobic drugs (e.g. PSs) in these polymeric micelles, determination of release profiles and *in vitro* efficacy studies

- ❑ modifying the polymers with fluorescent dyes, radiolabels and/or targeting ligands, to follow the trafficking of the micelles *in vitro* and *in vivo* and to introduce targetability
- ❑ stabilisation of the micellar structure to enhance blood circulation and tumour accumulation of drug-loaded micelles *in vivo*

Thesis outline

Chapter 2 is a literature review that discusses the various aspects required for the longevity, stability and stimuli-sensitivity of pharmaceutical polymeric micelles in depth.

Chapter 3 reports a step-by-step synthesis protocol of methacrylamidoalkyl-oligolactates as they are the building blocks for the biodegradable thermosensitive polymers described in this thesis. A synthesis route was developed to produce monomers on a large scale with a high purity.

The relative slow degradation kinetics of HPMAM-Lac₂ micelles initiated the search to a micellar system that dissociates within a day (**chapter 4**). The influence of the feed ratio of *N*-(2-hydroxyethyl)methacrylamide-oligolactates (HEMAM-Lac_n) monomers with different lactate chains lengths on the cloud point of poly(HEMAM-Lac_n) was studied and micelles based on this type of polymers were characterised with varying techniques (DLS, SLS, Cryo-TEM) to get insight into their composition, morphology and degradation kinetics.

In **chapter 5**, the formulation of a very hydrophobic photosensitiser (Si(sol)₂Pc) in mPEG-*b*-p(HPMAM-Lac₂) micelles was studied. The assembled particles were characterised, including their release kinetics, and the cellular uptake and photodynamic efficacy were explored *in vitro*.

In order to follow the fate of the polymers themselves during *in vitro* studies, a fluorescently labelled polymer was synthesised by covalent inclusion of a rhodamine moiety, as reported in **chapter 6**. After extensively purification to remove free label, confocal fluorescence microscopy experiments were performed to analyse micellar uptake in two different cell lines.

To enhance the physical stability of the micelles, to prevent premature dissociation upon i.v. administration and to retard the release of loaded drugs, a core crosslinking (CCL) strategy was applied (**chapter 7**). The stability of CCL micelles with respect to their non crosslinked (NCL) counterparts was evaluated upon addition of a destabilising agent and by decreasing the temperature below the CMT. The degradation profile of the NCL and CCL micelles upon incubation at physiological conditions (pH 7.4, 37 °C) was established.

In **chapter 8**, the biodistribution of empty and ^{14}C -paclitaxel-loaded CCL micelles versus NCL micelles in ^{14}C -tumour bearing mice after i.v. administration is reported.

Chapter 9 deals with the synthesis of a modified macroinitiator with the aim to develop shell functionalised micelles. Using this modified macroinitiator, block copolymers based on HPMAM-Lac_n were synthesised that presented a thiol-reactive moiety at the PEG terminus. After micelle formation, the reactivity of the coupling agent was demonstrated by a GPC method.

Chapter 10 summarises the results of this thesis and suggestions are given to further develop these very promising polymeric micelles towards advanced controlled drug delivery devices.

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chapter 2a

Pharmaceutical micelles: combining longevity, stability & stimu- sensitivity

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Abstract

During the last decades, polymeric micelles have been extensively studied as potential drug delivery systems, in particular for hydrophobic drugs. To achieve selective and tissue specific delivery of the encapsulated drugs after intravenous injection, these micellar carriers need to have long-circulating properties without premature loss of encapsulated agents. After accumulation at the target site via the enhanced permeation and retention (EPR) effect, controlled release of the drugs is required, preferably as a consequence of time- or site-specific degradation, or as a result of an external trigger. In this review, the various aspects encountered during the design of long-circulating, stable polymeric micelles are presented, including strategies to improve the longevity in the blood, such as surface coating and size adjustments. In the second part, a variety of methods to stabilise the polymeric micelles by physical or chemical crosslinking are described, and also the approach to improve drug retention by optimising the compatibility between the drug and the micellar core. Furthermore, the possibilities and mechanisms to obtain a triggered drug release from the polymeric micelles at the site of action are discussed. Various stimuli like temperature, pH, light and (enzymatic) degradation may increase the polarity of the hydrophobic block, thereby inducing micellar disintegration and drug release. Finally, the possibilities to co-encapsulated imaging agents in the micellar core to follow the fate of micelles *in vivo* are mentioned.

In conclusion, the enormous versatility of polymeric micelles and the many possibilities presented in this chapter indicate that polymeric micelles are very promising systems for the site specific delivery of drugs. It is anticipated that several attractive polymeric micelles will be further developed as delivery systems for hydrophobic drugs and will soon be clinically applied in the effective treatment of several diseases.

1 Introduction

Targeted drug delivery is of particular importance for the treatment of life-threatening diseases such as cancer, since the adverse effects of cytostatic drugs can be very detrimental^{43, 80, 230}. Nowadays, polymeric micelles are extensively studied as drug delivery systems to fulfil the requirements for selective and tissue specific drug delivery^{2, 7, 68, 115, 120, 128, 146, 150, 159, 197, 223, 242, 319, 322, 350}. The most attractive feature is their hydrophobic core with a relatively large capacity to accommodate hydrophobic agents, which are normally difficult to formulate. Polymeric micelles have been used to encapsulate a great variety of highly potent but hydrophobic drugs^{13, 37, 52, 176, 192, 193, 243, 244, 347, 349, 359}, such as doxorubicin (DOX)^{74, 93, 119, 170, 234, 266, 350}, paclitaxel (PTX)^{35, 83, 100, 135, 137, 147, 191, 298, 325}, amphotericin B¹⁶⁰ and photosensitisers (used for the photodynamic treatment of cancer)^{162, 330, 360}. Some of these micellar formulations have already entered clinical trials and showed promising results with regard to their therapeutic index in cancer patients^{20, 137, 215, 322}. A drug delivery system needs to fulfil several (pharmaceutical) requirements such as a significant increase in therapeutic effect with respect to the free drug, good biocompatibility and the possibility to scale-up the production of the micellar formulation. In addition, the ideal micellar system i) has long circulating properties and adequate stability in the blood stream, ii) has a high drug-loading capacity, iii) is able to selectively accumulate at the target site and iv) offers the possibility to control the release of drugs at the target site, for example by external stimuli^{7, 68, 115, 120, 146, 150, 223, 242, 319, 322, 323}. Other desirable properties of polymeric micelles are the ability to be (degraded and) excreted from the body after the drug is released and the option to track and trace the micellar structure by co-encapsulation of an imaging agent³²⁰.

The primary focus of this review is the description and discussion of longevity and stability of drug-loaded polymeric micelles after intravenous injection and the possibility to release their payload in a controlled manner upon local and/or external stimuli.

2 Longevity

Drug delivery systems such as polymeric micelles should deliver their payloads selectively at the target sites and therefore longevity in the blood circulation is a prerequisite. Provided that the encapsulated drug will remain associated with the nanocarrier, a long circulating nanocarrier will maintain the blood level of its loaded drug for a longer time, thereby enhancing the therapeutic effect of the drug as a result of the prolonged interactions in the target organ ^{223, 319, 322}. In addition, a long circulation time allows the accumulation of polymeric micelles themselves in pathological tissue via the so-called enhanced permeability and retention (EPR) effect. This EPR effect was proposed by Maeda *et al* in the eighties and is attributed to the higher permeability of the vasculature in diseased areas due to discontinuous endothelium and an impaired lymphatic drainage ^{68, 207, 208, 216, 319}. These two features enable extravasation of colloidal particles through the “leaky” endothelial layer into the tumour and inflamed areas and subsequent retention there. However, the human immune system rapidly recognises and eliminates foreign objects via adsorption of opsonic proteins onto their surface. Therefore, a key issue for prolonged circulation of colloidal drug carriers is to reduce the rate and extent of this opsonisation and the recognition by cells of the reticulo-endothelial system (RES). It has been shown that steric stabilisation, i.e. coating of the particle surface with hydrophilic polymers (e.g. poly(ethylene glycol), poloxamer) effectively reduces the interaction with opsonic proteins and thereby the uptake by the RES cells of the liver, spleen and bone marrow ^{102, 182, 225, 251, 304, 342}. Next to surface characteristics, the biodistribution of polymeric micelles depends on many other factors including predominantly particle size ^{68, 242, 323, 332} and particle rigidity ^{306, 332} as will be described in this section (vide infra).

2.1 Steric stabilisation

2.1.1 Poly(ethylene glycol)

Poly(ethylene glycol) (PEG) (also called poly(ethylene oxide) (PEO)) is the most frequently used hydrophilic segment of amphiphilic micelle-forming copolymers, for example in PEG-*b*-poly(propylene glycol) (PPO) ¹²⁰, PEG-*b*-polyesters ^{31, 82, 206, 296, 346}, PEG-*b*-phospholipids ^{204, 337} and various PEG-*b*-poly(meth)acrylamide derivatives ^{240, 276, 301, 318}. The ubiquitous use of PEG

results from its low toxicity and immunogenicity and FDA-approval in various pharmaceutical formulations. Moreover, it has unique physicochemical properties, such as excellent water solubility, high flexibility and a large exclusion volume, resulting in good 'stealth' properties^{2, 9, 174, 227, 327, 342}. Since the discovery of these 'stealth' properties and the positive effect of a PEG-coating on the circulation kinetics of colloidal drug delivery systems in the early nineties¹³⁸, the responsible mechanisms and factors that influence this effect have been extensively studied, but are not fully elucidated yet, as reviewed recently by Vonarbourgh *et al*³³². In general, the reduction of opsonisation by PEG is ascribed to shielding of surface charge and an increased hydrophilic surface, thereby preventing the two main driving forces for protein adsorption, i.e. electrostatic and hydrophobic interactions. In addition, reduction of the Van der Waals interactions, enhanced repulsive forces and the formation of an impermeable polymeric layer on the particle surface, but also the binding of dysopsonins (i.e. naturally occurring substances known to inhibit phagocytic ingestion) are considered to attribute to the protective effect^{251, 302, 322, 327, 332}. Prolonged circulation times as a result of effective blocking of opsonisation can only be achieved when the protective polymer layer is sufficiently thick. On the other hand, the PEG-chains should retain their flexibility for an optimal protection against recognition by the immune system. Both factors are related to the PEG molecular weight, conformation and the surface chain density^{68, 221, 251, 302, 332}.

The positive effect of a higher PEG molecular weight to reduce protein adsorption (Figure 1) and to prolong circulation times of colloidal particles was amongst others demonstrated for ¹⁴C-benzylamine labelled PEG-*b*-p(Asp) micelles with covalently bound doxorubicin. An increase in the molecular weight of PEG from 5000 to 12000 Da resulted in a fivefold increase of the blood level of the micelles at 4 hours post injection (13 versus 68 % of the injected dose) and decreased hepatosplenic uptake^{149, 155}. The favourable influence of a longer PEG-chain on the blood circulation times was also reported for a series of Pluronic® (PEG-PPO-PEG) block copolymers¹¹⁸. Although usually PEG with a molecular weight between 1000 and 15000 Da is used to design polymeric micelles for drug delivery, also smaller PEG-chains may be able to form a protective layer. For instance, coating of lipid nanocapsules with PEG with a molecular weight of 660 Da resulted in steric stabilisation and reduced protein opsonisation, which was ascribed to a high surface chain density³³³. Remarkably, regarding the PEG chain conformation on the surface, it was shown that the attachment of both ends of the PEG chain onto the

surface resulted in better protection than single chain end attachment, despite a lower chain mobility^{97, 258}. It was suggested that single chain end attachment allows easier penetration of the PEG-layer by proteins (Figure 1B) resulting in a less dense PEG-layer, when compared to double chain end attachment^{97, 258}.

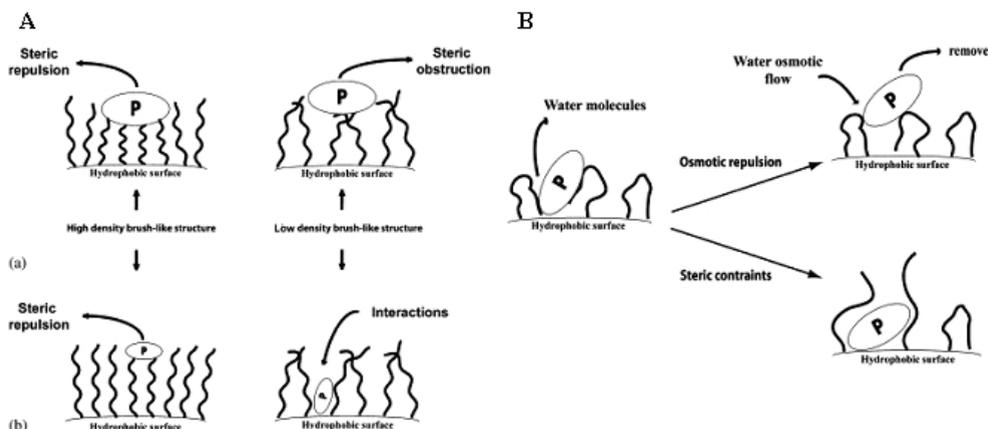


Figure 1 Effect of chain density (A, large (a) and small (b) proteins) and conformation (B) on the repulsion of opsonic proteins (P)³³²

Although nowadays PEG is considered to be the golden standard for the steric stabilisation of nanoparticles, it is not as inert as generally assumed. Several studies with PEGylated liposomes indicate the binding of blood proteins²²⁶ and disappearance of the stealth properties at low lipid doses and/or upon repeated administration^{32, 47, 104, 161}. The repeated administration of drug-loaded polymeric micelles has been reported in several studies. However, these studies focussed on the anti-tumour effect of the micellar formulation of a cytostatic drug, rather than the biodistribution of the carrier itself, or the loaded drug^{83, 135, 162, 244, 349, 351}. It is anticipated that the loss of the long circulation behaviour upon repeated administration or at low doses is not limited to PEGylated liposomes and that micelles with a hydrophilic PEG-shell may be subject to this phenomenon as well. This warrants the search for alternative protective coatings, as will be described in the next section.

2.1.2 Alternative coatings

Based on the above mentioned mechanistical aspects, alternative hydrophilic polymers (Table 1) have to be biocompatible, hydrophilic, water

soluble and highly flexible. Polymeric coatings that successfully prolonged the circulation times of liposomal carriers are based on poly(oxazoline)³⁴¹, poly(glycerol)²¹³, poly(*N*-vinyl pyrrolidone) (PVP)^{324, 326}, poly(acrylamide) (PAAm)³²⁶, poly(vinyl alcohol) (PVA)³¹¹, poly(*N*-(2-hydroxypropyl) methacrylamide) (pHPMAm)³³⁸ and poly(amino acids)^{220, 279}. Several of these polymers have also been used as hydrophilic part of amphiphilic block copolymers in the development of polymeric micelles, which will be described in this section.

polymer	abbreviation	chemical structure	references
poly(ethylene glycol)	PEG		31, 82, 120, 155, 204, 206, 240, 276, 296, 301, 318, 337, 346, 349
poly(<i>N</i> -vinylpyrrolidone)	PVP		22, 40, 162, 163, 177, 178, 205
poly(vinyl alcohol)	PVA		368
poly(2-ethyl-2-oxazoline)	PEOx		95, 133, 175, 177, 331
poly(<i>N</i> -(2-hydroxypropyl) methacrylamide)	pHPMAm		178
poly(acrylamide)	pAAm		7, 327
dextran	Dex		180, 281

Table 1 Various hydrophilic polymers used for the steric stabilisation of micelles

2.1.2.1 Poly(*N*-vinylpyrrolidone)

An attractive PEG-alternative is poly(*N*-vinylpyrrolidone) (PVP), which is highly hydrophilic, flexible and biocompatible, similar to PEG. In recent years, micelle formation has been reported of several amphiphilic PVP-containing block copolymers, for example copolymers with poly(lactic acid) (PLA)^{22, 162, 205}, PCL^{40, 177, 178} and pNIPAAm¹⁶³ and the effective encapsulation of hydrophobic drugs such as indomethacin²², chloro aluminium phthalocyanine¹⁶³ and paclitaxel (PTX)¹⁶² was demonstrated. It has been shown that PVP effectively prolongs the circulation time of liposomes^{324, 326}, but these data are not available for micellar systems yet. *In vivo* studies with PVP coated micelles focused on the biodistribution and tumour accumulation of micelles loaded with chloro aluminium phthalocyanine¹⁶³ or PTX¹⁶². For both drugs, no improvement in blood circulation times, tumour accumulation nor in therapeutic effect was observed when compared to a similar dose formulated in cremophor EL[®]. This may be related to a fast release of the drug from the micelles or disintegration of the micelles themselves. However, the maximum tolerated dose of PTX-loaded PVP-*b*-PLA micelles in mice was more than five times higher than that of Taxol[®] (PTX formulated in cremophor EL[®]). By using a higher dose, a better anti-tumour activity could be achieved¹⁶².

2.1.2.2 Polysaccharides

Another class of polymers used as PEG-alternative is the group of polysaccharides, which play a role in the surface characteristics of several cells, e.g. red blood cells. These cells effectively evade the immune system, which may be related to the presence of oligosaccharide groups on their surface^{179, 223}. It was demonstrated that dextran-*g*-PCL nanoparticles and dextran-coated PLA nanoparticles showed a lower protein adsorption than bare polyester nanoparticles^{180, 281}. Whereas these studies did not investigate the circulation kinetics of these dextran-coated nanoparticles, prolonged circulation times were reported for poly(methyl methacrylate) (pMMA) nanoparticles coated with dextran or heparin, compared to bare pMMA nanoparticles²⁵⁶. Recently, micelles of hydroxyethyl starch (HES) grafted with acyl chains were reported, which may also evade the RES²³. However, the pharmacokinetics of these HESylated nanoparticles *in vivo* has not been investigated yet. It was demonstrated that the conformation of polysaccharides is of high importance when minimising the interactions with plasma proteins¹⁸¹. In contrast to PEG (*vide supra*, section 2.1.1), a brush-like configuration conferred a more effective protection than the presence of dextran loops at

the surface. Although poly- and oligosaccharides present at the nanoparticles surface may protect against RES-uptake, saccharide-receptors are present in the membranes of several cells¹⁷⁹. This enables their use in active targeting approaches (section 2.4) and is illustrated by the recognition of galactose presenting nanoparticles by hepatocytes²¹².

2.1.2.3 Other hydrophilic blocks

Several other biocompatible hydrophilic polymers have been used as the shell-forming component in polymeric micelles, for example poly(*N,N*,dimethylamino-2-ethyl methacrylate) (pDMAEMA)²⁶, poly(ethylene imine) (PEI)²³⁵, poly(acrylic acid) (pAAc)^{7, 103} and poly(asparagine) (pAsp)¹⁰⁹. However, the longevity of these micelles *in vivo* is questionable, because of the charges present on the micellar shell surface. In addition, p(NIPAAM-co-*N,N*-dimethylacrylamide) (pDMAAm) was suggested as the shell-forming block¹³⁹. Since this copolymer displays thermosensitive behaviour, it can be used for the temperature triggered release of encapsulated drugs, as will be discussed further on in this chapter. Micelles composed of poly(2-ethyl-2-oxazoline)-*b*-polyester^{95, 133, 175, 177, 331}, pHPMAm-*b*-PCL¹⁷⁸, poly(acrylamide) (pAAm)-*b*-palmitate^{7, 327} and PVA-*b*-oleylamine³⁶⁸ may exhibit prolonged circulation times *in vivo*, since these hydrophilic blocks effectively protected liposomes against rapid RES-uptake (*vide supra*), but evidence is not yet obtained.

2.2 Micellar size

The size of micelles is another predominant parameter determining its fate after *i.v.* injection. Nanoparticles larger than 200 nm are removed by mechanical filtration by the interendothelial cell-slits in the spleen and particles with a molecular weight smaller than 50 kDa (a hydrodynamic diameter of 5 - 10 nm) are subject to renal excretion^{119, 150, 223, 224}. The size of polymeric micelles (10 - 100 nm) prevents elimination via these routes, but it was shown that the extent of RES uptake and tumour penetration were related to their size as well^{68, 118, 150, 242, 323}. For example shell crosslinked poly(*tert*-butylacrylate)-*b*-polystyrene (PBA-*b*-PSt) micelles with a size of 20 nm had significantly higher blood residence times than their two times bigger counterparts (50 % versus 5 % of the injected dose at 1 hour after injection)³⁰⁶ and the same trend was found with PEG-*b*-PHDCA micelles of 80 and 170 nm⁵⁹. It should be mentioned however that in the latter study the longer circulation times of the 80 nm micelles may be related to a higher surface density of PEG because of the smaller size. As pointed out in section 2.1.1, a higher PEG-

density results in more effective shielding. Furthermore, it was suggested that the particle size itself is of importance, since small particles have a higher curvature, thereby hampering the adsorption of proteins³³².

In contrast, Weissig *et al* demonstrated that i.v. injected PEG₅₀₀₀-*b*-distearoyl phosphatidyl ethanolamine (PEG-*b*-DSPE) micelles of 15 nm circulated shorter than 100 nm liposomes in mice with a subcutaneously established Lewis lung carcinoma. Remarkably, the tumour accumulation of the small micelles was much higher than that of the liposomes³³⁷. This is ascribed to the low cut-off size of the tumour vessel wall, which is different in each tumour type and determines the ability of nanoparticles to penetrate the tumour tissue^{91, 357}. Especially in solid tumours, such as Lewis lung carcinoma, the small size of micelles is indeed an additional advantage over e.g. liposomal and other bigger nanoparticulate systems. A different tumour penetration was also reported for doxorubicin loaded PEG-*b*-poly(aspartate hydrazone adriamycin) micelles of 65 nm and liposomes of 150 nm^{18, 242}. The micelles were found inside tumoural spheroids, whereas the 150 nm liposomes were found only in the periphery²⁴². Thus, small sized nanoparticles benefit more from the EPR effect as a result of their higher ability to penetrate into tumour tissue.

2.3 Other strategies to improve circulation times

Next to surface characteristics and particle size, the rigidity of the particle also influences the circulation kinetics. It has been reported that liposomes with a rigid lipid bilayer exhibit long circulation times despite the absence of a protective PEG-layer^{247, 291}. Sun *et al* compared the biodistribution of (PBA-*b*-PSt) micelles with a high T_g , glassy PSt core, to that of PBA-*b*-poly(methyl acrylate) (PMA) micelles with a low T_g fluid-like PMA core and demonstrated that a more rigid, glassy micellar core results in a longer blood retention. In addition to changing particle-related parameters such as surface characteristics, size and rigidity, another strategy to improve the circulation times of nanoparticles may be predosing with empty micelles to saturate the elimination mechanisms. In fact, this was the first strategy to improve the circulation time of liposomes¹, but so far it has not yet been investigated for micellar systems.

2.4 Longevity of actively targeted polymeric micelles

Although long circulation times promote the passive targeting of polymeric micelles and thus the delivery of the entrapped drug at its site of action, their delivery may be further improved by active targeting. Specific ligands such as internalising antibodies^{67, 325}, sugar moieties¹¹⁰, transferrin, RGD sequences and folate^{253, 344} have been coupled to the micellar shell to promote cellular recognition and internalisation of the drug carrier^{169, 198, 321, 344}. The delivery of drugs by actively targeted long circulating micelles is a promising approach to improve its site-specific action, but the properties of such carriers are paradoxal. On the one hand, the presence of a targeting ligand, especially antibodies or other proteins, on the micellar surface may enhance its recognition by the immune system and thereby removal from the circulation. On the other hand, the presence of a protective polymer such as PEG may interfere with the binding of the ligand to its target. A strategy to tackle the abovementioned paradox is the use of a 'shedtable' coating, i.e. a coating that is removed after arrival at the target site. Recently, a pH-sensitive deshielding of a TAT-peptide coupled to the surface of PEG-*b*-PLLA micelles was reported. The cationic TAT ligand was shielded at pH 7.4 by the anionic pH-sensitive poly(methacryloyl-sulfadimethoxine) (PSD) block of a PEG-*b*-PSD copolymer. Upon lowering the pH to 6.6, the complex was disrupted and TAT was exposed, resulting in enhanced cellular uptake and localisation at the surface of the nucleus²⁹³.

3 Micellar stability

Even with a prolonged circulation, selective drug accumulation can only take place if premature leakage of drug molecules from the micelles is prevented or the release of the drug is slow during the first few hours after administration. Essentially the core-forming segment determines the micellar stability, its drug loading capacity and drug release profile, which explains why so many core-forming mainly hydrophobic polymers have been investigated (Table 2) ^{6, 7, 82, 119, 150, 191, 319, 330, 349, 365}.

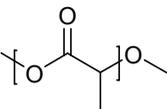
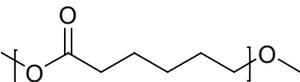
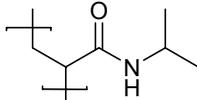
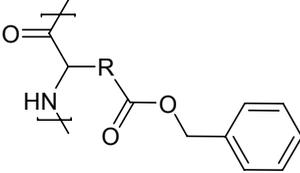
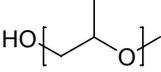
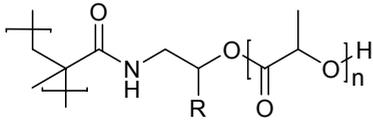
polymer	abbreviation	chemical structure	reference
poly(lactic acid)	PLA		53, 162
poly(ϵ -caprolactone)	PCL		31, 205, 296
poly(<i>N</i> -isopropylacrylamide)	pNIPAAm		241, 318
poly(γ -benzyl L-glutamate) or poly(β -benzyl L-aspartate)	PBLG or PBLA	 R = C ₂ H ₄ or CH ₂	110, 152, 336, 353
poly(propylene oxide)	PPO		119, 120, 267
poly(methacrylamide-oligolactates)	p(HEMAm-Lac _n) or p(HPMAm-Lac _n)	 R = H or CH ₃ ; n is 1 - 4	276, 301

Table 2 Hydrophobic polymers used as core-forming block in polymeric micelles

The stability of a polymeric micelle can be considered either thermodynamically or kinetically. Polymeric micelles are thermodynamically stable when the polymer concentration in the water is above the critical micelle concentration (CMC), also called the critical aggregation concentration (CAC). Below the CMC, amphiphilic block copolymers in water are present as single chains in the bulk and at the air-water interface. When the concentration is increased above the CMC, the Gibbs free energy (ΔG) of the system is minimised by the self-assembly of the amphiphiles, as a result of the hydrophobic interactions between the hydrophobic blocks^{11, 150}. Since polymeric micelles are subject to dilution in the circulation upon intravenous injection, it is important to know their CMC and to administer a sufficiently high dose^{7, 197}.

The kinetic stability of a micellar system is related to the exchange rate of single polymer chains between the micelles and the bulk and even upon dilution below the CMC, the micellar system may still be kinetically stable^{7, 11, 150, 154}. The rate of disassembly is related to the strength of the interactions in the micellar core, which depends on many factors, such as the physical state of the core-forming polymer (crystalline or amorphous), the presence of solvent (e.g. methanol or dioxane residues due to the preparation procedure) in the micellar core, the ratio between the hydrophilic and the hydrophobic block lengths of the copolymer and the encapsulation of hydrophobic compounds^{7, 68, 150, 349}. Preferably, polymeric micelles have a (semi-)crystalline or glassy core at body temperature and are composed of block copolymers with a low CMC^{7, 68, 150, 314}. In addition to micellar stability, several other factors influence the release of the loaded drug, such as the length of the core-forming polymer segment and the amount of loaded drug¹³⁶. Importantly, the compatibility between the core-forming polymer and the drug affects the drug loading and release^{7, 68}. By a proper selection of the block copolymer, the compatibility with the drug was optimised for various micellar systems, which is expected to increase the *in vivo* drug retention^{61, 83, 100, 149, 158, 162, 172, 195, 264, 319, 340}. However, the presence of blood components often leads to premature drug release, either by provoking micelle destabilisation, or by extraction of encapsulated drug from intact micelles^{142, 196, 203, 249, 288}. Therefore, much effort is currently undertaken to improve the thermodynamic and kinetic stability of drug-loaded micelles. Several strategies have been investigated, including modification of the micelle-forming polymers to reduce their CMC (section 3.1), physical and covalent crosslinking (sections 3.2 and 3.3) and improving the drug-polymer compatibility (section 3.4).

3.1 Reducing the CMC

The thermodynamic stability of polymeric micelles can be improved by reducing the CMC of the amphiphilic block copolymers. This is easily achieved by adjusting the sizes of the blocks^{7, 68}. Both a larger hydrophobic block and a smaller hydrophilic block result in a higher overall hydrophobicity, thereby reducing the CMC^{31, 119, 150, 162, 185}. In addition to size, the nature of the hydrophobic block is an important parameter determining the CMC. Chemical modification of the hydrophobic block, for example by the introduction of aromatic groups, has been demonstrated to effectively reduce the CMC^{31, 209, 248, 336, 353}. For instance, changing the amount of benzyl groups in the modified poly(β -benzyl L-aspartate) (PBLA_{mod}) block of PEG-*b*-PBLA_{mod} copolymer from 44 % to 75 % resulted in a ten-fold reduction of the CMC²⁴⁸. A 60 to 200-fold reduction was obtained by the end group modification of mPEG₇₅₀-*b*-oligo(ϵ -caprolactones) with a benzoyl or a naphthoyl moiety³¹. Similarly, an increase in the level of fatty acids¹⁵⁷ or hydrophobic oligolactates²⁷⁶ attached to the polymer backbone resulted in a reduced CMC.

3.2 Physical interactions

The introduction of aromatic groups does not only improve the thermodynamic stability by decreasing the CMC, but may also improve the kinetic stability of the micelles by strengthening the interactions inside the micellar core through π - π -stacking. Mahmud *et al* studied the viscosity of the core of PEG-*b*-PCL micelles and PEG-*b*-poly(α -benzyl- ϵ -caprolactone) micelles with fluorescence spectroscopy and found, besides a decreased CMC (*vide supra*), an increased rigidity of the micellar core as a result of the presence of aromatic groups²⁰⁹. The introduction of crystallinity or stereocomplex formation was shown to enhance the stability of micelles as compared to the amorphous counterparts^{127, 361}. An increase in physical interactions was also obtained by hydrogen bonding³⁵⁶. A summary of physical interactions that can play a role in the kinetic stability of micelles is illustrated in Figure 2. In addition to interactions between the core-forming polymers, the incorporation of a hydrophobic drug may also enhance the micellar stability. For example, Yokoyama *et al* demonstrated that the stability of PEG-*b*-p(Asp) micelles was not only increased by the amount of chemically bound doxorubicin (DOX), but also by the amount of physically entrapped DOX³⁴⁹.

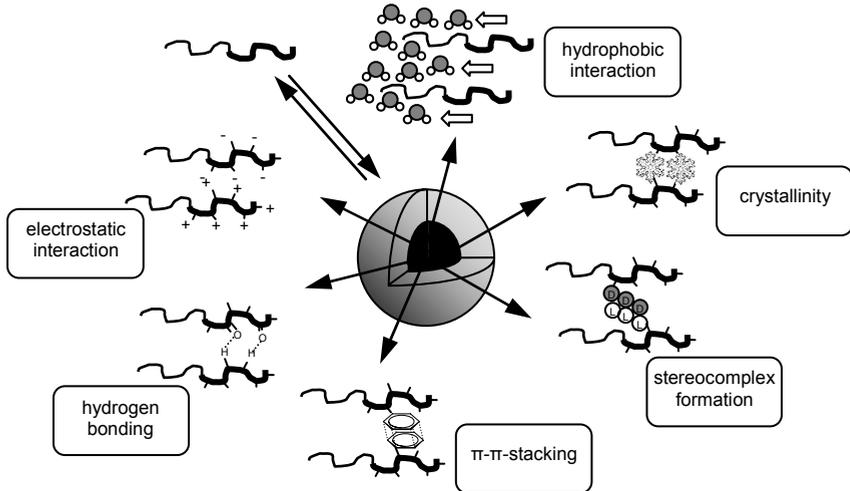


Figure 2 Interactions in the micellar core that enhance the kinetic stability of polymeric micelles

Micelle formation may also be driven by the electrostatic ionic interaction forces of oppositely charged block copolymers, to form a so-called polyion complex (PIC) or complex coacervate micelles (Figure 3)^{42, 86, 278}.

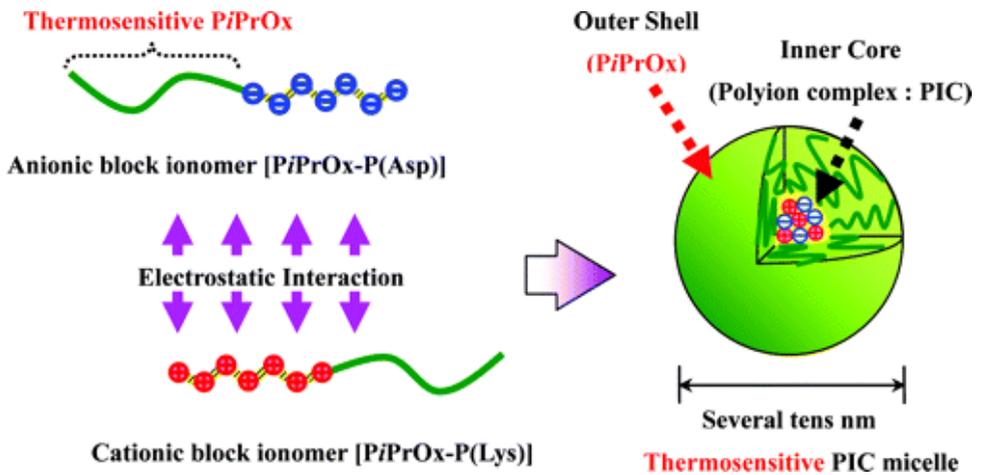


Figure 3 Formation of PIC micelles with a thermosensitive shell composed of poly(2-isopropyl-2-oxazoline) (PiPrOx) shell and a core composed of the anionic ionomer poly(aspartic acid) p(Asp) and cationic poly(L-lysine) (p(Lys))²⁵⁴

Examples of polyion-couples are PEG-*b*-poly(L-lysine) (p(Lys)) and PEG-*b*-poly(α,β -aspartic acid)^{85, 86}, PEG-*b*-poly(methacrylic acid) (PMAA) and poly(*N*-ethyl-4-vinylpyridinium) (PEVP)¹²¹, or PEG-*b*-poly(2-vinylpyridinium) (p2VP) and polystyrene sulphonate (PStS)⁷⁸. PIC micelles that comprise thermosensitive shells were described^{42, 254}, as well as PIC micelles composed of PEG-*b*-PMAA and Ca²⁺ with a crosslinked PMAA core²⁷. Polymers having negatively charged units, such as PMAA and p(Asp) (co)polymers, were used to form micelles with cationic drugs or peptides and polycations such as PEG-p(Lys) were used to form micelles with siRNA/DNA⁸⁷. The advantage of PIC micelles is their ease of preparation, i.e. simple mixing of aqueous solutions of drug and polymer. However, their application is limited due to the low stability in physiological saline and the drugs prerequisite to be hydrophilic, although this could be overcome by copolymerising phenylalanine in the polymer backbone, thereby enhancing the hydrophobic/aromatic interactions²⁶⁴. On the other hand, the saline induced micelle destabilisation can be utilised to control the release of the loaded drug. This concept was demonstrated for cisplatin-complexed PEG-*b*-p(glutamic acid), as will be discussed in section 4.7²⁴⁴.

3.3 Covalent crosslinking

In addition to the abovementioned physical means to enhance stability of micelles, chemical crosslinking of either the shell, the interfacial layer, or the core of the micelles has been used to prepare stable particles with a micellar morphology (Figure 4)^{87, 246, 278, 280}. The increased stability of covalently crosslinked micelles versus their non-crosslinked counterparts was proven amongst other techniques by the insensitivity of micelles towards a destabilising agent (e.g. sodium dodecyl sulphate (SDS))^{101, 134, 275}. While the micellar morphology was fixed by the crosslinking procedure, drug release could be controlled by the crosslink density⁹⁶ and stimuli responsiveness was retained (e.g. to pH, temperature, salt concentration)^{16, 24, 199, 275}. However, in contrast to physical crosslinking, the covalent crosslinking approach may adversely affect the overall degradability of the micelle and the structural integrity of the encapsulated drug (when the crosslinking procedure is performed in the presence of the drug).

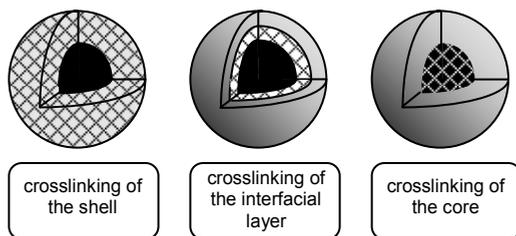


Figure 4 Covalent crosslinking of the micellar shell, the interfacial layer or the micellar core

3.3.1 Shell crosslinking

The hydrophilic shell of polymeric micelles has been covalently crosslinked by chemical or photo-induced reactions^{87, 111, 114, 117, 186, 188, 246, 277, 306}. For example, in polypeptide-*b*-polydiene micelles, covalent bonds were formed between either the amine or carboxylic acid groups in the hydrophilic polypeptide block using glutaraldehyde or a diamine, respectively, as crosslinking agents. Crosslinking by amide bond formation was induced by the addition of an activating agent (e.g. a water soluble carbodiimide)²⁷⁷. The shell consisting of poly(*N,N*-dimethylaminoethyl methacrylate) (pDMAEMA) was crosslinked by alkylation with a bifunctional alkyl iodide²⁶¹ and the shell of poly(4-vinylpyridine) (p4VP)-*b*-polystyrene was crosslinked by addition of a water soluble radical initiator, followed by UV-irradiation at 50 °C³¹⁵. Besides shape fixation, shell crosslinking also provides a tool to control the permeability of the micellar shell for drug molecules⁹⁶. The surface stabilisation can also be applied in stimuli-sensitive micelles to further control the drug release^{16, 188, 261} (see section 4). A major disadvantage of crosslinking the shell segments is that all reactions have to be performed at high polymer dilution, in order to selectively crosslink the micellar shell while avoiding the formation of intermicellar crosslinking²⁴⁶. Furthermore, the shell fixation may hamper the chain flexibility of the shell-forming polymers, thereby impairing the steric stabilisation.

3.3.2 Interfacial crosslinking

Alternatively, the interfacial layer between the micellar core and shell can be crosslinked by the introduction of a crosslinkable spacer between the hydrophobic and the hydrophilic block^{114, 200, 362}. This approach will leave both the micellar core and shell and consequently the loaded drug and steric stabilisation, respectively, unaffected, while it may provide a way to control

drug release. Examples of spacers used are poly(glycerol monomethacrylate) (pGMA) and poly(2-hydroxyethyl methacrylate) (pHEMA), which can be crosslinked by the addition of divinyl sulphone^{199, 200}, or by derivatising pGMA with cinnamoyl chloride followed by UV-irradiation of the aqueous micellar solution¹¹⁴.

3.3.3 Core crosslinking

Core crosslinked (CCL) micelles can be prepared using functional groups present at the chain end or along the core-forming block. Hydroxyl moieties present in the hydrophobic block are often functionalised with (meth)acrylate groups^{101, 189, 298}. After micelle formation, the hydrophobic blocks are crosslinked by thermal^{101, 189} or photo-induced polymerisation^{134, 272}. Other strategies to obtain CCL micelles from (meth)acrylate functional block copolymers are Michael addition with multifunctional thiol compounds¹⁶⁸, or formation of an interpenetrating network using a hydrophobic polyfunctional acrylate²⁵⁹. Another example are PIC micelles composed of anionic PEG-*b*-p(Asp) and the cationic protein trypsin, which are crosslinked by Schiff-base formation of glutaraldehyde with the protein. An interpenetrating network of crosslinked trypsin was formed in the core which was stable even at high ionic strength (0.6 M NaCl), indicating that, besides protein-protein crosslinks, also covalent bonds between the primary amino groups at the ω -end of the p(Asp) segments and aldehyde groups in glutaraldehyde were formed. Additionally, the protein retained its enzymatic activity¹⁰⁶. PIC micelles composed of PEG-*b*-PMAa/ Ca^{2+} were crosslinked by reaction of the carboxylic acid groups in PMAa with 1,2-diethylenediamine in presence of a carbodiimide^{24, 27}.

3.3.4 Cleavable crosslinks

A drawback of crosslinked micelles may be that covalent linkages in the shell, the core or the interfacial layer can negatively affect the biodegradability of the polymeric assemblies. The use of reversible or degradable crosslinks may (partly) circumvent this. Reversible crosslinked micelles were formed by the introduction of thiol groups on the lysine units in PIC micelles, followed by their oxidation to disulfide bonds¹²⁴. In addition, disulfide bonds were used to stabilise the interfacial layer of PEG-*b*-(poly(*N,N*-dimethylacrylamide)-stat-(*N*-acryloxysuccinimide))-*b*-pNIPAAM micelles. The resulting particles were susceptible to reduction by agents such as glutathione or dithiothreitol. Therefore, the reducing environment in the cytoplasm of cells is also a potential trigger for disintegration and drug release from these

particles¹⁸⁸. Recently, hydrolysable CCL micelles were developed via introduction of methacrylate moieties at degradable oligolactate grafts of a core-forming polymer backbone. These crosslinked micelles showed a superior physical stability with respect to their non crosslinked counterparts and the degradation time could be controlled by their crosslink density²⁷⁵.

3.3.5 Effects crosslinking on drug loading & release

The above described crosslinking strategies of the shell, interfacial layer or micellar core not only fixed the micellar morphology, but also retarded the release of the loaded drug^{96, 112, 188, 194}. In addition, crosslinking can influence the drug loading capacity. An eight-fold higher amount of triclosan was encapsulated in PEG-lipid micelles after polymerisation of the chain ends, which was attributed to a higher stability of core crosslinked compared to unmodified micelles³¹⁶. Obviously, one should always be aware that the structural integrity of the loaded drug molecules should be preserved upon the chemical crosslinking of the core. To avoid unwanted modification of entrapped drug molecules, the micelles can be crosslinked first and subsequently loaded with drugs. For example, mPEG-*b*-PLA micelles were crosslinked by thermally initiated polymerisation of methacrylate groups in the core. Next, via a microemulsion method and subsequent evaporation of the organic solvent, paclitaxel (PTX) was loaded into these CCL micelles. A loading capacity of 3 to 6 weight percent (% w/w) was achieved, equal to non crosslinked micelles¹³⁴. Crosslinked PEG-*b*-poly(methacrylic acid) micelles were loaded with cisplatin by 48 hours of incubation with an aqueous drug solution. Subsequently, the unbound cisplatin was removed by ultracentrifugation and a drug loading of 22 % (w/w) was obtained²⁴.

3.4 Micellar core - drug compatibility

Even at high micellar stability, the retention of the loaded drug cannot be guaranteed. Upon contact with blood, extraction and redistribution of the drug between the micellar core and blood components might take place^{142, 196}. The retention and release of a drug is related to the amount of drug loaded, the size of the core¹³⁶, the compatibility between the micellar core and the drug and the effect of external stimuli (*vide infra*). The compatibility between the polymer and the drug can be quantified and predicted by the Flory-Huggings interaction parameter:

$$\chi_{sp} = (\delta_s - \delta_p)^2 \frac{V_s}{RT}$$

where χ_{sp} is the interaction parameter between the drug (solubilise, s) and the core-forming polymer (p), δ_s is the Scatchard-Hildebrand solubility parameter of the drug and δ_p that of the polymer and V_s is the molar volume of the drug^{7, 319}. To obtain an optimal compatibility between the drug and the core-forming polymer, χ_{sp} should be as low as possible. This means that there is not a universal micellar system that can be used for all drugs, but an optimal combination has to be found for each drug to improve its retention^{7, 61, 68, 83, 100, 149, 155, 158, 162, 172, 195, 264, 319, 336, 340, 349, 352, 353}. This approach was proven for example by Liu *et al* who compared the interaction parameters of various core-forming polymers with a drug (ellipticine). It was demonstrated that in this way a good selection could be made, resulting in high drug loading capacities and slow release¹⁹⁵. Similarly, the core-forming polymer can be modified to get a better compatibility with the drug. For example, the loading efficiency in PEG-*b*-poly(B-benzyl L-aspartate) (PBLA) micelles and the *in vivo* therapeutic effect of the aromatic drug camptothecin was improved by increasing the number of aromatic groups on the polymer backbone, which was ascribed to aromatic interactions between the benzyl groups and the drug^{336, 353}. A similar approach was used to design a micellar system for doxorubicin (DOX)¹²⁹ and paclitaxel (PTX)⁸³. Chemical modification of the drug can be an alternative way to increase the compatibility with the micellar core. Forrest *et al* synthesised prodrugs of the anticancer drug geldanamycin and indeed demonstrated that a higher encapsulation could be obtained when the chemical structure of the prodrug was matched with the core forming segment⁶¹.

The drug is stably retained in the micellar core when the drug is chemically attached to the micelle-forming polymer. This approach was applied by Yokoyama *et al*, who covalently bound DOX to the p(Asp) block of PEG-*b*-p(Asp) copolymers. The resulting PEG-*b*-p(Asp)-DOX conjugate formed micelles^{149, 349, 352}. In addition to the bound DOX, large amounts of free DOX could be loaded through π - π stacking in these micelles and encapsulation efficiency depended on the amount of conjugated drug³⁴⁹. *In vivo* studies demonstrated that these DOX-loaded micelles had a considerably higher anti-tumour activity compared to free DOX in C26-bearing mice after i.v. injection²³⁴. A phase I clinical trial was conducted with this formulation (NK911, Figure 5) in 23 patients with metastatic or recurrent solid tumours refractory to conventional chemotherapy. It was found that the toxicity profile of NK911 was similar to

free DOX. However, NK911 exhibited longer half-lives, a lower clearance and a larger AUC, suggesting prolonged circulation times compared to free DOX. A phase II clinical trial is currently ongoing ²¹⁵.

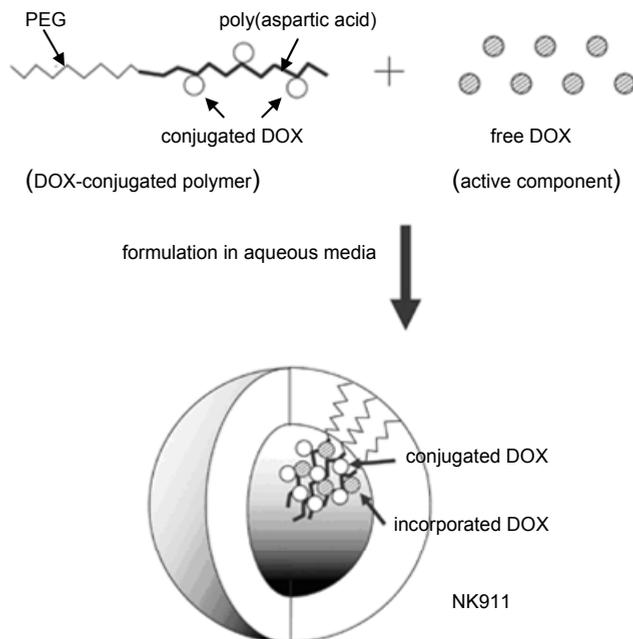


Figure 5 PEG-*b*-poly(aspartic acid) micelles with covalently bound and physically entrapped doxorubicin (DOX) also known as NK911 ²¹⁵

Ideally the micelles should circulate long and reach the target site intact, with the drug still loaded in the micellar core. However, this should not be confused with completely inert, non-degrading and non-releasing micelles since this would cause long-term accumulation in the body, especially after repeated administration. Moreover, the drug should eventually be released to interact with the therapeutic target. Therefore, ideally the encapsulated agents should be selectively released at the target site and the micelles should dissociate into single block copolymer chains or even chain fragments, with a molecular weight less than 50 kDa to enable clearance via the renal pathway ^{159, 294}. At the target site the drug can be released by degradation of the carrier system, but preferably via a more controlled mechanism, i.e. being the result of specific stimuli as will be discussed in the next sections of this chapter.

4 Stimuli-sensitivity

As pointed out in the previous sections, in an ideal micellar system, the drug is stably retained in the micelle during circulation and after accumulation in the targeted tissue, only here the drug is released as a result of environmental triggers. A release mechanism utilising the locally different conditions in pathological tissue compared to healthy tissue is attractive to achieve a high concentration of the drug in the target tissue. Besides, the loaded drug can be released by an external trigger including temperature, light, or ultrasound. Micelles which are destabilised as a result of either physiological or external triggers are referred to as ‘stimuli-sensitive micelles’. After micelle formation, stimuli-sensitive micelles disassemble only after certain triggers, for example as a result of changed polymers properties (e.g. polarity). Moreover, the originally stably encapsulated drug is expected to be released concomitantly with the disintegration of the micelles. A variety of triggers has been investigated to destabilise drug-loaded polymeric micelles, including temperature (section 4.1), pH (section 4.2), hydrolysis (section 4.3), enzymatic reactions (section 4.4), redox processes (section 4.5), light (section 4.6), others (e.g. ultrasound in section 4.7), as well as combinations hereof (section 4.8) (Figure 6). These approaches have been described for micelle-forming amphiphilic block copolymers²⁷⁸ and for peptide amphiphiles²¹⁰. A sophisticated stimuli-sensitive release system is obtained by co-loading of an imaging agent, which enables tracking of the micelles *in vivo* as discussed in section 4.9.

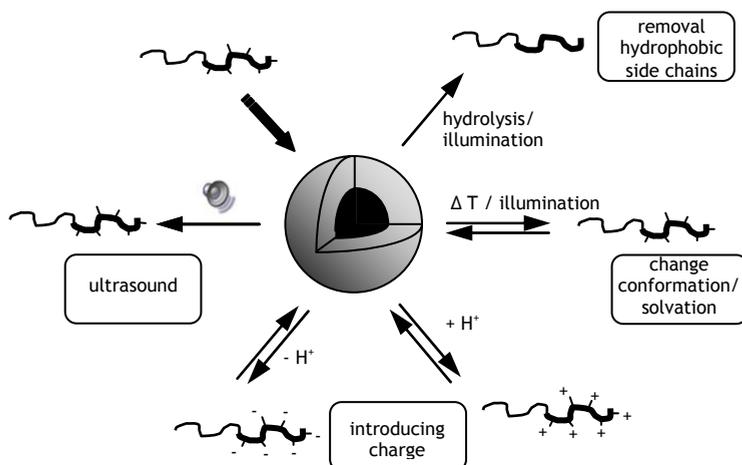


Figure 6 Stimuli-sensitive destabilisation mechanisms of polymeric micelles²⁷⁴

4.1 Thermosensitive polymeric micelles

An aqueous solution of a thermosensitive polymer is characterised by a so-called cloud point (CP). Below the CP, the polymer is hydrated and intra- and interpolymer interactions are prevented, thus rendering the polymer water-soluble. Once the polymer solution is heated above the CP, the hydrogen bonds between the water molecules and the polymer chain are disrupted and water is expelled from the polymer chains. Interactions between the hydrophobic moieties of the polymer chain can now take place, which is associated with the collapse of the polymer and finally results in phase separation (aggregation/precipitation of the polymer). Various thermosensitive block polymers are presently under investigation for the development of polymeric micelles for pharmaceutical applications^{38, 92, 107, 118}. Poly(*N*-isopropylacrylamide) (pNIPAAm) is the most extensively studied thermosensitive polymer with a CP of 32 °C^{90, 257, 290}. The CP of a thermosensitive polymer can be tailored by copolymerisation with hydrophobic or hydrophilic comonomers, resulting in a decreased or increased CP, respectively^{60, 241, 255, 276, 297, 300}. Via this strategy, polymers with a CP around body temperature were designed to create polymeric micelles, which are suitable for temperature-induced micelle dissociation. Thermosensitive copolymers can be used either as a hydrophilic, shell-forming segment (for example p(NIPAAm-co-DMAAm) and poly(2-isopropyl-2-oxazoline)^{139, 254}) or as a hydrophobic, core-forming segment of block copolymers (for example pNIPAAm and pHPMAm-Lac_n^{301, 318}) (Figure 7). The advantage of thermosensitive core-forming segments is that micelles are simply prepared by heating an aqueous polymer solution (above the CMC) till above the CP of the thermosensitive part; i.e. no organic solvents are required. The heating rate is a critical factor for the ultimate size of the formed nanoparticles; a fast heating rate results in smaller micelles than when a slow heating rate is applied^{240, 265, 367}. A major drawback for the first generation of thermosensitive polymeric micelles based on non-degradable polymers (e.g. pNIPAAm) is that thermal treatment (hyperthermia or hypothermia) is required for their destabilisation and concurrent drug release, which is not always feasible in clinical practice. Therefore, thermosensitivity is frequently combined with other stimuli-responsive mechanisms, such as pH- or light-sensitivity and degradability (section 4.8).

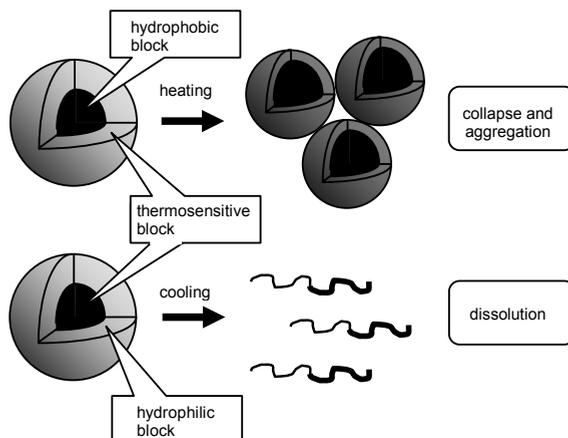


Figure 7 Drug loaded block copolymer micelles comprising a thermosensitive block either as the hydrophilic shell below the CP (top) or as the hydrophobic core above the CP (bottom). Heating or cooling will accomplish distortion of the micellar structures with concomitant release of the loaded drug²⁷⁴

4.2 pH-sensitive polymeric micelles

The mildly acidic pH encountered in tumour and inflammatory tissues (pH ~ 6.8) as well as in the endosomal and lysosomal compartments of cells (pH ~ 5 - 6), provides a potential trigger for destabilisation of a pH-sensitive carrier^{58, 81}. The major mechanism to induce pH-sensitivity is changes of charges in (polyion complex) micelles; pH dependent cleavage and destabilisation will be discussed in section 4.3. Typically, block copolymeric micelles that contain basic groups such as L-histidine (His)^{169, 171}, pyridine²¹¹ and tertiary amine groups^{166, 313} are pH-sensitive. The block copolymers assemble into micelles at a pH one unit above the pKa of the amines, where the pH-sensitive block is essentially uncharged and hydrophobic, thereby forming the core of the micelles. Decreasing the pH below the pKa results in protonation of the polymer, this in turn leads via an increased hydrophilicity and electrostatic repulsions to destabilisation of the micelles. The transition pH can be controlled by mixing different block copolymers. For instance, a mixture of PEG-*b*-p(His) and PEG-*b*-PLA formed stable micelles at pH 7.4 and dissociated at pH 6.0 to 7.2, depending on the ratio of the two block copolymers in the micelles^{169, 171}. Another example of pH-sensitive micelles is the sulphonamide-containing nanoparticles which collapsed upon protonation of the sulphonamide units (pKa = 6.1), thereby releasing the loaded doxorubicin

at $\text{pH} < 7$ ^{231, 232}. Block copolymers that are in their protonated (= water-soluble) form at $\text{pH} < \text{pKa}$ can be easily loaded with drug. Upon increasing the pH of an aqueous solution of 2-(methacryloyloxy)ethyl phosphorylcholine (PMPC)-*b*-2-(diisopropylamino)ethyl methacrylate (PDPA) block copolymers from pH 3 to pH 7, the PDPA block (pKa 6 - 7) became deprotonated (i.e. hydrophobic) and self-assembly took place. In presence of a model compound (dipyridamole, only soluble below pH 5.8), this neutralisation resulted in the formation of dipyridamole-loaded micelles⁷². Furthermore, tamoxifen and paclitaxel could be stably encapsulated in these PMPC-*b*-PDPA micelles at pH 7.4. Lowering of the pH below the pKa of the PDPA block resulted in a fast release of the drugs. This pH-triggered release might be advantageous when loaded micelles permeate into relatively acidic tumour tissue, or when they are taken up via the endocytotic pathway¹⁹⁰.

4.3 Chemical hydrolysis to induce micellar disintegration

Micellar disintegration and concomitant drug release can be established by chemical hydrolysis, which includes degradation of the polymer backbone (discussed in 4.3.1), cleavage of side groups (discussed in 4.3.2) and hydrolysis of covalent bonds between drug and polymer in micelle-forming polymer-drug conjugates (as is discussed in 4.3.3).

4.3.1 Chemical hydrolysis of the polymeric backbone

Backbone hydrolysis of the hydrophobic block of an amphiphilic block copolymer is a frequently applied method to destabilise micelles used for drug delivery¹⁴⁸ (Table 3). For example, the chemical degradation of the polyester block in PEG-*b*-poly(DL-lactic-co-glycolic acid) (PLGA)³⁶⁹, PCL-*b*-PEG-*b*-PCL⁹⁸ and mPEG-*b*-oligocaprolactones³³ was associated with changes in particle size, indicating micelle destabilisation. Furthermore, transition of PEG-*b*-PCL worm-like micelles into spherical micelles was observed upon hydrolysis of the ester bonds in the PCL block⁶⁹. The ester hydrolysis is pH dependent and the ester bonds in oligolactates⁴⁹ and oligocaprolactones³³ displayed an optimal stability at $\text{pH} \sim 4\text{-}5$. However, even at physiological pH and temperature, chemical hydrolysis of caprolactone based polymers and oligomers is slow and will hardly play a role *in vivo*. It is anticipated that in the body these polymers will be mainly cleaved by enzymatic degradation (vide infra)³³. Moreover, when the drug should be released in the mildly acidic tumour tissue and endosomal compartments of cells and, consequently, degradation of micelles is desired at relatively low pH, other type of polymers such as poly(ortho esters) (POE) have

a better degradation profile⁸⁸ (Table 3). Indeed, PEG-*b*-POE micelles displayed a higher stability at pH 7.5 than at pH 5.5⁸⁹. The effect of hydrolytic degradation on micelle stability has been extensively studied and although it is generally believed that micelle destabilisation leads to the release of the loaded drugs, experimental data on the relation between degradation and drug release are scarce. One of the few examples was described by Geng *et al*, who correlated the degradation-induced transition of PEG-*b*-PCL worm-to-sphere micelles with the release of the loaded paclitaxel (PTX). PTX release from these micelles was caused by a reduction of the drug carrying capacity, since spherical micelles have a smaller volume-to-surface ratio than worm-like micelles⁷⁰.

degradable group	structure	degradation products	reference
ester		$R_1-OH + \begin{array}{c} O \\ // \\ R_2-OH \\ \\ OH \end{array}$	33, 276
orthoester		+ $\begin{array}{c} R_2 \\ \\ C=O \\ \\ R_1-O \end{array}$ or R_1-OH	88
acetal		+ $\begin{array}{c} O \\ // \\ R_3 \\ \\ R_2 \end{array}$	73
hydrazone		$\begin{array}{c} R_1 \\ \\ C=O \\ \\ R_2 \end{array} + \begin{array}{c} H_2N \\ \\ HN-C=O \\ \\ R_3 \end{array}$	17, 18, 93

Table 3 Biodegradable moieties embedded in polymeric micelles

4.3.2 Cleavable side chains

When hydrophobic side chains in the core-forming block, which are contributing to the stability of the micelles, are removed by hydrolysis, the hydrophilicity of the micellar core will increase and micelles undergo destabilisation. A good example is PEG-*b*-p(Asp) that is stabilised by cyclic

benzylidene acetals in the hydrophobic core via π - π stacking. The micelles were stable at physiological pH, whereas hydrolysis of the acetal bonds at pH 5 generated the more polar diols (Table 3). The overall hydrophilicity of the polymer increased, resulting in micellar dissolution and release of an encapsulated hydrophobic dye⁷³. A similar mechanism was applied for linear-dendritic block copolymers. *In vitro*, these polymeric micelles displayed an accelerated release of entrapped DOX at acidic pH as a result of micelle disruption^{74, 77}. Thermosensitive (block co)polymers containing biodegradable side chains will be discussed in section 4.8.2.

4.3.3 Cleavage of polymer-drug conjugates

In the case of polymer-drug conjugates, drug release from polymeric micelles can be established by the acid-catalysed cleavage of a labile linkage by which the drugs are attached to the polymer. For example, the acid labile hydrazone linkage between DOX and PEG-*b*-p(Asp) resulted in an accelerated release of DOX at acidic pH *in vitro* (Table 3). In comparison to free DOX, these pH-sensitive DOX-hydrazone-micelles had a 15-fold greater AUC_{blood}, a higher anti-tumour activity and a reduced toxicity *in vivo*. Moreover, micelles in which DOX was bound via a non-degradable amide bond did not exert any anti-tumour activity^{17, 18, 93}. In a recent study, a triblock copolymer ((PLA-*co*-glycolic acid-*alt*-glutamic acid)-*b*-PEG-*b*-(PLA-*co*-glycolic acid-*alt*-glutamic acid)) was used to couple PTX via an acid labile ester linkage and the resulting micelles displayed a three-fold higher release of PTX at pH 4.2 than at 7.4³⁴³.

4.4 Enzymatic triggered destabilisation of polymeric micelles

The abundant presence of certain enzymes in pathological tissues has been applied as an environmental trigger to destabilise (drug-loaded) polymeric micelles. Similar to hydrolytic degradation, enzymes may cleave either the backbone of the hydrophobic block, their side chains, or the bonds between the polymer and drug. It was demonstrated that polyesters are not only degraded hydrolytically, but they are also susceptible to enzymatic degradation, for example by lipases. This lipase-catalysed degradation was shown for PEG-*b*-poly(3-hydroxybutyrate)(PHB)-*b*-PEG micelles³⁶, PEG-*b*-PCL nanoparticles⁶⁴, accompanied by the release of encapsulated pyrene and for PEG-*b*-oligo(ϵ -caprolactone) micelles³³. Furthermore, the peptide bonds in poly(amino acid)s, used as hydrophobic blocks, can be cleaved by proteases, as demonstrated for example for poly(γ -glutamic acid)-*g*-L-phenylalanine (PGA-*g*-L-PEA) micelles⁵.

4.5 Oxidation- & reduction-sensitive polymeric micelles

The reduction of disulphide bonds in polymeric assemblies by intracellular glutathione can be used for micellar decrosslinking (vide supra, section 3.3) or for full destabilisation^{71, 124, 125, 188}. Furthermore, the reversible redox reactions of organometal compounds, e.g. viologen and ferrocene, are an attractive trigger to alter the charge density and thus to change the solubility of viologen or ferrocene containing polymers¹⁰. Oxidation of redox-active micelles, containing a hydrophobic ferrocenylalkyl moiety in the block copolymer, was demonstrated to shift the hydrophobic/hydrophilic balance and the micelles disintegrated into water soluble unimers. The release of a model hydrophobic drug (perylene) from these micelles was precisely controlled by a selective electrochemical oxidation of the ferrocenylalkyl moiety and zero-order kinetics could be realised³¹⁰. Selective drug release at pathogenic sites may be accomplished via externally applied electric current or by taking advantage of the accumulation of activated macrophages in inflamed tissues and certain tumours. These macrophages release oxygen-reactive species, which may also trigger the transformation of redox-sensitive micelles. However, so far, these redox-sensitive polymeric micelles have not yet been investigated *in vivo*.

4.6 Light-induced micellar deformation

Light-responsive polymeric micelles ideally release their entrapped guest molecules only upon either ultraviolet (UV), visible (VIS) or (near-)infrared ((N)IR)-light exposure. The use of NIR is of particular interest for biomedical applications because of its deeper tissue penetration and minimal detrimental effects on healthy cells.

4.6.1 Irreversible reactions upon illumination (photolysis)

Light-induced micellar disruption can be applied using UV- or IR-illumination to cleave photolabile hydrophobic side chains. An amphiphilic block copolymer composed of PEG and a polymethacrylate bearing photolabile pyrene methyl esters in the side chain (PPy) as the hydrophobic core-forming domain was synthesised^{112, 113}. Upon illumination, these ester side groups were split off, thereby transforming the hydrophobic micellar block into a hydrophilic poly(methacrylic acid) (PMAa) block, which brought about dissociation of the micelles. A controlled release of encapsulated Nile Red could be accomplished, since the dissociation kinetics were controlled by the intensity of the light¹¹³.

Moreover, upon core crosslinking of these micelles, the photolysis-induced micellar destabilisation was prevented by the crosslinks. Nevertheless, the overall hydrophilicity of the polymer increased upon illumination and the micelle swelled, thereby still releasing the loaded hydrophobic guests, although at a lower rate ¹¹².

In addition, irreversible rearrangements upon illumination are used to eradicate the hydrophobic micellar interaction forces. For example, hydrophobic 2-diazo-1,2-naphthoquinone derivatives were attached to alkyl-PEG chains, which self-assembled into micelles. The so-called Wolff rearrangement (Figure 8) that takes place upon illumination of these chromophores drastically changed their polarity, destabilised the micelles and released the encapsulated Nile red ⁷⁹.

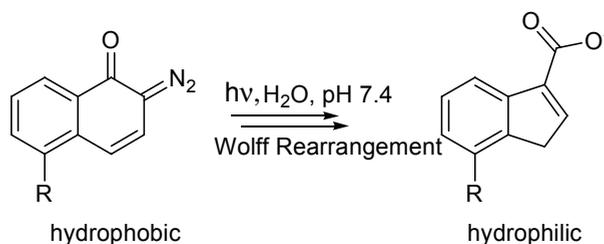


Figure 8 Solubility change of 2-diazo-1,2-naphthoquinone derivatives after Wolff rearrangement to 3-indenecarboxylate in buffered water

4.6.2 Light-induced reversible changes

Besides irreversible micellar disintegration, environmental light can induce reversible and non-destructive destabilisation. Several photoactive groups that undergo reversible structural changes upon illumination have been attached to amphiphilic block copolymers, thereby mainly shifting the hydrophobic/hydrophilic balance. Chemical entities that display photochemical induced transitions include azobenzenes (change in dipole moment) ³¹⁷, cinnamoyl (isomerisation into a more hydrophilic residue or photodimerisation) ¹¹¹, spirobenzopyran (formation of zwitterionic species) ¹⁴¹ and triphenylmethane leucohydroxide (generation of charges) ¹⁴⁴, as recently reviewed in detail ²⁷⁴. For instance, exposure of azobenzenes-containing methacrylate-*b*-(*tert*-butyl acrylate-*co*-acrylic acid) polymers to UV-light results in a *trans*-to-*cis* isomerisation and a more hydrophilic polymer is generated, causing dissolution of the assemblies ³¹⁷. Another example is the reversible *trans*-to-*cis* photoisomerisation upon UV-irradiation of cinnamoyl-containing

PEG-*b*-poly(methacrylate) polymers, which creates compounds with an increased hydrophilicity or leads to reversible photodimerisation (Figure 9)^{111, 114}. Some of these photosensitive moieties were also embedded in thermosensitive block copolymers as will be discussed in section 4.8.3.

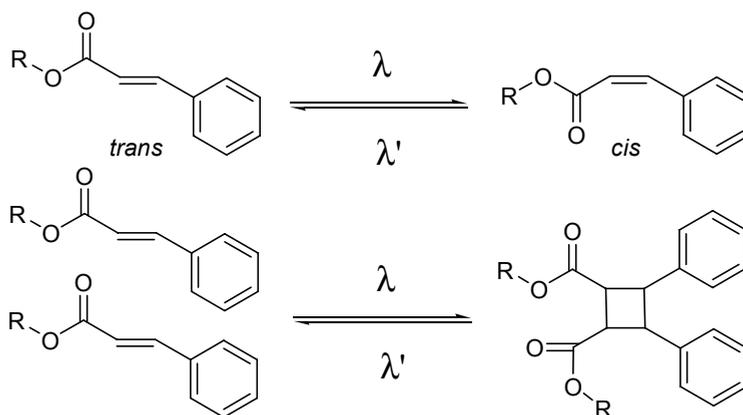


Figure 9 Reversible *trans*-to-*cis* isomerisation (top) and photodimerisation (bottom) of the cinnamoyl photoreactive group upon irradiation with UV-light

4.7 Other physical triggers to destabilise polymeric micelles

Besides the above mentioned temperature, pH, hydrolysis and light triggers, ultrasound and ion exchange have also been explored to induce drug release. The use of ultrasound as an external trigger to release drug from Pluronic[®] micellar systems *in vitro* and *in vivo* was extensively studied by Rapoport *et al*^{66, 268, 269}. The cellular cytotoxicity of DOX-loaded micelles in combination with ultrasound was 66 %, without ultrasound 53 % while free DOX without applying ultrasound resulted only in 15 % cell death²⁶⁸. An increased uptake of both free drug (in PBS) and DOX-loaded Pluronic[®] micelles was observed for sonicated tumour cells *in vivo*^{66, 269}. The mechanisms of the ultrasound effect might be: i) enhanced permeability of blood vessels results in extravasation of the carriers; ii) dissociation of micelles into unimers with concomitant drug release, iii) accelerated diffusion in the interstitial and tumour and iv) enhanced membrane permeability, which all lead to increased cellular uptake of the drug^{66, 268, 269}.

Ion-sensitive polymer-metal micelles were formed by complexation of cisplatin to the carboxylate groups of a PEG-*b*-p(glutamic acid) block

copolymer. In 0.15 M NaCl, ion exchange reactions occurred, thereby slowly releasing cisplatin from the micelles, accompanied by the dissociation of the micellar structure (Figure 10). Intravenous injection of these micelles led to a significantly increased plasma level and tumour accumulation of cisplatin as compared to the free drug ²⁴⁴. Cellular uptake and release of cisplatin was recently also demonstrated with cisplatin-loaded crosslinked PIC micelles ²⁴.

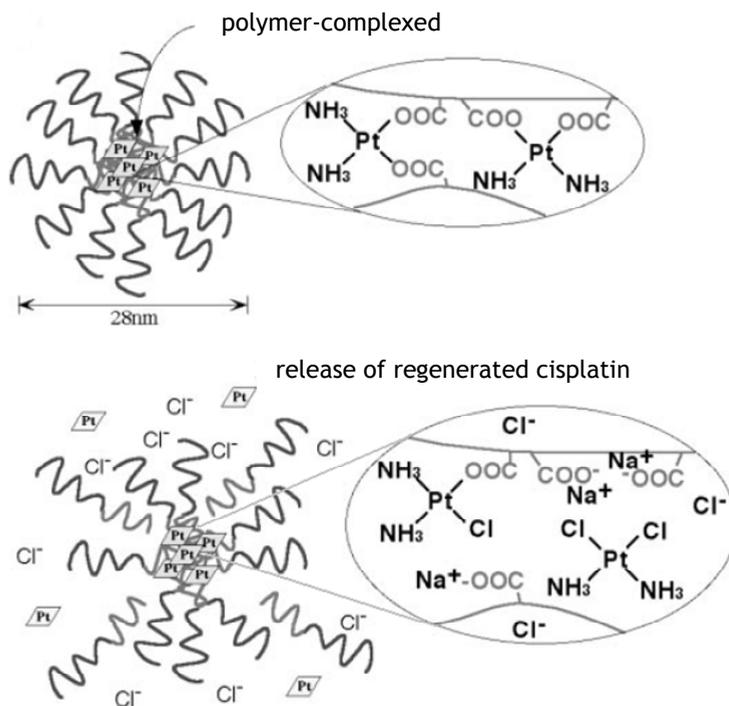


Figure 10 Cisplatin complexation with carboxylate containing polymers (PEG-*b*-p(glutamic acid)) results in stabilised micelles (above), while ligand exchange reactions in saline lead to gradual release of cisplatin (below) ²⁴⁴

Magnetic micelles (220 - 430 nm) were obtained in water by coating iron oxide nanoparticles with peptide-based polymers (polybutadiene-*b*-poly(glutamic acid)). It is anticipated that the micellar shape is manipulable in a magnetic field but experimental data are not available yet. These superparamagnetic self-assembled hybrids might be of interest for future drug delivery systems but also as contrast agents in MRI ^{164, 165} as described in the section 4.9.

4.8 Polymeric micelles sensitive to a combination of triggers

4.8.1 pH- and temperature sensitivity

Random copolymers based on NIPAAm, *N,N*-dimethylacrylamide and 10-undecenoic acid displayed a CP that was not only dependent on the copolymer composition but also on the pH. The polymer was designed in such a way that the polymer was below its CP and thus highly hydrated at pH 7.4 and 20 °C, with the hydrophobic undecenoic acids side chains clustering together to form core-shell morphologies of approximately 200 nm. The hydrophobic drug DOX could be loaded in the undecenoic acid core by a dialysis method. Lowering the pH to 6.6 caused protonation of the undecenoic carboxylate group and decreased the CP below 20 °C. Thereby, the micelles disintegrated which was accompanied by the release of the encapsulated DOX^{198, 303}. A similar effect was seen at pH 7.4, when increasing the temperature to above the CP of 40 °C³⁰³. Furthermore, a broad range of other block copolymeric assemblies that use this pH dependency to control the temperature sensitivity have been reported in literature^{201, 219, 262, 284, 348}.

4.8.2 Biodegradable temperature sensitive polymers

Dual sensitive micellar systems that are based on biodegradable thermosensitive (block co) polymers have been described^{167, 238, 241, 276, 295, 301}. The polymers self-assemble into micelles in aqueous solution above their critical micelle temperature (CMT), which is the temperature above which phase separation of the thermosensitive block takes place. pH-dependent cleavage of hydrophobic side chains resulted in a 'hydrophobic-to-hydrophilic' conversion of the micellar core. Consequently, the CMT gradually increases, which ultimately results in micelle destabilisation and polymer dissolution. In casu, our department designed biodegradable thermosensitive polymers that have methacrylamide backbones with oligolactates attached via hydrolytically sensitive ester bonds (e.g. 2-hydroxypropyl methacrylamide lactate (HPMAm-Lac_n, n is the number of lactic acid units in the oligolactate chain)^{167, 276, 300}. The CMT of these methacrylamide-oligolactate copolymers is precisely tailored by the monomer feed ratio. The CMT of copolymers of HPMAm-Lac₁ with HPMAm-Lac₂ covers a temperature range of 10 to 63 °C (corresponding to 0 to 100 % HPMAm-Lac₁ respectively). The slightly more hydrophilic homopolymer poly(*N*-(2-hydroxyethyl)methacrylamide-dilactate) (p(HEMAm-Lac₂)) has a CP of 22 °C, which could be lowered by copolymerisation with HEMA-Lac₄. Generally, the CMT of the polymers can be tuned to be below the temperature

at which micelles are wanted (e.g. ambient or body temperature). The block copolymer mPEG-*b*-p(HPMAm-Lac₂) (CMT 8 °C) displayed a transient stability at physiological conditions: after one week a sufficient amount of lactate side chains were hydrolysed to increase the CMT to above body temperature, which resulted in polymer dissolution and thus the micelles disintegrated (Figure 11)²⁹⁹. A much shorter destabilisation time of 8 hours was obtained with mPEG-*b*-p(HEMAm-Lac_n) micelles since the hydrolysis of HEMA_n-Lac_n was much faster than that of HPMA_n-Lac_n²⁷⁶.

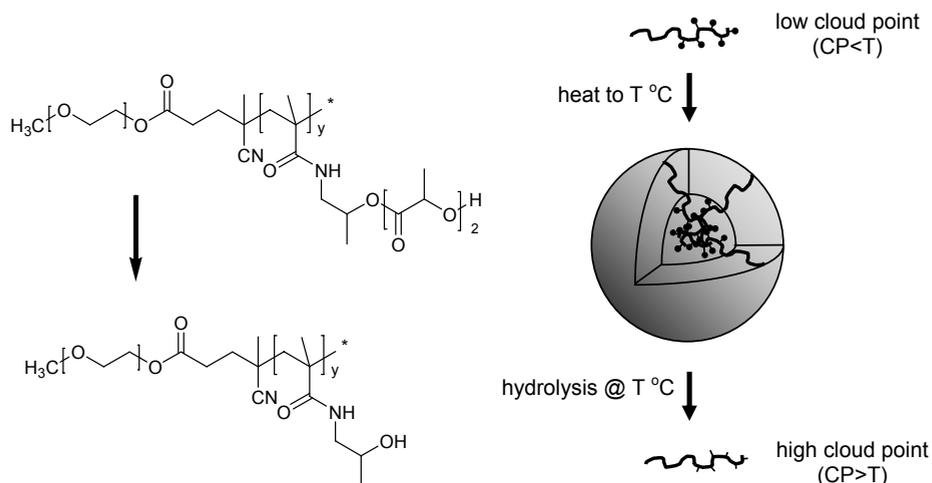


Figure 11 Hydrolysis of mPEG-*b*-p(HPMAm-Lac₂) (left) causes an increase of the critical micelle temperature of the block copolymer by removal of the hydrophobic lactic acid groups (black dots; right). Thus, micelles formed above the cloud point of mPEG-*b*-p(HPMAm-Lac₂) destabilise when the CMT passes the incubation temperature²⁷⁴

Recently, other biodegradable thermosensitive polymers with different hydrolysable groups were reported. A cyclic ester is the degradable moiety in poly(NIPAAm-*co*-dimethyl- γ -butyrolactone acrylate)⁴⁶, whereas hydrazone bonds in poly(NIPAAm-hydrazone-alkyl_n)⁹⁴ or orthoesters in poly(*N*-(2-(*m*)ethoxy-1,3-dioxan-5-yl)methacrylamide)⁹⁹ have been used as acid-labile groups. It is anticipated that by applying these types of polymers in block copolymer architectures, a second generation of controlled biodegradable thermosensitive micelles can be created.

4.8.3 Various

Photoresponsive thermosensitive copolymers do not only respond to temperature, but also display a photo-induced change in CP. UV-illumination of a thermosensitive copolymer that contains light-sensitive compounds (such as those mentioned in section 4.6.2) can result in increased hydrogen bonding capacity and consequently an increased CP^{105, 156, 305}. In the case of poly(2-(dimethylamino)ethylmethacrylate)-*b*-poly(6-(4-phenylazo)phenoxy)-hexyl-methacrylate) micelles, the application of only light was not sufficient to induce dissociation, since the light induced trans-to-cis isomerisation did not overcome the hydrophobic interactions²⁷⁰. Additionally, pH-sensitivity could be obtained by the introduction of carboxylic acid units⁵⁰. The photodimerisation of cinnamoyl units was used to design crosslinked polymeric micelles that responded to pH, temperature and ionic strength³⁰⁷.

So-called schizophrenic micelles were developed by Armes *et al* and are based on the pH-induced micellar inversion of zwitterionic diblock copolymers assemblies. For example, a diblock copolymer composed of 2-(diethylamino)ethyl methacrylate (pDEA) and 2-(*N*-morpholino)ethyl methacrylate (pMEMA) is fully dissolved at pH 6 and 20 °C, since the pDEA is protonated and the neutral pMEMA is hydrophilic. At pH 8.5 the pDEA block is deprotonated and micelles are formed with pDEA as the core-forming part. Lowering the pH and addition of sufficient electrolyte causes the pMEMA block to be selectively salted out to yield pMEMA-core micelles²⁹. An overview of these type of micelles bearing pH and ionic strength dependency was recently published³⁰.

The combination of enzyme and temperature sensitivity was introduced in a pNIPAAm-based polymer with peptide side chains. Upon phosphorylation of the peptide by protein kinase A, the CP of the copolymers increased (from 36.7 to 40 °C) because of the hydrophilisation of the peptide chains¹³⁰. At physiological conditions and due to the action of this enzyme, the polymers gradually dissolved in time. However, this concept is not used to design responsive micelles so far.

4.9 Imaging-guided drug delivery

Encapsulation of drugs in stimuli-sensitive polymeric micelles aims at achieving an optimal therapeutic effect, e.g. drug release as a result of (external) stimuli after accumulation of the loaded nanocarrier at its site of action. The possibility to detect the presence of the drug-loaded micelles at

their aimed site of action by *in vivo* imaging and to subsequently trigger the release of the loaded drug would be an important new feature³³⁹.

For imaging purposes, polymeric micelles were loaded with either magnetic resonance imaging (MRI) contrast agents (mainly Fe, Mn, or Gd), γ -emitting radiolabels (such as ¹¹¹indium or ^{99m}technetium)³²⁸, heavy elements (e.g. I, Br and Ba) for CT imaging, or with quantum dots (QDs)^{250, 320, 323}. Importantly, encapsulation of these agents in micelles is favourable since in that form they are less prone to renal clearance while their accumulation in tumour tissue (via the EPR-effect) will enhance the signal strength and specificity, respectively^{4, 233}.

Co-loading diacyllipid micelles with Fe₃O₄ and a photosensitiser (a drug that is activated via illumination) enabled to monitor the *in vitro* cellular uptake in real time⁴¹. Furthermore, applying an external magnetic field to cells incubated with these magnetic nanoparticles resulted in a so-called magnetophoretic control of the cellular uptake^{41, 355}. Future *in vivo* administration of iron and drug-loaded micelles in combination with a local magnetic field might thus increase the concentration of the drug at the target site. Another sophisticated carrier for imaging-guided drug delivery was developed by Reddy *et al* PEGylated poly(acrylamide) nanoparticles were loaded with both iron-oxide and photofrin (photosensitiser) and also a targeting ligand directing to tumour vasculature was coupled onto the micellar shell. *In vivo*, a higher photodynamic therapeutic effect (i.e. killing of tumours) was observed when compared to non-targeted nanoparticles or free photofrin. The multifunctional targeted nanoparticles were internalised while the iron-oxide enabled to monitor the tissue localisation of the micelles in real-time by MRI and the optimal time for illumination could be chosen^{145, 271}.

Semiconductor quantum dots also have a potential for image-guided drug delivery using polymeric micelles. Several markers can be probed simultaneously since QDs absorb over a very broad spectral range, while the extreme high photostability of QDs enables real-time monitoring over long periods of time^{250, 282}. Furthermore, since QDs generate highly reactive free radicals upon illumination, these QDs containing micelles can be used for diagnostic as well as for (photodynamic) therapeutic purposes (Figure 12)²¹⁸. The most extensively studied QD in biology is CdSe, which has been encapsulated in phospholipids⁵⁵ and in antibody decorated multiblock copolymeric micelles. The latter type of micelles gave a clear imaging signal *in vivo* and enabled very precise tracking of the active tumour uptake⁶⁵.

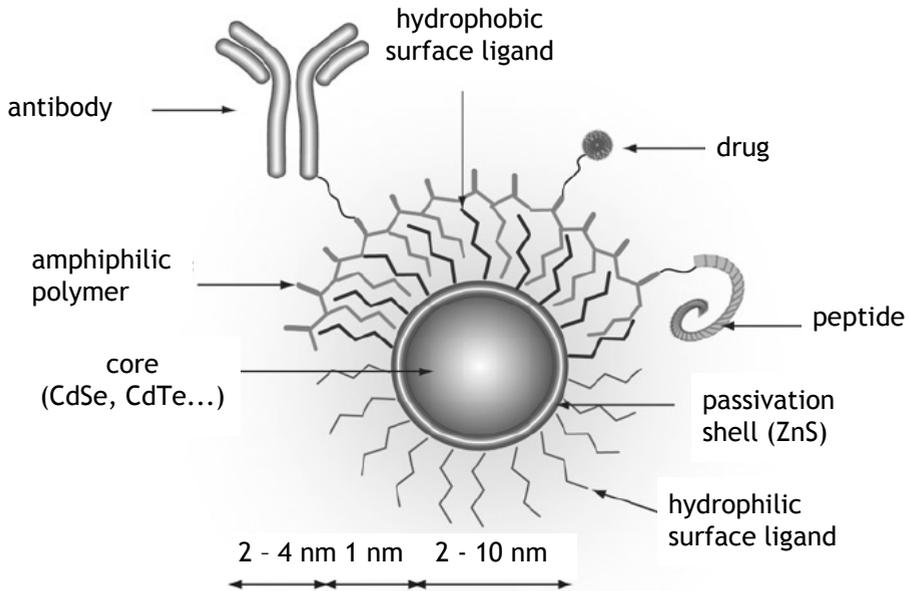


Figure 12 Multifunctional quantum dots containing micelles ²¹⁸

5 Combining longevity, stability & stimuli-sensitivity

In conclusion, polymeric micelles are very attractive drug delivery carriers for hydrophobic drugs in particular because of their unique morphology, high versatility and high drug loading capacity. The ideal micellar system should be able to stably encapsulate drugs, also when circulating *in vivo*. Several strategies have been investigated to improve the circulation times ('longevity') of the micellar carriers, their stability and the retention of the loaded drugs in the micellar core. Once the polymeric micelle has reached the aimed target site, the desired release of the entrapped drug poses conflicting requirements on the micellar building blocks. Internal or external triggers create possibilities to develop transiently stable polymeric micelles from which the time and site of release of entrapped drug can be precisely tailored.

The most popular approach to combine longevity and triggered release properties is the use of building blocks that contain a hydrophilic PEG-block in combination with a degradable or stimuli-sensitive hydrophobic block, accounting for both desired properties, respectively. However, only a few systems combine additional stabilising strategies with stimuli-triggered drug release. For example, biodegradable PEG-*b*-PCL micelles were stabilised either by reducing the CMC^{31, 209} or by core crosslinking²⁹⁸. Furthermore, thermosensitive PEG-*b*-p(HEMA-Lac_n) micelles were core crosslinked and demonstrated transient stability upon pH-dependent degradation²⁷⁵. A third example is PEG-*b*-p(Asp) micelles with covalently coupled doxorubicin, which increased the compatibility of the micellar core with additional physically encapsulated doxorubicin³⁴⁹.

Despite all promising aspects, so far, only a few micellar formulations have entered clinical trials; well-known concepts are Genexol-PM[®] (paclitaxel-loaded mPEG-*b*-PLA micelles) and PEG-*b*-p(Asp) micelles with either encapsulated paclitaxel (NK105) or covalently bound and physically entrapped doxorubicin (NK911)^{131, 137, 215}. Phase I studies with these formulations indicated prolonged circulation times when compared to the conventional formulations^{131, 215} or a better toxicity profile, allowing higher dosing¹³⁷.

In conclusion, it is anticipated that a proper combination of long circulating properties with sophisticated stabilising strategies will generate highly stable micelles that are able to reach the target site in their intact form. Introduction of stimuli-sensitive building blocks will control the release behaviour while further improvement is obtained by attaching targeting ligands. Incorporation of imaging agents allows detection of the drug-loaded

micelles at the target site and application of the external trigger at the appropriate site and time. The building blocks of polymeric micelles are synthetic polymers, which offer almost unlimited possibilities to tailor and optimise the micellar structures towards the desired morphology, drug compatibility and drug release profile. It is therefore expected that within the coming years their favourable properties will be exploited to successfully encapsulate various hydrophobic compounds and that these drug-loaded micelles will play superior performance in the treatment of several diseases.

chapter 2b

Triggered destabilisation of polymeric micelles & vesicles by changing polymers polarity: an attractive tool for drug delivery

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Abstract

Polymeric micelles and vesicles have emerged as versatile drug carriers during the past decades. Furthermore, stimuli-responsive systems are developed whose properties change after applying certain external triggers. Therefore, a triggered release of drugs from stimuli-sensitive micelles and vesicles has become an interesting challenge in the pharmaceutical field. Polymeric micelles or vesicles are mainly composed of amphiphilic block copolymers that are held together in water due to strong hydrophobic interactions between the insoluble hydrophobic blocks, thus forming a core-shell or bilayer morphology. Consequently, destabilisation of these assemblies is induced by increasing the polarity of the hydrophobic blocks. Preferably, this process should be the consequence of an external trigger, or take place in a certain time frame or at a specific location. A variety of mechanisms has recently been described to accomplish this transition, which will be reviewed in this paper. These mechanisms include the destabilisation of polymeric micelles and vesicles by temperature, pH, chemical or enzymatic hydrolysis of side chains, oxidation/reduction processes and light.

1 Introduction

Most systemically administered drugs exert their biological effects not only at their target sites but also at non-target sites, which often results in undesired side effects and hampers their therapeutic potential. This emphasises the importance of drug delivery systems (DDS) which deliver biologically active compounds selectively to the pathological area. Targeted drug delivery is of particular importance for the treatment of life-threatening diseases such as cancer since the adverse effects of cytostatic and other drugs can be very detrimental^{43, 80, 230}. Therefore, one of the main challenges in DDS research is the development of drug carriers enabling selective and tissue specific drug targeting. For this purpose, drug carriers should preferably have a high drug loading capacity, adequate stability in the bloodstream, long circulating properties, selective accumulation at the site of action with concomitantly a suitable drug release profile and good biocompatibility. Extensive research for the ideal, universal drug carrier system in the last quarter of the last century has resulted in a great variety of nanoscopic drug carriers^{45, 57}. Nanosized drug carriers have, as their name implies, nanoscale dimensions (typically 10 to 200 nm) and can be categorised into particulate systems and water soluble macromolecular systems. The first category includes lipid based systems such as liposomes^{44, 54}, systems based on surfactants such as emulsions^{312, 345} and systems based on synthetic polymers such as nanoparticles^{19, 252}, polymeric micelles^{81, 128, 150} and polymeric vesicles^{3, 51}. The second category of nanoscopic drug carrier systems encompasses polymer-drug conjugates^{245, 287} and dendrimers^{12, 76}. Some of these systems are presently on the market and many clinical evaluations are in progress to demonstrate their potential for the treatment of patients^{323, 359}.

The present review paper will be introduced in the first sections, which summarise relevant general issues related to polymeric micelles and vesicles with references to some recent review papers on these issues. The second part of this paper is limited to the application of triggered destabilisation of polymeric micelles and vesicles resulting in an increased polarity of the constituent block copolymers, aiming at the release of encapsulated compounds and partly retrieves new information not yet covered by other review papers.

1.1 The features of polymeric micelles & vesicles

Polymeric micelles are self-assembled core-shell nanostructures formed in an aqueous solution consisting of amphiphilic block copolymers (Figure 1).

Typically, the hydrophobic blocks of the copolymers form the core of the micelles by hydrophobic interaction ^{62, 273}, although other interactions such as electrostatic interaction ⁸⁶ and stereocomplex formation ¹²⁷ can also be utilised as the driving force for the core formation. The hydrophilic blocks of the copolymers form the shell of the micelles and stabilise the micellar structure. Depending on the polymer composition and the preparation conditions, amphiphilic block copolymers can also form vesicular assemblies (Figure 1). These are commonly called ‘polymersomes’ and reflect the structure of liposomes in the way that a bilayer structure is present which encloses an aqueous interior. As opposed to lipid vesicles, polymersomes are considered to be more rigid, stable and versatile ⁵¹. Polymersomes can be used as carriers of hydrophobic drugs (in the bilayer) as well as hydrophilic drugs (in the interior).

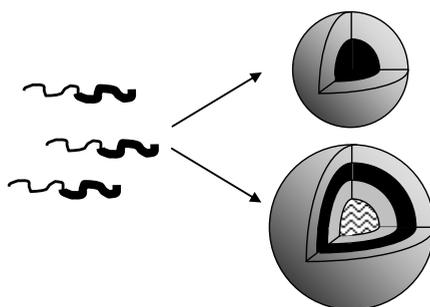


Figure 1 Formation of core-shell micelles (top) or bilayer vesicles (bottom) from amphiphilic block copolymers

The application of polymeric micelles as drug delivery system was pioneered by the group of H. Ringsdorf in 1984 ¹⁵ and subsequently used by Kataoka in the early 1990's through the development of doxorubicin-conjugated block copolymer micelles ³⁵⁰. Kabanov was the first to describe polymeric micelles with non-covalently incorporated drug ¹²². Nowadays polymeric micelles are extensively studied as a promising nanoscopic drug carrier because of their attractive features to fulfil the requirements for selective drug delivery ^{2, 7, 115, 159, 319}. Most notably, the hydrophobic core has a large capacity to accommodate hydrophobic drugs. Recently, polymeric micelles are also investigated as oral drug delivery system ^{214, 286}, but originally they were considered to be most suitable for intravenous (i.v.) administration. Due to their submicron size (typically between 10 and 100 nm), i.v. administered polymeric micelles provide possibilities to reach the pathological sites while

avoiding biological barriers in the human body upon oral administration, such as limited gastrointestinal absorption and high hepatic first-pass effect.

Formation of micelles or vesicles in aqueous solution occurs when the concentration of the block copolymer increases above a certain concentration, named as the critical aggregation concentration (CAC) or critical micelle concentration (CMC). At the CAC or CMC, hydrophobic segments of block copolymers start to associate to minimise the contact with water molecules, leading to the formation of a vesicular or core-shell micellar structure. The CMC of polymeric micelles is typically of the order of 10^{-6} to 10^{-7} M, while that of low molecular weight surfactant micelles is of the order of 10^{-3} to 10^{-4} M². This indicates that polymeric micelles, compared to surfactant micelles, are less prone to dissociation at low concentrations. Since polymeric micelles are subject to dilution upon i.v. administration this low CMC is advantageous as to maintain the micellar structure that facilitates prolonged circulation in the bloodstream.

After i.v. administration, polymeric micelles should deliver their payloads selectively at the target sites. It is known that the so-called enhanced permeability and retention (EPR) effect plays an important role for such targeting to e.g. tumour tissues^{208, 216}. The EPR effect was proposed by Maeda *et al* in the 1980's and is attributed to two factors. Firstly, the angiogenic tumour vasculature, as well as blood vessels in other pathological tissues, has a higher permeability compared to normal ones due to its discontinuous endothelium. Secondly, it has been shown that lymphatic drainage is not fully developed in tumours. These features lead to the fact that colloidal particles (polymeric micelles) extravasate through the 'leaky' endothelial layer in tumour and other inflamed tissues and are subsequently retained there. It should be noticed that to achieve this "passive" targeting, the polymeric micelles have to circulate in the bloodstream for sufficient time. In this respect, the surface properties of polymeric micelles are important factors since they determine their biological fate. A key issue for prolonged circulation is to reduce the rate of non-specific recognition and uptake by the reticulo-endothelial system (RES). It has been shown that grafting of hydrophilic polymers (e.g. poly(ethylene glycol) (PEG), poloxamer) on the surface of particles is effective to oppose opsonisation and subsequent uptake by the RES cells of the liver, spleen and bone marrow^{220, 304}. However, the exact biodistribution and accompanying pharmacokinetics of polymeric micelles is depending on many factors, such as the particle size, the surface layer characteristics and the particle rigidity^{14, 149, 239, 306}. Poly(ethylene glycol)-*b*-

poly(D,L-lactide) micelles showed proper circulation times (25 % of the injected dose was still detected in the blood after 24 hours ³⁴⁶), but in general, polymeric micelles are cleared from the systemic circulation of experimental animals within the first 8 to 10 hours after intravenous administration ^{223, 319}.

Once reached the targeted tissue, the virus-like size of the polymeric micelles enables their internalisation into the cells via e.g. fluid-state endocytosis even without any targeting ligand present on their surface ^{266, 289, 298}. However, 'active' targeting is accomplished by attaching a specific ligand (e.g. antibodies) to drug-loaded polymeric micelles. Through ligand-receptor interactions with the target cells, enhanced internalisation of the loaded vehicles delivers the drugs directly into the cell ⁸. Furthermore, introducing stimuli-sensitive segments in the block copolymers generates nanoparticles with environmentally controlled release mechanisms (*vide infra*). Concerning their toxicity, it must be noted that polymeric micelles finally dissociate in the body into single block copolymer chains. Block copolymers for micellar applications generally have a molecular weight less than 50,000 Da and are therefore subject to renal clearance, preventing their long-term accumulation in the body ^{159, 294}. Also biodegradable building blocks can be used to design polymeric micelles, e.g. poly(lactic acid) or poly(ϵ -caprolactone) which facilitates their elimination. Lastly, for pharmaceutical development it is advantageous that polymeric micelles can be easily sterilised by filtration through a 0.22 μm filter.

1.2 The building blocks

Generally, amphiphilic block copolymers of the A-B type, where A represents a hydrophilic block and B represents a hydrophobic block, are used to design polymeric micelles and vesicles. Other examples include A-B-A triblock copolymers ¹¹⁹ and graft copolymers ^{63, 126}. PEG is most commonly used as the hydrophilic segment of the copolymers, since it is a non-toxic polymer with FDA-approval as component of various pharmaceutical formulations and its unique physicochemical properties (high water-solubility, high flexibility and large exclusion volume) provide good "stealth" properties ^{173, 227, 342}. Also other polymers can be used as the shell forming segment, e.g. poly(*N*-vinyl-2-pyrrolidone) (PVP) ²² and poly(acrylic acid) ¹⁰³. In contrast, a larger variety of polymers has been studied as the hydrophobic segment in polymeric micelles: poly(propylene glycol) (PPO, Pluronic®) ¹¹⁹, poly(aspartic acid) with chemically conjugated doxorubicin (pAsp(DOX)) ³⁴⁹, poly(β -benzyl-L-aspartate) (pBLA) ¹⁵³ and poly(ester)s such as poly(lactic acid) (PLA) ^{82, 191}, poly(ϵ -caprolactone) (PCL) ^{6, 185} and poly(trimethylene carbonate) (pTMC) ³⁶⁵. The choice of the core-

forming segment is the major determinant for important properties of polymeric micelles such as stability, drug loading capacity and drug release profile (described in more detail in the next section) and explains why so many core-forming hydrophobic polymers have been used for the development of polymeric micelles.

1.3 Drug loading, retention and release by polymeric micelles

Polymeric micelles have been loaded with an extensive variety of drugs such as doxorubicin (DOX)^{74, 93, 119, 170, 234, 266, 350}, paclitaxel (PTX)^{34, 83, 100, 135, 137, 147, 191, 298, 325}, cisplatin^{243, 244}, amphotericin B¹⁶⁰, indomethacin^{52, 193}, photosensitisers^{163, 330, 360} and new cytotoxic drug candidates, e.g. KRN 5500^{217, 354}. Drugs can be incorporated into polymeric micelles by physical entrapment or by chemical conjugation. However, chemical conjugation of a drug to the core-forming block has several disadvantages: i) it requires the presence of reactive groups on the drug, ii) the coupling reaction might be detrimental for the drugs activity, iii) the drug should be released preferably at the target site, e.g. by enzymatic or chemical hydrolysis, iv) the final polymer properties and micellar characteristics depend on the type of conjugated drug, while it is more desirable to have a platform technology where micellar behaviour is determined by the polymer backbone only and independent of the drug that is to be encapsulated. There are several means for loading drugs physically into the core of polymeric micelles. In most cases, loading procedures with organic solvents are utilised, such as o/w emulsion¹¹⁵, dialysis³⁰⁹ and solid dispersion^{135, 363}.

A mechanism to release the content of a polymeric micelle exclusively at the target site will only be effective when premature release of the drug from the micelles is minimised before reaching their target site (Figure 2). The release of physically entrapped drugs from polymeric micelles is controlled by diffusion of the drug through the micellar core and the partition coefficient of the drug over the micellar core and the aqueous phase, provided that the micelles remain intact. The release of pyrene from polymeric micelles with a glassy core such as poly(styrene) ($T_g = 100\text{ }^\circ\text{C}$) and PBA ($T_g = 40 - 43\text{ }^\circ\text{C}$) was slow, indicated by very small diffusion constants of 10^{-18} to $10^{-16}\text{ cm}^2/\text{s}$ ³¹⁴. On the other hand, the release of pyrene from poly(2-vinylpyridine)-*b*-PEG, whose core is liquid-like under the experimental conditions, was too fast to be assessed³¹⁴. Consequently, polymeric micelles have preferably a solid-like, glassy, core at body temperature if their aim is targeted drug delivery.

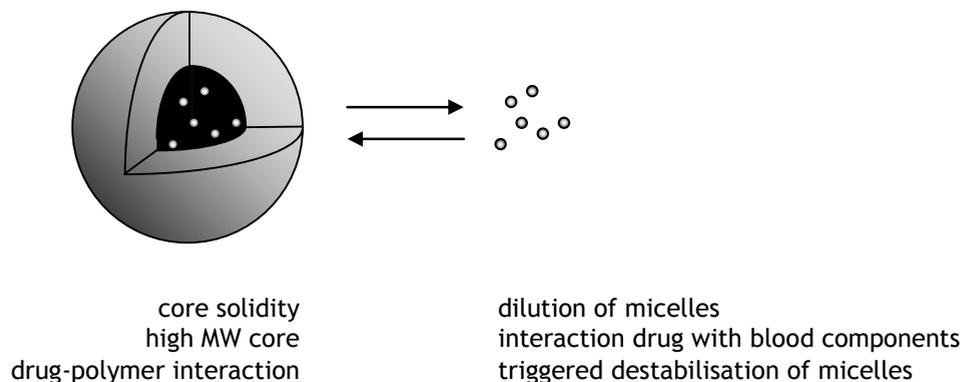


Figure 2 Summary of properties that favours retention (left) and release (right) of drugs in polymeric micelles

Other factors influencing drug release are the length of the core-forming polymer segment, the affinity between the drug and the core (i.e. partition coefficient between the core and the aqueous phase) and the amount of the loaded drug. It was shown that the release rate of indomethacin from PCL-*b*-PEG micelles decreased as the molecular weight of the PCL block and the amount of entrapped indomethacin increased¹³⁶. Most stable drug loading can be achieved when the affinity (e.g. assessed by the Flory-Huggins interaction parameter⁷) between the drug and the core-forming segment of the block copolymer is high. For example, the partition coefficient of pyrene is of the order of 10^2 for Pluronics[®]¹²³, 10^4 for PBLA-*b*-PEG¹⁵¹ and 10^5 for PSt-*b*-PEG^{340, 366}. The affinity of a drug for the hydrophobic core can be optimised by a proper selection of the block copolymer. It has been shown that PTX-loaded polymeric micelles with a PLA core did not show improved target distribution of PTX *in vivo* compared to Taxol^{28, 162}, while those with a core of pAsp modified with 4-phenyl-1-butanol significantly increased the plasma AUC (90-fold vs. Taxol) and the tumour AUC (25-fold vs. Taxol) of PTX⁸³. These results would suggest that a micellar core which contains more hydrophobic and more compatible groups to PTX (e.g. phenyl groups) is preferable for strong interaction with PTX and its stable retention *in vivo*. Other examples where the compatibility between drug and polymer has been optimised include poly(*N*-(6-hexylstearate)-L-aspartamide)-*b*-PEG (pHSA-*b*-PEG) for amphotericin B^{157, 158}, poly(aspartic acid-co-phenylalanine)-*b*-PEG for diminazene aceturate²⁶⁴ and poly(C16-benzyl-L-

aspartate)-*b*-PEG for KRN 5000³⁵⁴. Covalently linking a drug to the core-forming block creates another possibility to improve the physical entrapment of the same drug. This was demonstrated for p(Asp-DOX)-*b*-PEG which stably incorporated DOX by π - π stacking between conjugated and non-conjugated molecules^{349, 352} and this formulation (NK911) is presently under clinical evaluation. Park *et al* recently reported an interesting approach to the design of a polymer with a core-forming block that has a high affinity for the drug to be entrapped. They first screened a large number of hydrotropic agents to identify their ability to enhance the solubility of PTX in water and *N,N*-diethylnicotinamide (NNDENA) was found to be the most effective hydrotropic agent for PTX¹⁷². Next, monomers containing NNDENA, 2-(4-(vinylbenzyloxy)-*N,N*-diethylnicotinamide (DENA monomer) and their block copolymers with PEG were synthesised. The resulting pDENA-*b*-PEG block copolymer solubilised PTX up to 37 % (w/w) to form polymeric micelles with a size of 100 nm, which were stable for months without leakage and subsequent precipitation of PTX¹⁰⁰.

2 Polymeric particles with controlled instability

The ideal polymeric micellar system stably retains the entrapped drug in the bloodstream and releases the drug, preferably in a relatively short time, only after reaching the site of action. To balance these conflicting requirements (retention and release, see Figure 2), polymeric micellar systems have been developed from which the release of the entrapped drugs occurs in a controlled way. One sophisticated and rational approach is the conversion of the core-forming segment of polymeric micelles from hydrophobic to a more hydrophilic state under physiological or pathological conditions or by an external stimulus, which causes the hydration of the amphiphilic block copolymer and eventual dissolution of the micelles (Figure 3). Ideally, the drug is stably associated with the hydrophobic core and the release of the drug is expected to occur concurrently with the destabilisation of the micelles. Such change in polarity can be induced by cleaving covalent bonds, which is generally irreversible (section 2.3). On the other hand, a reversible change in polarity can be the result of changes of the hydrogen bonding capability (temperature sensitive polymers, section 2.1), by protonation (pH-sensitive polymers, section 2.2), or by changes of the redox potential (section 2.4). Another strategy concerns the use of light as an external stimulus, which can induce both reversible and irreversible changes in the polarity of block copolymers (section 2.5).

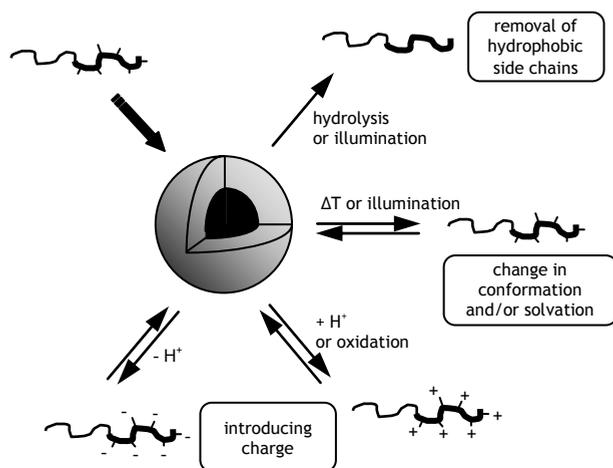


Figure 3 Examples of mechanisms to increase the hydrophilicity of the core forming block to destabilise polymeric micelles

2.1 Thermosensitive polymeric nanoparticles

Thermosensitive polymers are presently under investigation for biomedical and pharmaceutical applications^{38, 92, 107, 119}. Typically, an aqueous solution of a thermosensitive polymer is characterised by a so-called lower critical solution temperature (LCST) or cloud point (CP). Below this CP, water is bound to the polymer and prevents intra- and interpolymer interactions thus rendering the polymer water-soluble. Once the polymer solution is heated above the CP, the hydrogen bonds between the water molecules and the polymer chain are disrupted and water is expelled from the polymer chains. Interactions between the hydrophobic moieties of the polymer chain can now take place, which is associated with the collapse of the polymer and finally results in phase separation and aggregation/precipitation of the polymer. Poly(*N*-isopropylacrylamide) (pNIPAAm), which has a reversible and sharp phase transition around 32 °C in water, has been most extensively investigated among a variety of thermosensitive polymers^{90, 257, 290}. Since the CP of pNIPAAm in water is slightly below body temperature, it is very attractive for pharmaceutical use and currently widely applied for the design of thermosensitive drug delivery systems such as hydrogels¹⁰⁸, nanoparticles^{228, 229}, films¹³² and surface-modified liposomes^{143, 183}. The CP of a thermosensitive polymer can be modulated by copolymerising with hydrophobic or hydrophilic comonomers. Hydrophobic comonomers decrease the CP whereas hydrophilic comonomers have an opposite effect^{60, 297}. This makes it possible to design polymers with their CP around body temperature.

pNIPAAm (and its random copolymers with other monomers) can be used either as a hydrophilic segment or as a hydrophobic segment of polymeric micelles (Figure 4). In the former case, pNIPAAm functions as the hydrophilic outer shell of micelles below the CP. Okano *et al* prepared DOX-loaded polymeric micelles of poly(butyl methacrylate)-*b*-pNIPAAm (PBMA-*b*-pNIPAAm) and PSt-*b*-pNIPAAm, which showed a core-shell micellar structure below the CP of pNIPAAm³⁹. Upon heating above the CP, a rapid release of DOX from the PBMA-*b*-pNIPAAm micelles was observed as a result of the structural distortion of the relatively flexible PBMA core (T_g of PBMA is 20 °C) due to the collapse of the pNIPAAm shell. In contrast, PSt-*b*-pNIPAAm micelles did not show any enhanced DOX release after increasing the temperature above the CP because the rigid PSt core (T_g of PSt is 100 °C) was insensitive for the collapse of pNIPAAm (Figure 5). Since pNIPAAm is in its precipitated form at body temperature, this system is not suitable for *in vivo* application without

modification. By copolymerising NIPAAm with the hydrophilic dimethylacrylamide (DMAAm), the resulting random copolymer (poly(NIPAAm-co-DMAAm)) showed a CP of 40 °C, which is slightly above body temperature¹³⁹. The release of DOX from PLA-*b*-poly(NIPAAm-co-DMAAm) micelles was very slow at 37 °C, while an increase in DOX release rate was observed at 42.5 °C, suggesting that this system might be appropriate for hyperthermia treatment.

Recently, hollow pNIPAAm nanospheres were prepared by crosslinking polymerisation of pNIPAAm at the surface of poly(ϵ -caprolactone) (PCL) nanoparticle templates. The PCL core was then enzymatically degraded by lipase, leaving hollow spheres that exhibited reversible swelling and de-swelling upon changing the temperature of the medium around the CP. These nanospheres are potentially suitable to release hydrophilic biomacromolecules and drugs in a controlled manner³⁶⁴.

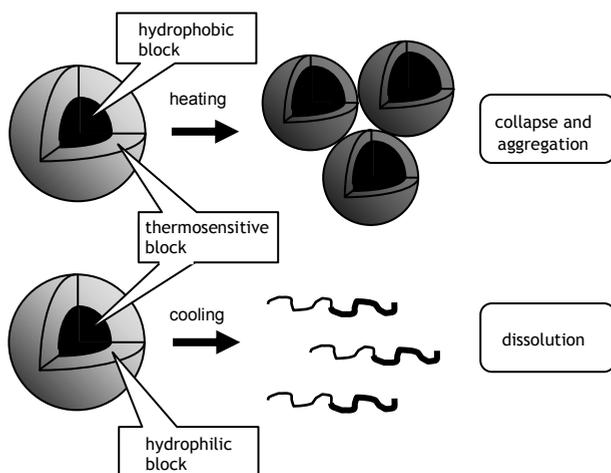


Figure 4 Different ways to use thermosensitive block copolymers with LCST behaviour to accomplish distortion of the micellar core for drug release purposes. Block copolymer micelles contain either thermosensitive blocks as the hydrophilic shell below the LCST (top) or as the hydrophobic core above the LCST (bottom). Heating or cooling will lead to micellar collapse or dissolution, respectively and concomitantly drug release in both cases.

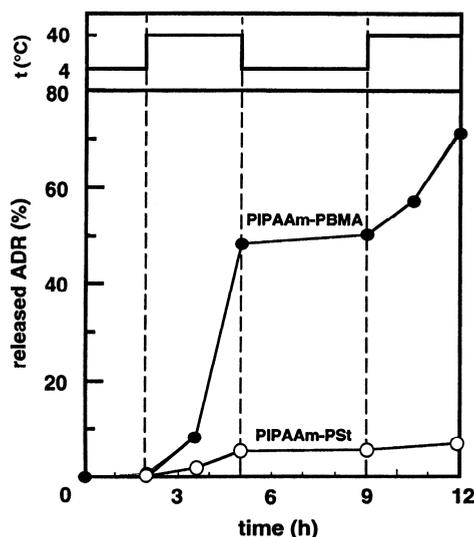


Figure 5 Adriamycin (ADR) release from PSt-*b*-pNIPAAm (open circles) and PBMA-*b*-pNIPAAm (closed circles) micelles in response to temperature switching between 4 and 40 °C ³⁹

Using pNIPAAm as the hydrophobic segment of polymeric micelles requires coupling of pNIPAAm to a hydrophilic polymer e.g. PEG (Figure 4) ³¹⁸. The pNIPAAm-*b*-PEG block copolymer is hydrophilic and soluble in aqueous solution below the CP of pNIPAAm, but above this temperature it forms polymeric micelles with a collapsed pNIPAAm core and a PEG outer shell. The temperature at which micelles are formed is called the critical micelle temperature (CMT). The advantage of pNIPAAm-*b*-PEG system is that polymeric micelles can be simply prepared by heating an aqueous polymer solution of sufficient concentration (above the CMC) to above the CP of the pNIPAAm block. Thus, in contrast to other methods to prepare polymeric micelles, pNIPAAm-*b*-PEG micelles can be prepared without using organic solvents. The heating rate is a critical parameter for the size of pNIPAAm-*b*-PEG micelle. A fast heating rate resulted in micelles with a smaller size than when a slow heating rate was applied ^{265, 367}. The formation of thermosensitive micelles upon heating is a competitive process between intrapolymer coil-to-globule transition (collapse) of thermosensitive segments and interpolymer association (aggregation) of polymers. A higher heating rate causes a rapid dehydration of the thermosensitive segments and therefore the subsequent collapse of these segments precedes the aggregation between polymers. As a result, micelles

with a well-defined core-shell structure are formed. Our group further found that an extremely rapid pass through the CMT (“rapid heating procedure”) leads to the formation of pNIPAAm-*b*-PEG micelles with a size around 50 nm²⁴⁰.

A major disadvantage of pNIPAAm-based systems is that thermal treatment (hyperthermia or hypothermia) is required for the controlled destabilisation of the micelles and concurrent drug release, which is not always feasible in clinical practice. However, local heating can also be applied by secondary external triggers, such as light. Sershen *et al* developed a photothermally modulated hydrogel using thermally responsive NIPAAm-*co*-acrylamide polymers in combination with photoactive gold nanoshells. The nanoshells strongly absorbed near IR-irradiation (1064 nm) and converted it to heat resulting in collapse of the hydrogel. Sequential laser irradiation led to a controlled pulsatile release of methylene blue and proteins of varying molecular weights²⁹².

For other drug delivery purposes, thermosensitivity has frequently been combined with other stimuli-responsive behaviour of polymers, such as pH-sensitivity, light-sensitivity and degradability. Various examples are included in the following sections.

2.2 pH-sensitive polymeric nanoparticles

The mildly acidic pH encountered in tumour and inflammatory tissues (pH ~ 6.8) as well as in the endosomal and lysosomal compartments of cells (pH ~ 5-6), provides a potential trigger for the release of systemically administered drugs from a pH-sensitive carrier, since blood and normal tissues have a pH of 7.4^{58, 81}. On the other hand, drug delivery systems that are stable at low pH (i.e. within the stomach) and degrade near physiological pH (i.e. at the intestines) are interesting for controlled release upon oral administration. Therefore, we consider here two types of block copolymers (Figure 3): (i) those with basic core monomeric units (e.g. amines) which are uncharged and thus hydrophobic at high pH and become hydrophilic upon protonation at low pH and (ii) those with acidic core units (e.g. carboxylic acids) which are uncharged when protonated at low pH and become negatively charged at relatively high pH. In general, polymers of type (i) can be used for intratumoural or intraendosomal release, while polymers of type (ii) are interesting for intestinal release. Other types of pH-controlled release from polymer micelles, including pH-sensitive polymer-drug conjugates^{75, 329} and polyion complex micelles^{27, 56, 264} are not discussed in this review.

Relatively many examples of “protonation” approaches to trigger destabilisation of micelles have been reported and include block copolymers which contain L-histidine^{169, 171}, pyridine²¹¹ and tertiary amine groups^{166, 313} in their hydrophobic segments. Polymeric micelles are formed from such block copolymers at a pH above the pK_a of the protonatable group, where the hydrophobic segment essentially is uncharged. As the pH decreases below the pK_a , the ionisation of the polymer causes increased hydrophilicity and electrostatic repulsions of the polymers, leading to the destabilisation of the micelles. The control of the transition pH is possible by a combination of different block copolymers. It has been shown that poly(L-histidine)-*b*-PEG (pHis-*b*-PEG) micelles destabilised at physiological pH (7.4)¹⁷¹, whereas mixed polymeric micelles consisting of pHis-*b*-PEG and PLA-*b*-PEG showed improved micellar stability at pH 7.4 and dissociated at pH 6.0 to 7.2, depending on the blended amount of PLA-*b*-PEG¹⁶⁹.

Poly(2-vinylpyridine-*b*-ethylene glycol) (p2VP-*b*-PEG) formed vesicles under neutral and alkaline conditions. Upon lowering the pH value below 5, the p2VP block was protonated and the vesicles dissolved. The vesicles showed rapid release of a fluorescent dye loaded in the aqueous core when the vesicles were exposed to pH 4²⁵. Polypeptide-based vesicles composed of poly(*N*-2-(2-(2-methoxyethoxy)ethoxy)acetyl-L-lysine) as the hydrophilic block and poly(L-leucine-*co*-L-lysine) as the pH-sensitive block also formed vesicles with similar pH response. At pH 10.6, the pH-sensitive block adopted a hydrophobic α -helical conformation, while protonation of the lysine residues at pH 3.0 caused a helix-to-coil conformation and destabilisation of the vesicle structure with instantaneous release of an encapsulated dye²¹.

Another interesting approach uses a combination of a block copolymer and a drug both having similar pK_a ^{72, 190}. Both the polymer and the drug are protonated and soluble at $pH < pK_a$ and they self-assemble coincidentally to form the drug-loaded micelles when the solution is neutralised. This simple drug loading procedure avoids the use of non-aqueous (co)solvents when preparing the micelles. Furthermore, acid-triggered release was shown for tamoxifen and paclitaxel-loaded micelles (Figure 6)¹⁹⁰.

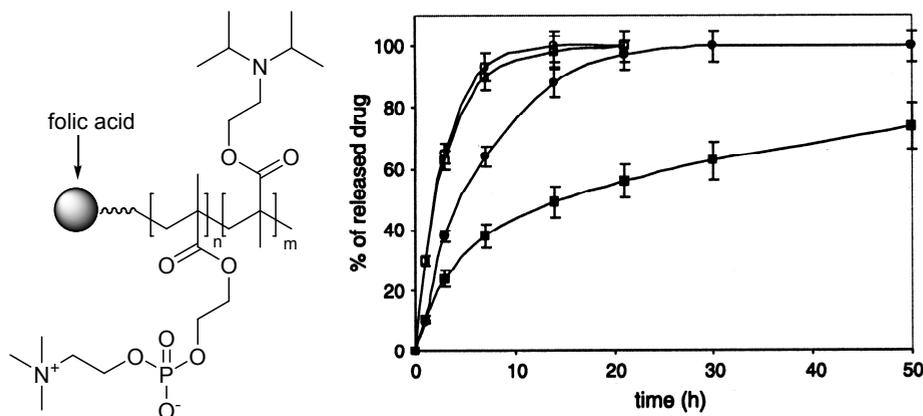


Figure 6 *In vitro* tamoxifen (circles) and paclitaxel (squares) release profiles (right) from folate conjugated poly(2-methacryloyloxyethyl phosphorylcholine)-*b*-poly(2-(diisopropylamino)ethyl methacrylate) (left) micelles at pH 7.4 (closed symbols) and pH 5.0 (open symbols), at 37 °C ¹⁹⁰

Parameter	FNB micelles	Lipidil Micro®	FNB suspension
C_{\max} ($\mu\text{g}/\text{mL}$)	$11.79 \pm 1.17^*$	$9.41 \pm 0.54^*$	3.99 ± 0.34
T_{\max} (h)	$1.25 \pm 0.33^{**}$	2.0 ± 0.45	1.83 ± 0.4
$t_{1/2}$ (h)	5.49 ± 0.33	5.54 ± 0.38	5.49 ± 0.34
$AUC_{0-24\text{ h}}$ ($\mu\text{g h}/\text{mL}$)	$70.08 \pm 4.95^*$	$61.08 \pm 4.51^*$	27.37 ± 2.80

* $P < 0.05$ vs. FNB suspension

** $P < 0.05$ vs. Lipidil Micro®

Table 1 Pharmacokinetic parameters after oral administration of fenofibrate (FNB)-loaded pH-sensitive micelles, Lipidil Micro® and fenofibrate control suspension to rats (mean \pm SEM, $n = 6$) ²⁸⁶

Two types of block copolymer micelles with an opposite pH response, i.e. which release drugs at relatively high pH, have been developed by Leroux *et al* for oral administration ^{116, 286}. Diblock copolymers of PEG as the hydrophilic block and poly(alkyl acrylate-*co*-methacrylic acid) as the pH-sensitive core block form micelles and aggregates of micelles of ca. 120 - 350 nm, depending on the polymer composition, at pH < 4.5. Enhanced release of progesterone from the micelles was observed when raising the pH from 1.2 to 7.2 ²⁸⁵. Furthermore, fenofibrate-loaded micelles showed improved pharmacokinetics upon oral administration when compared to Lipidil Micro®

suspensions and free fenofibrate (Table 1) ²⁸⁶. Star block copolymers of poly(ethyl acrylate-co-methacrylic acid) and poly(ethylene glycol) methacrylate have been investigated for this purpose as well ¹¹⁶. These so-called 'unimolecular micelles' showed similar in vitro pH-dependent release profiles for progesterone as for the diblock copolymers mentioned above.

The pH-sensitive micelles mentioned above release their contents upon dissociation of the micelles. Another type of micelles was designed to release their contents upon collapse/aggregation of the particles. The change in the micellar morphology when changing the pH is then responsible for the release. For example, Leroux *et al* prepared random copolymers of *N*-isopropylacrylamide (NIPAAm) and methacrylic acid (MAAc, typically 3 - 5 mol %), substituted with alkyl chains either at the terminal chain ends or randomly distributed over the copolymer chain (by means of 2 - 4 mol % octadecyl acrylate as a comonomer) to induce micelle formation ^{56, 183, 309}. These micelles were loaded with AlClPc, a photosensitiser used for the photodynamic treatment of cancer. It was shown that the presence of 5 mol % MAAc in the copolymers caused the micelles to precipitate and the hydrophobic core to distort upon neutralisation of the MAAc as the pH decreased below 5.7-5.8 at 37 °C ³⁰⁹. This phenomenon was supposed to cause the release of the entrapped photosensitiser and change the intracellular localisation of the drug in a favourable way to become more photoactive. Indeed, the endosomal decrease in pH played a role as observed by the enhanced photoactivity ³⁰⁸. In the presence of chloroquine, a weak base that is known to raise the internal pH of acidic organelles, the activity of the drug-loaded in these pH-sensitive micelles decreased whereas it remained unchanged in the case of a Cremophor EL formulation.

A similar concept was used by others, e.g. Soppimath *et al* synthesised thermosensitive copolymers of NIPAAm, *N,N*-dimethylacrylamide and 10-undecenoic acid ³⁰³. These polymers self-assembled to form nanoparticles in aqueous solution, which collapsed and aggregated upon heating above the CP. The polymer composition was adjusted such that the polymer was below its CP at pH 7.4 and the nanoparticles collapsed upon protonation at pH 6.6 as a result of the decrease in CP. This phase transition was accompanied with the release of encapsulated doxorubicin. Na and Bae studied nanoparticles composed of sulphonamide-derivatised pullulan acetate ^{231, 232}. These nanoparticles collapsed upon protonation of the weakly acidic sulphonamide units ($pK_a = 6.1$), resulting in a concomitant release of doxorubicin at pH < 7.

2.3 Polarity change by side chain hydrolysis

Backbone degradation of amphiphilic block copolymers, such as in PEG-*b*-polyester block copolymers, is the most generally applied method to destroy micelles used for drug delivery. However, this does not result in a dramatic change in polarity of the core-forming block within a short time period and will therefore not be included in this review. Interested readers are referred to other comprehensive reviews covering this subject^{148, 191}. This section focuses on polymers that display increasing hydrophilicity in a relatively short time due to the (irreversible) cleavage of side chains present in the hydrophobic segments. Such degradation mechanism may broaden the spectrum of accessible release profiles as compared to the backbone-degradable polymers alone.

2.3.1 Biodegradable thermosensitive (block co)polymers

An early example of a polymer that displays degradation controlled increase of hydrophilicity was described by Couvreur *et al*¹⁸⁴. They prepared nanoparticles of poly(methylidene malonate). Incubation of these nanoparticles at basic pH released ethanol from the nanoparticles due to the hydrolysis of the ester bond in the side chains. The resulting polymers were more hydrophilic than the starting polymers owing to the presence of free carboxyl groups, leading to the eventual solubilisation of the nanoparticles.

Later, a similar biodegradation approach was applied to thermosensitive polymers. Copolymers of NIPAAm with monomers containing hydrolytically sensitive side chains, including acryloxy succinimide²⁹⁵, (2-hydroxyethyl)methacrylate lactate (HEMA-Lac_n)^{167, 238} and *N*-(2-hydroxypropyl)-methacrylamide lactate (HPMAm-Lac_n) (where *n* is the number of lactic acid units per side chain)²⁴¹ have been prepared (Figure 7). Side chain hydrolysis of these thermosensitive copolymers gave rise to hydrophilisation of the whole polymer and consequently increased the CP. Next, when the CP of the polymer increases from below to above the temperature of the medium, the copolymer converts from the collapsed to the dissolved state in time. For example, when 35 mol % HPMAm-Lac₃ was copolymerised with NIPAAm, the CP of poly(NIPAAm-co-HPMAm-Lac₃) was around 15 °C in phosphate buffered saline and thus below body temperature. The ester bond of HPMAm-Lac_n monomer is hydrolytically sensitive and, importantly, the resulting HPMAm is a more hydrophilic monomer than NIPAAm. Indeed, the CP of the hydrolysed polymer, poly(NIPAAm-co-

HPMAm) was 45 °C. As a result, the polymer gradually dissolved upon side chain hydrolysis at body temperature ²⁴¹.

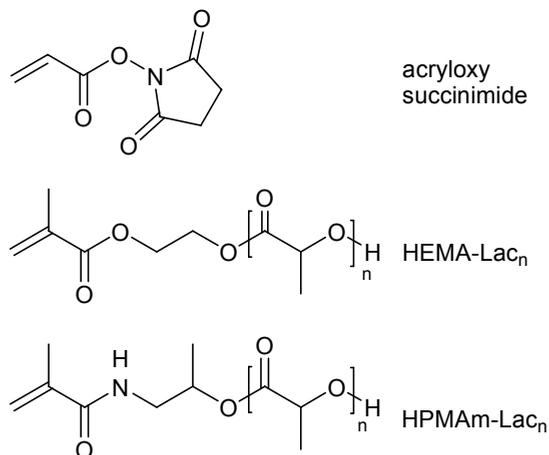


Figure 7 Structural formulas of monomers used for the preparation of hydrolytically and temperature sensitive polymers

Recently, we found that also p(HPMAm-Lac_n) itself, i.e. without NIPAAm, showed very nice thermosensitive behaviour ³⁰⁰. Interestingly, the CP of this new class of polymers can be fine-tuned by the number and length of the lactate side chains. For example, the CP of a copolymer composed of HPMAm-Lac₁ and HPMAm-Lac₂ varies between 13 °C and 65 °C in water, depending on the ratio of the two monomers present in the copolymer. Since poly(HPMAm-Lac₂), having a CP of 13 °C, is converted in time into the more hydrophilic and biocompatible poly(HPMAm), this polymer is supposed to be highly suitable for “hydrophobic-to-hydrophilic” conversion at body temperature. As described above, “hydrophobic-to-hydrophilic” conversion of the core of polymeric micelles is an interesting strategy to destabilise polymeric micelles and release their payload in a controlled way. This idea led our group to develop novel thermosensitive block copolymers of PEG and poly(HPMAm-Lac_n) with or without NIPAAm as a comonomer (poly(NIPAAm-co-(HPMAm-Lac_n))-b-PEG) (Figure 8) ^{241, 301}. Owing to their unique degradation property, polymeric micelles formed with these block copolymers showed controlled instability at body temperature. In other words, the block copolymers formed polymeric micelles with a core of poly(NIPAAm-co-(HPMAm-Lac_n)), since the CP of this block is below 37 °C. Then, due to the hydrolysis of

the lactic acid side chains, the CP of the thermosensitive block increased in time and when the CP came above body temperature, the polymers became soluble in water and dissolution of the micelles occurred. It was shown that the dissolution time of empty micelles of poly(HPMAm-Lac₂)-*b*-PEG was approximately 1 week at physiological conditions (37 °C, pH 7.4)²⁹⁹. This may be too long for drug delivery *in vivo* and therefore we designed similar block copolymer micelles with a much shorter destabilisation time based on poly(*N*-(2-hydroxyethyl)methacrylamide-oligolactates) (poly(HEMAm-Lac_n)-*b*-PEG)²⁷⁶. The characteristics of these micelles were comparable with the poly(HPMAm-Lac₂)-*b*-PEG, i.e. the critical micelle temperature was 6 °C and the average particle size was 80 nm. The hydrolysis of HEMAm-Lac_n was however much faster than HPMAm-Lac_n, which resulted in complete micelle disintegration within only eight hours at pH 7.4 and 37 °C.

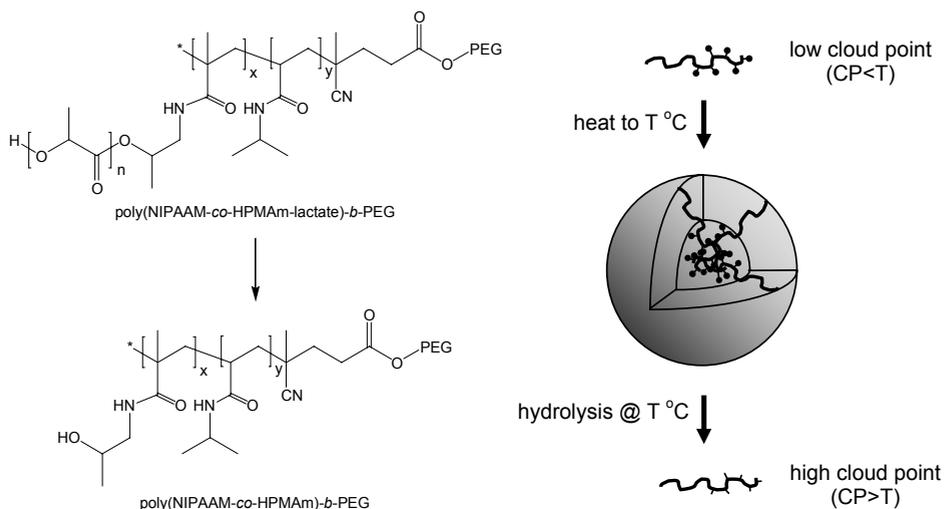


Figure 8 Hydrolysis of poly(NIPAAm-co-(HPMAm-lactate))-*b*-PEG (left) causes an increase of the cloud point of the block copolymer by removal of the hydrophobic lactic acid groups (black dots in the schematic picture on the right). Thus, micelles formed above the cloud point of poly(NIPAAm-co-(HPMAm-lactate))-*b*-PEG are destabilised when the increasing cloud point passes the incubation temperature²⁴¹

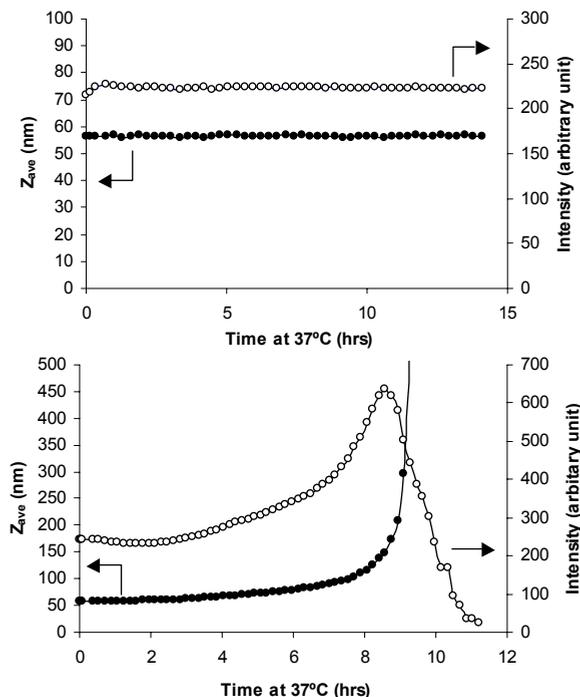


Figure 9 Z_{Ave} (size, closed symbols) and scattering intensity (open symbols) of paclitaxel-loaded poly(HPMAm-Lac₂)-*b*-PEG micelles at 37 °C and at pH 5 (left) and pH 8.8 (right). Increase and subsequent decrease in scattering intensity at elevated pH is due to aggregation and precipitation of paclitaxel, respectively ²⁹⁹

The poly(HPMAm-Lac₂)-*b*-PEG based micelles were loaded with paclitaxel (PTX), thereby taking advantage of the thermosensitivity of poly(HPMAm-Lac_n). The loading method was very straightforward: heating an ice-cold polymer-drug mixture till above the CMT resulted in micelles of 60 nm with a maximum loading capacity of 22 % (w/w). Incubation of the drug-loaded micelles showed a stable incorporation of PTX in the micelles at non-degrading conditions (i.e. pH 5), whereas precipitation of PTX occurred at elevated pH coincident with the time point at which the dissolution of the micelles started (Figure 9) ²⁹⁹. *In vitro* cytotoxicity studies showed that PTX-loaded micelles were comparably toxic with respect to the commercial paclitaxel delivery vehicle (Taxol®). The advantage was however that the empty micelles were far less toxic than the Cremophor EL vehicle used in Taxol.

2.3.2 Acid-labile biodegradable (block co)polymers

As already mentioned (section 2.2), acid-sensitive DDS are interesting because of the mildly acidic pH encountered in tumour and inflammatory tissues as well as in the endosomal and lysosomal compartments of cells. Frechet *et al* recently reported on smart block copolymers of PEG and poly(aspartic acid) functionalised with trimethoxybenzylidene acetals as acid-labile linkages ⁷³. Cyclic benzylidene acetals increased the hydrophobicity of the core by stacking of the aromatic rings and masked the polarity of the diol by the acetal groups. These micelles were quite stable at physiological pH, but once the acidity of the solution was lowered to pH 5, hydrolysis of acetal bonds occurred. The generation of diols increased the hydrophilicity of the polymers and dissolution of the micelles occurred, followed by the release of a hydrophobic dye. The same group also synthesised linear-dendritic block copolymers of PEG and a polylysine or a polyester dendron, to whose periphery cyclic benzylidene acetals were attached (Figure 10) ^{74, 77}.

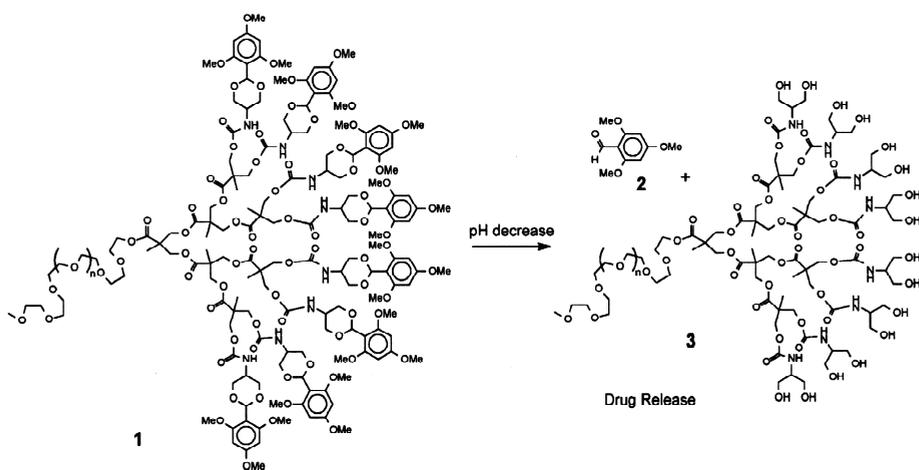


Figure 10 Structure of a linear-dendritic block copolymer of a PEG and a third generation polyester dendrimer with cyclic acetals of 2,4,6-trimethoxybenzaldehyde attached to the periphery (1). Hydrolysis of the cyclic acetals on the dendrimer periphery releases 2,4,6-trimethoxybenzaldehyde (2) and reveals polar 1,3-diol moieties on the dendrimer periphery to provide (3) ⁷⁴

The dendritic block copolymers formed polymeric micelles with a size of 20 to 50 nm ⁷⁷ and an accelerated release of entrapped doxorubicin (DOX) at

2.4 Oxidation- & reduction-sensitive polymeric micelles or vesicles

Hubbell *et al* synthesised amphiphilic A-B-A block copolymers consisting of the hydrophobic poly(propylene sulphide) and PEG (PEG-*b*-PPS-*b*-PEG), which formed polymeric vesicles in water²³⁷. Upon exposure to oxidative agents, the thioethers in the PPS block are oxidised to poly(propylene sulfoxide) and eventually to poly(propylene sulphone), leading to the hydrophilisation of the originally hydrophobic block (Figure 12). Accordingly, the vesicles destabilised upon incubation with H₂O₂, as evidenced by Cryo-TEM. The oxidative conversion was also achieved by encapsulating glucose oxidase (GOx) into these vesicles²³⁶. When the GOx-containing polymersomes were incubated in 0.1 M glucose solution, glucose which diffused across the vesicular membrane into their inner spaces was oxidised by GOx to produce H₂O₂, leading to the dissolution of the GOx-encapsulated polymeric vesicles.

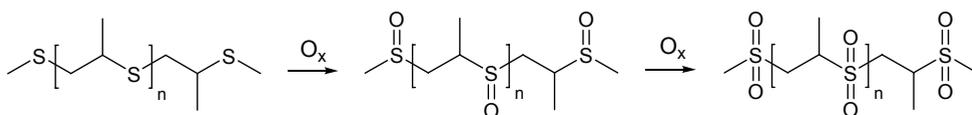


Figure 12 Reaction scheme of the oxidation reaction for poly(propylene sulphide) to poly(propylene sulfoxide) and to poly(propylene sulphone)²³⁷

Another option is to take advantage of the redox tuneability of metal-containing compounds. One of the first redox-active copolymers was synthesised by Laschewsky *et al* and contained viologen and ferrocene. These compounds underwent reversible redox reactions in water, either chemically or electrochemically induced. This altered the number of charges on the polymers which drastically changed their solubility; i.e. the hydrophilic-hydrophobic balance was adjusted by redox processes¹⁰. Redox-active micelles were built from amphiphilic block copolymers bearing a hydrophobic ferrocenylalkyl moiety (FPEG). Oxidation of the ferrocenyl moiety (to FPEG⁺) changed the hydrophobic/lipophilic balance and the micelles were disrupted into the (water-soluble) unimers. Takeoka *et al* investigated these micelles with perylene as a model hydrophobic drug for an electrochemical controlled drug release. The release of perylene from these redox-active FPEG micelles was precisely controlled by applying selective and electrochemical oxidation of FPEG by which initially zero-order kinetics could be realised³¹⁰.

Electrochemically triggered destabilisation was not only examined for micelles but is also reported for vesicles. A block copolymer with a hydrophilic polyferrocenylsilane (PFS) block and a hydrophobic polydimethylsiloxane (PDMS) self-assembled into redox-active vesicles (~ 85 nm) with the metal-containing block on the outside. Reversible oxidation of the ferrocene units was observed and indicated that these vesicular assemblies can be applied as intelligent switching material ²⁶³.

Application of redox-sensitive polymeric micelles may be found in the selective release of drugs by externally applied electric current. On the other hand, inflamed tissues and certain tumours appear to be populated with activated macrophages that release oxygen-reactive species, which may also trigger the transformation of redox-sensitive micelles thereby enabling selective release at pathogenic sites. This possibility has not been investigated so far *in vivo* to the best of our knowledge.

Another type of reduction-sensitive material is the class of polymeric assemblies containing disulphide bonds. Intracellular glutathione will reductively cleave these links which lead to system destabilisation ^{210, 283}. However, this is not really destabilisation by hydrophilisation and will therefore not be further discussed in this review.

2.5 Light-induced polarity change

The attractive feature of stable but light-responsive polymeric particles is that the release of the entrapped carrier guest can be induced at the specific time and the location of light-exposure. Either ultraviolet (UV), visible (VIS) or (near-) infrared ((N) IR) light can be applied as the trigger. However, the use of NIR is of particular interest for biomedical application due to its deeper tissue penetration and minimal detrimental effects to healthy cells.

2.5.1 Light-induced irreversible polarity changes

In general, this concept comprises the application of light to photosensitive micelles to cleave side chains which concomitantly induces micellar destabilisation. As micellar building block, an amphiphilic block copolymer was reported composed of PEG and a polymethacrylate bearing a photolabile pyrene chromophore (PPy) moiety in the side chain as the hydrophobic core-forming domain ¹¹³. Upon UV-irradiation, the pyrenyl methyl esters were cleaved and the hydrophobic micellar block was transformed into a hydrophilic poly(methacrylic acid) (PMA) block, which brought about

dissociation of the micelles. The micelle disrupting process reached completion after approximately 5 minutes but displayed dependency on the intensity of the UV-light. The hydrophobic PPy block in the core encapsulated 6 % Nile Red, which was rapidly released after UV-exposure and disintegration of the loaded micelles ¹¹³.

The same authors developed another dye-containing block copolymer, i.e. a PEG-*b*-poly(2-nitrobenzyl methacrylate) to be used for photocontrolled delivery applications. Cleavage of 2-nitrobenzyl moieties occurred either via one-photon UV (365 nm) or two-photon NIR (700 nm) excitation. This light-induced disruption of the micelles appeared to be independent of the hydrophobic block length; the underlying mechanism is photolysis which is an intramolecular process. The formation of carboxylic acid upon irradiation shifted the hydrophilic/hydrophobic balance and resulted in full dissociation of the micelles after approximately 5 minutes of UV-irradiation; NIR-irradiation required much longer time. In absence of irradiation, encapsulated Nile Red was stably retained in the micellar core; the kinetics of the photoinduced release of Nile Red was again controlled by the intensity of the light. In a second approach, the carboxylic acid groups that were formed in the core of the micelles after (partial) photolysis were crosslinked with a diamine. Upon re-irradiation with UV-light, the photolysis-induced micellar destabilisation was prevented by the crosslinks. Nevertheless, the overall hydrophilicity of the polymer increased and the micelle swelled, thereby still able to release encapsulated hydrophobic guests although at a lowered rate ¹¹².

Initially, Goodwin *et al* were the first to use IR-light to release Nile Red from micelles of 7 nm diameter consisting of a short PEG chain connected to hydrophobic 2-diazo-1,2-naphthoquinone (DNQ) via an alkyl spacer ⁷⁹. Irradiation of these DNQ derivatives with either UV (350 nm) or IR (795 nm) laser light led to a so-called Wolff rearrangement. The polarity of the chromophore containing block increased drastically (Figure 13) thereby eliminating the driving forces to keep the micelles together. After IR-irradiation for 30 minutes most encapsulated Nile Red had been released due to disintegration of the IR-light-sensitive micelle. The destabilisation of these micelles takes place at wavelengths that are potentially useful for the photoactivation of drug carriers within a living system.

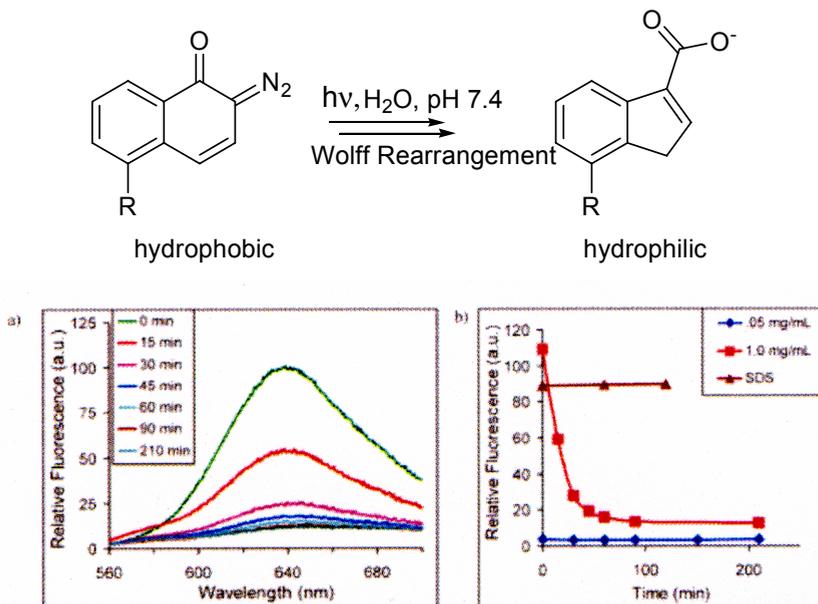


Figure 13 Top: Solubility change of 2-diazo-1,2-naphthoquinone (DNQ) derivatives after Wolff rearrangement to 3-indenecarboxylate in buffered water upon illumination. Bottom: a) Release of Nile Red from DNQ micelles as observed from the decreasing fluorescence during illumination of the micelles (squares in Figure b). Controls are a surfactant concentration below the CMC (diamonds) and micelles that are insensitive to IR-light (SDS, triangles) ⁷⁹

2.5.2 Light-induced reversible polarity changes

The ability to destabilise aggregates in response to environmental light can, besides by the above described irreversible ways, also be achieved via non-destructive mechanisms. The exposure of photoactive groups to light can generate reversible structural changes, thereby changing the hydrophilic - hydrophobic balance without the addition of further reagents. Units that display photochemical induced transitions include azobenzenes (change in dipole moment), cinnamoyl (isomerisation into more hydrophilic residue or photodimerisation), triphenylmethane leucohydroxide (generation of charges) and spirobenzopyran (formation of zwitterionic species).

2.5.2.1 Azobenzene-derivatives

Key to the application of azobenzenes is the readily induced and reversible isomerisation of the azo-bond between the *trans* (E) and *cis* (Z)

^{84, 202}. Li *et al* prepared nanospheres of an amphiphilic azobenzene-containing random copolymer in THF - water mixtures. At low water content, UV-illumination disrupted the spheres. When the water content in the dispersion medium was high (> 80 %), the colloidal structures were “frozen” through the polymeric chain collapse and entanglement and the *trans*-to-*cis* photoisomerisation could no longer cause full disintegration ¹⁸⁷.

Thermosensitive polymers were modified with azobenzenes and they displayed a photo-responsive change in CP. Upon illumination, the higher polarity of the *cis*-isomer increased the CP due to increased hydrogen bonding capacity ³⁰⁵. Additionally, pH sensitivity was added to the polymer composed of NIPAAm, partially grafted with an azobenzene derivative ⁵⁰. Other thermo-, pH and photosensitive amphiphilic block copolymers based on poly(2-(dimethylamino)ethylmethacrylate)-*b*-poly(6-(4-phenylazo)phenoxy)hexylmethacrylate) (pDMAEMA-*b*-pPPHM) self-assembled into micelles in aqueous solutions. The equilibrium surface tension of the micellar solution was slightly decreased upon illumination, but the observed CMC values were about the same for the *cis* and *trans* isomers. Apparently, the hydrophilic effect induced by the *cis* conformation was not able to dominate the hydrophobic aggregation effects in this case ²⁷⁰.

2.5.2.2 Cinnamoyl derivatives

Irradiation of cinnamate with UV-light of ~ 280 nm, like all derivatives of unsaturated carboxylic acids, undergoes either *trans*-to-*cis* photoisomerisation thereby generating cinnamate residues with an increased hydrophilicity or leads to reversible photodimerisation (Figure 15). Since either the *cis* or *trans* isomers are thermally stable, proper selection of the wavelengths and appropriate conditions enables a selective photosensitive *trans*-to-*cis* switching. Partial modification of the non-ionic poly(HPMAm) by cinnamate (9 mol %), produced intelligent polymers that responded to temperature and light. Exposure of the polymer in aqueous solution to UV-light resulted in an increase of the CP by 6 °C due to the above mentioned *trans*-to-*cis* isomerisation. The increase in CP depended on the polymer concentration, the amount of cinnamate moieties per polymer chain and the extent of isomerisation ¹⁵⁶. A high content of cinnamate (21 %) was supposed to maximise the polarity change (with further increase of the CP) but led to photocrosslinking, indicating the delicate balance between isomerisation and dimerisation upon UV-illumination. In other studies, photodimerisation of cinnamoyl units was purposely used to prepare crosslinked polymeric micelles

that responded to pH, temperature and ionic strength³⁰⁷, or to generate microcapsules with a photocrosslinked shell³⁵⁸. In the latter system, cyclodextrin was encapsulated and subsequently released upon UV-illumination as a result of photocleavage and microcapsular disintegration.

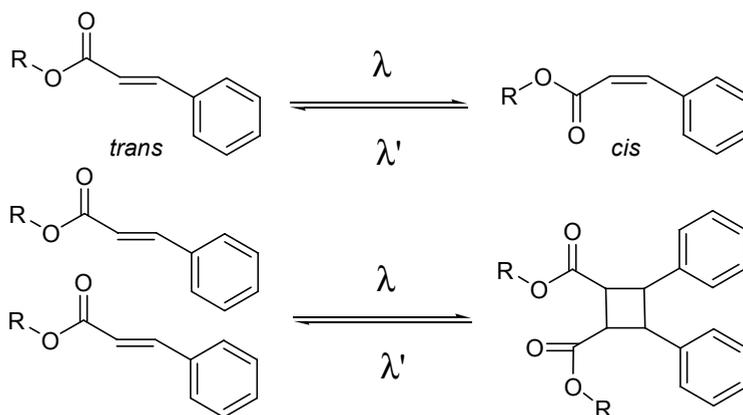


Figure 15 Reversible *trans*-to-*cis* isomerisation (top) and photodimerisation (bottom) of the cinnamoyl photoreactive group upon irradiation with UV-light

2.5.2.3 Triphenylmethane leucohydroxide

Polyelectrolyte complex capsules were built of partly crosslinked poly(acrylic acid)-poly(ethylenimine) and poly(acrylic acid-*co*-bis(4-(dimethylamino)phenyl) (4-vinylphenyl)methyl leucohydroxide). Upon UV-irradiation, the triphenylmethane derivative reversibly dissociates into an ion pair, thereby generating charges (Figure 16). The permeation of encapsulated *p*-toluenesulfonate through the membrane was thereby significantly enhanced¹⁴⁴.

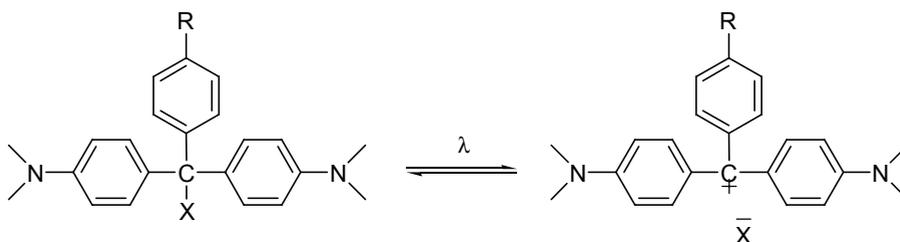


Figure 16 Dissociation of triphenylmethane leucohydroxide derivatives into an ion-pair under ultraviolet irradiation

2.5.2.4 Spirobenzopyran derivatives

Upon UV-irradiation, the neutral spirobenzopyran (SBP) undergoes reversible isomerisation into a zwitterionic merocyanine (Figure 17). Merocyanine, when attached to a polymer, provides intra- and interchain association of the polymer chains resulting from attractive electrostatic dipole-dipole interaction of the zwitterionic moieties. For example, HPMAM copolymers bearing various amounts of spirobenzopyran moieties aggregated into large clusters after UV-exposure in demineralised water due to zwitterionic formation, with a stable size (~ 400 nm) after 20 minutes illumination. Upon exposure to visible light, the reversal of the metastable zwitterionic form to the neutral form (and thus cluster disintegration) went rapidly (20 min) while the thermal recovery process was very slow¹⁴⁰. However, this photoreversible cluster formation was completely opposite in 1M NaCl. Since the neutral form of the copolymer had a low solubility at high ionic strength, clusters were formed already during the dissolution step in 1M NaCl. Subsequent UV-illumination induced disintegration of the clusters; the generated zwitterions now increased the polymers solubility and since the charges were masked by the NaCl ions, any possible electrostatic attractions were fully suppressed. Unfortunately, the authors did not discuss the possibilities to use this cluster formation with UV-induced destabilisation as a tool for controlled drug delivery¹⁴¹.

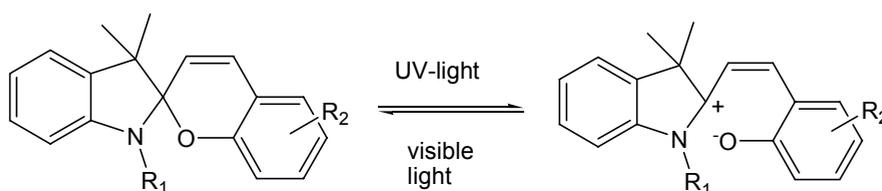


Figure 17 Reaction scheme of MSBP photoisomerisation, R_1 = methacryloyloxyethyl, R_2 = 6-Br

A photoresponsive thermosensitive copolymer was obtained when NIPAAm was copolymerised with a methacryloyl derivative of SBP (MSBP). The non-irradiated copolymer displayed its CP in water in the temperature range of 30 - 50 °C. UV-irradiation of the aqueous solution caused photoinduced transformation of MSBP units into their coloured merocyanine forms and concomitant lowering the CP of the irradiated copolymer with circa 10 degrees. During a long-term exposure to daylight (20 days) the copolymer gradually

elapsed back to its colourless spiropyran form with a higher CP. This phenomenon allows control of the CP by its exposure to light ¹⁰⁵.

2.6 Other environmental triggers

The approach to use enzymes as environmental triggers to influence polymeric properties was employed by Katayama *et al*, who synthesised a thermosensitive polymer with peptide side chains, i.e. a copolymer of NIPAAm and *N*-methacryloyl-GLRRASLG (*N*-methacryloylpeptide). The GLRRASLG peptide was phosphorylated by protein kinase A, which resulted in an increase of the CP of the copolymers from 36.7 to 40 °C due to the hydrophilisation of the peptide chains ¹³⁰. This suggests that the polymers gradually dissolve in time when incubated between those two temperatures in the presence of the enzyme. These polymers were not yet applied to build responsive micelles or vesicles.

Glucose-responsive hollow poly-electrolyte multilayer capsules (approximately 10 µm) based on poly(3-acrylamidophenylboronic acid)-*co*-poly(dimethylaminoethylacrylate) copolymers were recently prepared. The phenylboronic acid units are partially uncharged in an aqueous medium. Through complexation with glucose the equilibrium is shifted towards the charged state, thereby rendering the entire polymer more hydrophilic. Only at glucose concentrations higher than 2.5 mg/ml (which is above healthy levels), the complexation between the charged phenylborates and glucose was great enough to result in disassembly of the capsules within 5 minutes ⁴⁸.

3 Perspectives

The destabilisation of polymeric micelles or vesicles by conversion of the constituent block copolymers from an amphiphilic to a fully hydrophilic state is a valuable tool to trigger the release of encapsulated molecules. A variety of mechanisms to acquire transiently stable particles has been discussed in this review and combinations of destabilisation mechanisms can be used to further fine-tune the release properties. The role of internal or external triggers (temperature, pH, light, chemical or enzymatic degradation) is to be able to precisely tailor the time and site of drug delivery *in vivo*. Although polymeric micelles and vesicles are intrinsically more stable than those from low molecular weight surfactants, stable incorporation of drugs in those systems is frequently encountered as a major hurdle in the application of polymeric micelles and vesicles for drug delivery, especially for targeted delivery. The release of drugs at the site of action can only be accomplished if the drugs are stably encapsulated inside the nanoparticles during circulation and no premature leakage of the drug molecules takes place. This may be one of the reasons why not so many *in vivo* data have been published so far using transiently stable nanoparticles. Therefore, much effort is currently undertaken to improve the stability of drug-loaded micelles/vesicles, including the use of favourable drug-polymer interactions, as discussed in section 1.3 of this chapter and the use of crosslinked systems. The feasibility of *in vivo* application of triggered destabilisation of polymeric micelles and vesicles by changing core polarity will strongly benefit from these developments. Furthermore, more complex particles are desired whose fate can be followed *in vivo*, e.g. nanoparticles loaded with MRI contrast agents or with quantum dots. This enables optimal use of the external trigger: only once enough nanoparticles are accumulated in the targeted region, the trigger is applied. This will then in turn result in a high concentration of the free drug at its site of action and in an optimal therapeutic effect.

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chapter 3

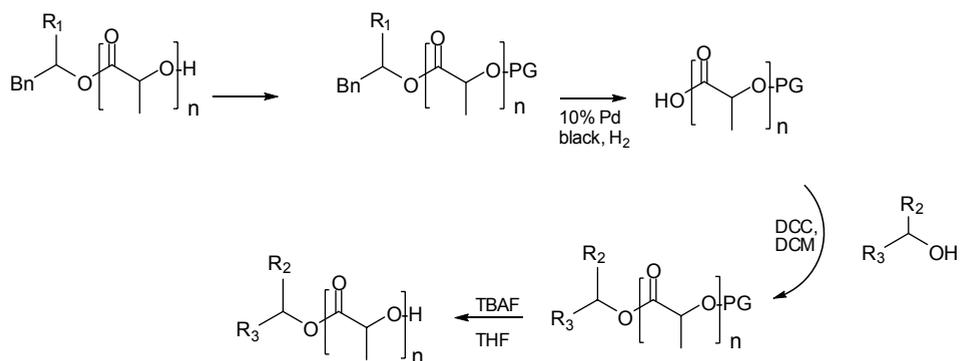
Step-by-step synthesis of monodisperse methacrylamidoalkyl- oligolactates

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Abstract

This chapter describes the facile step-by-step synthesis to graft lactic acid or lactoyl lactate onto primary as well as secondary alcohol derivatives of methacrylamides via esterification. Elongation of the chains is achieved by repeating the coupling reactions with either monolactate-PG (with PG = protecting group) or dilactate-PG. This synthesis route generates monodisperse oligolactates of predetermined lengths to be used as building blocks for biodegradable polymeric material.



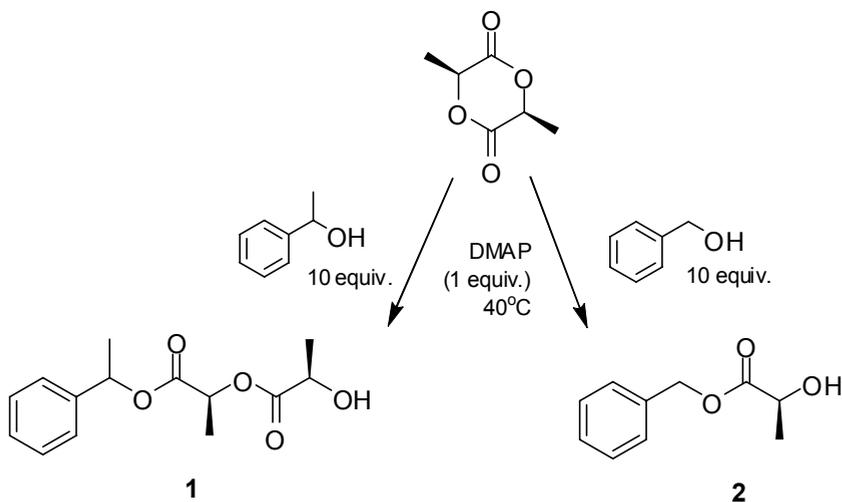
Introduction

Monodisperse oligolactate derivatives are applied as biodegradable components in hydrogels^{1, 5, 17, 18, 20}, as building blocks for nanoparticles², or to investigate the hydrolytic degradation kinetics of lactic acid chains^{13, 19}. Furthermore, polymers based on methacrylamide monomers with lactic acid side chains display thermosensitive behaviour which is transient under physiological conditions due to hydrolysis of the side chains^{8, 11, 14, 15}. The properties of these polymers (e.g. lower critical solution temperature (LCST) or degradation kinetics) depend to a large extent on the exact length of the lactic acid side chain and the type of methacrylamide. The ability to synthesise monomers with defined lactic acid side chains enables the formation of polymeric material with tailored properties.

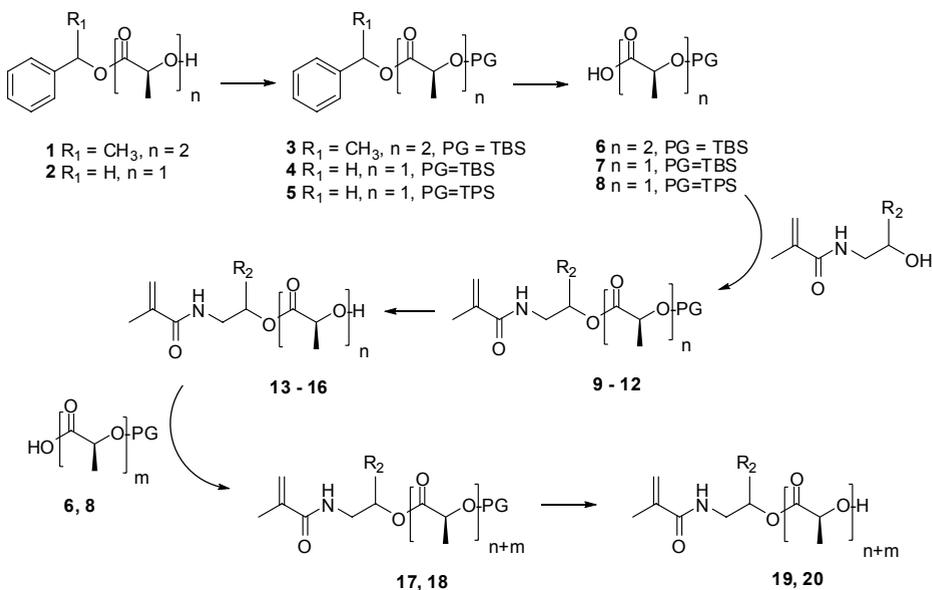
Oligolactate chains can be grafted onto a methacrylamide with an alcohol terminus (*N*-(2-hydroxypropyl)methacrylamide (HPMAm) or *N*-(2-hydroxyethyl)methacrylamide (HEMAm) in the melt using stannous 2-ethyl hexanoate (SnOct₂) as a catalyst by ring-opening polymerisation of the cyclic diester L-lactide with the alcohol^{1, 11, 14, 15, 18}. The average lactate chain length is dependent on the ratio of L-lactide versus methacrylamide. Nevertheless, due to unequal stoichiometry, transesterification reactions and statistical variation, the obtained product is polydisperse. To obtain well-defined polymeric material, separation of the monomers into the monodisperse methacrylamidoalkyl-oligolactates is required and can be achieved by preparative HPLC^{1, 11, 14}. However, this method is rather laborious, has a low capacity and is therefore not very useful for large scale preparations. Consequently, we designed another route to synthesise monodisperse methacrylamidoalkyl-oligolactates.

Ring-opening of the L-lactide by alcohols can also be catalysed by the nucleophilic compound 4-(dimethylamino)pyridine (DMAP). It has been reported that lactic acid esters of a primary alcohol (e.g. benzylalcohol) are susceptible towards transesterification in the presence of DMAP while esters of secondary alcohols (e.g. sec-phenethyl alcohol) are dormant towards transesterification with the ring-opened products (Scheme 1)^{9, 10}. Based on this knowledge, the following reaction strategy was designed towards the synthesis of monodisperse methacrylamidoalkyl-oligolactates (Scheme 2). After protection of the hydroxyl function of the lactate-ester and deprotection of the carboxylic acid by catalytic hydrogenation, the mono- or dilactic acid is coupled to the hydroxyl function of methacrylamide derivatives. After cleavage of the protecting group,

these reactions were repeated to yield monodisperse methacrylamidoalkyl-oligolactates with a defined lactic acid chain length.



Scheme 1 Selective transesterification of L-lactide after DMAP catalysed ring-opening by primary alcohols in contrast to secondary alcohols



Scheme 2 Step-by-step synthesis strategy for monodisperse methacrylamidoalkyl-oligolactates

Materials & methods

General

All reagents and solvents (HPLC grade quality) were used without purification, unless stated otherwise. Tetrahydrofuran (THF) was freshly distilled over sodium/benzophenone before use. Dichloromethane (DCM) was dried over molecular sieves (0.4 nm). Pd/C and Pd black were purchased from Aldrich. Reactions were monitored by TLC. R_f values were obtained on silica coated plastic sheets (Merck silica gel 60 F₂₅₄) with the indicated eluent. Analysis of the compounds was performed with UV light (254 nm) and/or detection after dipping into a 10 % (w/w) solution of ammonium molybdate in 2M sulfuric acid followed by heating. Column chromatography was accomplished with Acros silica gel (0.030 - 0.075 mm) using ethyl acetate (EtAc) and/or hexane (Hex) as eluent. ¹H NMR and ¹³C-NMR spectra were recorded in CDCl₃ with a Gemini 300 MHz spectrometer, the values for chemical shifts (δ) given in ppm relative to TMS and coupling constants (J) in Hertz (Hz). The central line of CDCl₃ was used as internal reference line; 7.26 ppm for ¹H-NMR and 77.0 ppm for ¹³C-NMR. Mass spectra were recorded on a LCT MS (Micromass, electrospray positive mode).

HPLC analysis was carried out on a Waters system (Waters Associates Inc., Milford, MA, USA) consisting of a pump Model 600, an auto-injector Model 717, a variable wavelength absorbance detector Model 996 and an analytical reversed phase column LiChrosphere 100 RP-18 (5 μ m, 125 x 4 mm i.d.) with a RP-18 guard column (4 x 4 mm) (Merck). The injection volume was 50 μ L and the detection wavelength was 254 nm. After 5 minutes isocratic flow of water/acetonitrile = 95:5 (w/w), (eluent A), a gradient was run using 100 % eluent A to 100 % acetonitrile/water = 95:5 (w/w), (eluent B) in 30 minutes with a flow rate of 1.0 mL/min. The methacrylamides *N*-(2-hydroxyethyl)methacrylamide (HEMAm) and *N*-(2-hydroxypropyl)-methacrylamide (HPMAm) were synthesised as described in literature^{12, 14, 16}.

Compound 1: 1-oxo-1-(1-phenylethoxy)propan-2-yl 2-hydroxypropanoate (Benzyl-Lac₂)

DMAP (43 g, 350 mmol) was dissolved in *sec*-phenethyl alcohol (440 mL, 3.50 mol) and heated at 40 °C. L-lactide (51 g, 350 mmol) was added and the mixture was stirred at 40 °C for 20 min. Subsequently, the mixture was diluted with 500 mL of ethyl acetate and DMAP was removed by flash chromatography using ethyl acetate as the eluent. Vacuum distillation (Bp. (0.6 mbar) = 105 -

110 °C) resulted in 62 g of a colourless oil (**1**, 67 %). $^1\text{H-NMR}$ (CDCl_3) δ (ppm) 7.33 (m, 5H, Ph), 5.93 (q, $J = 6.6$, 1 H), 5.21 (q, $J = 7.1$ Hz, 1H), 4.35 (q, $J = 6.9$ Hz, 1H), 1.40-1.60 (m, 9H); $^{13}\text{C-NMR}$ (CDCl_3) δ (ppm) 16.6 (CH_3), 20.2 (CH_3), 21.7 (CH_3), 66.5 (CH), 69.1 (CH), 73.4 (CH), 125.8 (CH), 128.0 (CH), 128.3 (CH), 140.5 (C_q), 169.4 (C_q), 174.8 (C_q); MS (ES) calculated $[\text{M} + \text{Na}]^+$: 289.1052, measured M^+ : 289.0987.

Compound 2: benzyl 2-hydroxypropanoate (Benzyl-Lac₁)

Benzyl 2-hydroxypropanoate was essentially synthesised as **1**, only benzylalcohol was used and the reaction time was extended to 24 hours to achieve full transesterification. Vacuum distillation yielded 63 g of a colourless oil (**2**, 62 %, Bp (0.15 mbar) = 81 °C). $^1\text{H-NMR}$ (CDCl_3) δ (ppm) 7.35 (m, 5H, Ph), 5.20 (s, 2H), 4.31 (q, $J = 6.9$ Hz, 1H), 1.42 (d, $J = 6.9$ Hz, 3H); $^{13}\text{C-NMR}$ (CDCl_3) δ (ppm) 20.0 (CH_3), 66.6 (CH), 66.8 (CH_2), 128.0 (CH), 128.2 (CH), 128.4 (CH), 135.1 (C_q), 175.2 (C_q); MS (ES) calculated $[\text{M} + \text{Na}]^+$: 203.0684, measured $[\text{M} + \text{Na}]^+$: 203.0634.

Compound 3: 1-oxo-1-(1-phenylethoxy)propan-2-yl 2-(tert-butyldimethylsilyloxy)-propanoate (Benzyl-Lac₂-TBS)

Compound **1** (115 g, 432 mmol) was dissolved together with imidazole (32 g, 476 mmol) in 230 mL of DMF. *Tert*-butyldimethylsilylchloride (TBS-Cl, 72 g, 476 mmol) was added and the mixture was stirred overnight at room temperature under a nitrogen atmosphere. After addition of 500 mL water, the mixture was extracted with diethyl ether (3 x 500 mL). The combined organic layers were dried with MgSO_4 . After filtration, the organic solvent was removed under reduced pressure to yield 158 g of **3** (96 %) as a colourless oil. Compound **4** and **5** were synthesised via a similar procedure, compound **5** was protected with a *tert*-butyl diphenylsilyl (TPS) group. $^1\text{H-NMR}$ (CDCl_3): δ (ppm) 7.33 (m, 5H, Ph), 5.90 (q, $J = 6.6$ Hz, 1H), 5.12 (q, $J = 7.1$ Hz, 1H), 4.37 (q, $J = 6.9$ Hz, 1H), 1.40-1.60 (m, 9H), 0.89 (s, 9H), 0.06 (s, 6H); $^{13}\text{C-NMR}$ (CDCl_3) δ (ppm) -5.4 (CH_3), 16.8 (CH_3), 21.2 (CH_3), 21.9 (CH_3), 25.6 (CH_3) 68.0 (CH), 68.8 (CH), 73.4 (CH), 125.9 (CH), 128.0 (CH), 128.5 (CH), 140.8 (C_q), 169.6 (C_q), 173.3 (C_q); MS (ES) calculated $[\text{M} + \text{Na}]^+$: 403.1917, measured $[\text{M} + \text{Na}]^+$: 403.1783.

Compound 4: benzyl 2-(tert-butyldimethylsilyloxy)propanoate (Benzyl-Lac₁-TBS)

Analogous to the synthetic procedure for **3**, the TBS group was coupled to **2** and yielded 99 % of **4**. $^1\text{H-NMR}$ (CDCl_3) δ (ppm) 7.35 (m, 5H, Ph), 5.15 (dd,

$J = 12.1$ Hz, $J = 9.9$ Hz, 2H), 4.36 (q, $J = 6.6$ Hz, 1H), 1.41 (d, $J = 6.9$ Hz, 3H), 0.88 (s, 9H), 0.06 (s, 6H). $^{13}\text{C-NMR}$ (CDCl_3) δ (ppm) -5.1 (CH_3), 18.5 (C), 21.5 (CH_3), 25.9 (CH_3), 66.6 (CH_2), 68.6 (CH), 128.5 (CH), 128.7 (CH), 128.9 (CH), 136.0 (C_q), 174.0 (C_q); MS (ES) calculated $[\text{M} + \text{Na}]^+$: 317.1549 measured $[\text{M} + \text{Na}]^+$: 317.1064.

Compound 5: benzyl 2-(tert-butyldiphenylsilyloxy)propanoate (Benzyl-Lac₁-TPS)

Following the procedure as described for the synthesis of compound **3**, a *tert*-butyl diphenylsilyl (TPS) group was coupled to **2**. Purification with column chromatography using ethyl acetate/hexane (1:30) as eluent ($R_f = 0.25$) resulted in a white solid (**5**; 75 %). $^1\text{H-NMR}$ (CDCl_3) δ (ppm) 7.66 (m, 4H, Ph), 7.40 (m, 6H, Ph), 7.33 (m, 5H, Ph), 5.01 (dd, $J = 12.4$ Hz, $J = 6.3$ Hz, 2H), 4.34 (q, $J = 6.9$ Hz, 1H), 1.39 (d, $J = 6.6$ Hz, 3H), 1.09 (s, 9H); $^{13}\text{C-NMR}$ (CDCl_3) δ (ppm) 19.2 (C_q), 21.2 (CH_3), 26.8 (CH_3), 66.3 (CH_2), 69.0 (CH), 127.5 (CH), 127.6 (CH), 128.1 (CH), 128.3 (CH), 129.7 (CH), 133.2 (C_q), 135.5 (C_q), 135.7 (CH), 173.5 (C_q); MS (ES) calculated $[\text{M} + \text{Na}]^+$: 441.1862, measured $[\text{M} + \text{Na}]^+$: 441.1703

Compound 6: 2-(2-(tert-butyldimethylsilyloxy)propanoyloxy)propanoic acid (Lac₂-TBS)

Catalytic hydrogenation of 199 mmol of **3** (76 g) was performed overnight in 175 mL of ethanol with addition of 7.5 g Pd/C (10 % w/w) using a Parr equipment (40 PSI hydrogen pressure). Filtration over hyflo gave 54 g of **6** (98 %) as colourless oil. No further purification was necessary. Compound **7** and **8** were obtained by the same procedure. $^1\text{H-NMR}$ (CDCl_3): δ (ppm) 5.10 (q, $J = 7.1$ Hz, 1H), 4.38 (q, $J = 6.9$ Hz, 1H), 1.53 (d, $J = 7.1$ Hz, 3H), 1.43 (d, $J = 6.6$ Hz, 3H), 0.88 (s, 9H), 0.08 (s, 6H); $^{13}\text{C-NMR}$ (CDCl_3) δ (ppm) -5.4 (CH_3), 17.0 (C_q), 18.2 (CH_3), 21.1 (CH_3), 25.6 (CH_3) 68.0 (CH), 68.4 (CH), 173.5 (C_q), 176.0 (C_q); MS (ES) calculated $[\text{M} + \text{Na}]^+$: 299.1291, measured $[\text{M} + \text{Na}]^+$: 299.1182.

Compound 7: 2-(tert-butyldimethylsilyloxy)propanoic acid (Lac₁-TBS)

A large variety of catalytic hydrogenation conditions were assessed to convert **4** into **7** (Parr equipment versus balloon, different grades of palladium, different solvents and concentrations) but the product readily decomposed into 2-hydroxypropanoic acid during hydrogenation, upon purification, during chromatography as well as on storage at ambient temperature.

Compound 9: 1-(2-methacrylamidoethoxy)-1-oxopropan-2-yl 2-(tert-butyl-dimethylsilyloxy)-propanoate (HEMAm-Lac₂-TBS)

HEMAm (8.0 g, 62 mmol) and compound **6** (34 g, 124 mmol) was dissolved in 300 mL dry dichloromethane. Subsequently, the reaction mixture was cooled in an ice bath and DCC (32 g, 155 mmol) was added. The resulting solution was slowly heated to room temperature and stirred for 46 h. After filtration and flash chromatography with ethyl acetate/hexane (1:1, $R_f = 0.30$), 20 g (85 %) of **9** was obtained as a colourless oil. Coupling dilactate-TBS via this route was also achieved for compounds **10** and **17**. ¹H-NMR (CDCl₃): δ (ppm) 5.73 (s, 1H), 5.32 (s, 1H), 4.97 (q, $J = 7.1$ Hz, 1H), 4.36 (q, $J = 6.9$ Hz, 1H), 4.26 (m, 2H), 3.57 (q, $J = 4.7$ Hz, 2H), 1.94 (s, 3H), 1.50 (d, $J = 7.1$ Hz, 3H), 1.42 (d, $J = 6.9$ Hz, 3H), 0.88 (s, 9H), 0.07 (s, 6H); ¹³C-NMR (CDCl₃) δ (ppm) -5.4 (CH₃), 16.6 (CH₃), 18.2 (C), 18.5 (CH₃), 21.2 (CH₃), 25.6 (CH₃), 38.7 (CH₂), 64.0 (CH₂), 67.9 (CH), 69.3 (CH), 120.1 (CH₂), 139.4 (C_q), 168.4 (C_q), 170.7 (C_q), 174.1 (C_q); MS (ES) calculated [M + Na]⁺: 410.1975, measured [M + Na]⁺: 410.1799.

Compound 10: 1-methacrylamidopropan-2-yl 2-(2-(tert-butyl)dimethylsilyloxy)-propanoate (HPMAm-Lac₂-TBS)

After coupling of HPMAm (7.5 g, 52 mmol) to **6** (29 g, 105 mmol) using DCC (27 g, 131 mmol) as coupling agent, purification (EtAc/Hex 1:1; $R_f = 0.29$) gave 21 g of a mixture of diastereoisomers of **10** (99 %, colourless oil). ¹H-NMR (CDCl₃): δ (ppm) 5.75 (s, 1H), 5.70 (s, 1H), 5.32 (s, 1H), 5.30 (s, 1H), 5.10 (m, 1H), 4.96 (q, $J = 7.1$ Hz, 1H), 4.87 (q, $J = 7.1$ Hz, 1H), 4.36 (q, $J = 6.9$ Hz, 1H), 3.68 (m, 1H), 3.25 (m, 1H), 1.94 (d, $J = 4.3$ Hz, 3H), 1.49 (d, $J = 6.9$ Hz, 3H), 1.41 (d, $J = 6.6$ Hz, 3H), 1.24 (d, $J = 6.6$ Hz, 3H), 0.88 (s, 9H), 0.07 (s, 6H); ¹³C-NMR (CDCl₃) δ (ppm) -5.5 (CH₃), 16.4 (CH₃), 17.3 (CH₃), 18.1 (C_q), 18.5 (CH₃), 21.0 (CH₃), 25.5 (CH₃), 43.8 (CH₂), 67.8 (CH), 69.4 (CH), 71.2 (CH), 119.9 (CH₂), 139.5 (C_q), 168.5 (C_q), 170.5 (C_q), 174.3 (C_q); MS (ES) calculated [M + Na]⁺: 424.2132, measured [M + Na]⁺: 424.2247.

Compound 11: 2-methacrylamidoethyl 2-(tert-butyl)phenylsilyloxy)-propanoate (HEMAm-Lac₁-TPS)

Compound **4** (3.5 g, 12 mmol) was hydrogenated in 60 mL THF also containing 10 % (w/w) palladium black (350 mg, H₂-balloon). The reaction was monitored with TLC and was completed after seven hours. The mixture was filtered over hyflo. A catalytic amount of DMAP (approximately 20 mg) was added together with 1.1 equivalent of HEMAm and the solution was cooled down till 0 °C. After addition of 13.2 mmol DCC (2.7 g), the reaction was

stirred under nitrogen for 20 hours at ambient temperature. Filtration and subsequent column chromatography (EtAc/Hex 1:1, $R_f = 0.40$) yielded 2,4 g of **11** as a colourless oil (45 %). $^1\text{H-NMR}$ (CDCl_3) δ (ppm) 7.65 (m, 4H, Ph), 7.37 (m, 6H, Ph), 5.93 (s, 1H), 5.57 (s, 1H), 5.29 (s, 1H), 4.31 (q, $J = 6.9$ Hz, 1H), 4.07 (m, 2H), 3.42 (m, 2H), 1.89 (s, 3H), 1.38 (d, $J = 6.6$ Hz, 3H), 1.08 (s, 9H); $^{13}\text{C-NMR}$ (CDCl_3) δ (ppm) 18.5 (CH_3), 19.2 (C_q), 21.3 (CH_3), 26.8 (CH_3), 39.0 (CH_2), 63.4 (CH_2), 68.9 (CH), 118.1 (CH_2), 119.7 (CH_2), 127.6 (CH), 130.0 (CH), 133.2 (C_q), 135.6 (CH), 139.6 (C_q), 168.2 (C_q), 174.0 (C_q); MS (ES) calculated $[\text{M} + \text{Na}]^+$: 462.2077, measured $[\text{M} + \text{Na}]^+$: 462.1520.

Compound 12: 1-methacrylamidopropan-2-yl 2-(tert-butyldiphenylsilyloxy)-propanoate (HPMAm-Lac₁-TPS)

Analogous to the previous procedure for **11**, two mmol **4** (899 mg) was hydrogenated in THF and coupled to HPMAm (2.2 mmol, 315 mg) using DCC (2.2 mmol, 453 mg). Purification by column chromatography ($R_f = 0.45$) yielded 632 mg of a mixture of diastereoisomers of **12** (colourless oil, 70 %). $^1\text{H-NMR}$ (CDCl_3) δ (ppm) 7.66 (m, 4H, Ph), 7.35 (m, 6H), 6.02 (s, 1H), 5.96 (s, 1H), 5.61 (s, 1H), 5.56 (s, 1H), 5.29 (s, 1H), 4.94 (m, 1H), 4.30 (q, $J = 6.6$ Hz, 1H), 3.38-3.28 (m, 2H), 1.89 (d, $J = 7.4$ Hz, 3H), 1.37 (d, $J = 6.6$ Hz, 3H), 1.15 (d, $J = 7.7$ Hz, 3H), 1.09 (s, 9H); $^{13}\text{C-NMR}$ (CDCl_3) δ (ppm) 17.4 (CH_3), 18.5 (CH_3), 19.2 (C_q), 21.3 (CH_3), 26.8 (CH_3), 44.2 (CH_2), 69.0 (CH), 70.6 (CH), 118.4 (CH_2), 119.7 (CH_2), 127.6 (CH), 129.9 (CH), 133.2 (C_q), 135.7 (CH), 139.7 (C_q), 168.3 (C_q), 173.9 (C_q); MS (ES) calculated $[\text{M} + \text{Na}]^+$: 476.2233, measured $[\text{M} + \text{Na}]^+$: 476.1896.

The protecting group of compounds **9**, **10**, **11**, **12**, **17** and **18** was cleaved with tetrabutylammonium fluoride trihydrate (TBAF) which was dissolved in THF (1 M), dried for three days over molecular sieves (0.4 nm) and neutralized prior to use by adding a threefold equivalent of glacial acetic acid. The protected compound was dissolved in THF (1M) and a twofold molar excess of the TBAF solution was added. The reaction mixture was stirred for 24 hours under a nitrogen atmosphere and yielded the deprotected compound **13**, **14**, **15**, **16**, **19** or **20**, respectively, after purification by flash chromatography with ethyl acetate.

Compound 13: 1-(2-methacrylamidoethoxy)-1-oxopropan-2-yl 2-hydroxypropanoate (HEMAm-Lac₂)

HEMAm-Lac₂ (**13**, 10 g, 70 %) was obtained from **9** as a light yellow oil after purification ($R_f = 0.25$). ¹H-NMR (CDCl₃): δ (ppm) 5.70 (s, 1H), 5.34 (s, 1H), 5.09 (q, $J = 7.1$ Hz, 1H), 4.32 (m, 3H), 3.58 (m, 2H), 1.95 (s, 3H), 1.51 (d, $J = 7.1$ Hz, 3H), 1.46 (d, $J = 6.9$ Hz, 3H); ¹³C-NMR (CDCl₃) δ (ppm) 16.5 (CH₃), 18.3 (CH₃), 20.1 (CH₃), 38.5 (CH₂), 63.8 (CH₂), 66.5 (CH), 69.2 (CH), 120.0 (CH₂), 139.3 (C_q), 168.6 (C_q), 170.3 (C_q), 175.0 (C_q); MS (ES) calculated [M + Na]⁺: 296.1110, measured [M + Na]⁺: 296.1090. HPLC analysis of HEMAm-Lac₂, (Figure 1) showed that the purity was more than 99 %. This was also the case for all the other end products (data not shown).

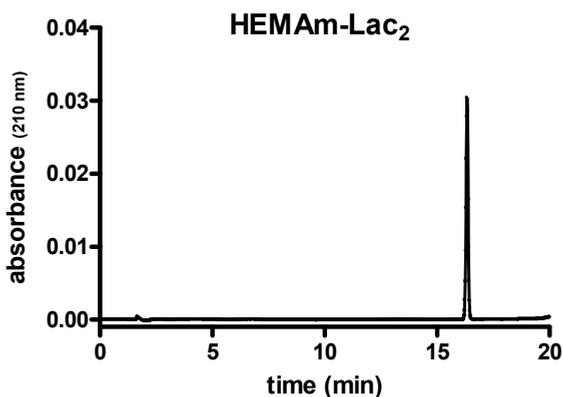


Figure 1 HPLC analysis of HEMAm-Lac₂ (UV-detection at 210 nm)

Compound 14: 1-methacrylamidopropan-2-yl 2-(2-hydroxypropanoyloxy)propanoate (HPMAm-Lac₂)

Workup of **10** after the TBAF-reaction ($R_f = 0.29$) yielded HPMAm-Lac₂ (**14**, light yellow oil, 99 %) as a mixture of diastereoisomers. ¹H-NMR (CDCl₃): δ (ppm) 5.70 (s, 1H), 5.33 (s, 1H), 5.10 (m, 2H), 4.33 (q, $J = 7.1$ Hz, 1H), 3.60 (m, 1H), 3.34 (m, 1H), 1.95 (m, 3H), 1.55 (m, 6H), 1.24 (d, $J = 6.6$ Hz, 3H); ¹³C-NMR (CDCl₃) δ (ppm) 16.5 (CH₃), 17.4 (CH₃), 18.5 (CH₃), 20.3 (CH₃), 43.9 (CH₂), 66.7 (CH), 69.7 (CH), 71.5 (CH), 119.9 (CH₂), 139.6 (C_q), 168.6 (C_q), 169.9 (C_q), 175.3 (C_q); MS (ES) calculated [M + Na]⁺: 310.1267, measured [M + Na]⁺: 310.1132.

Compound 15: 2-methacrylamidoethyl 2-hydroxypropanoate (HEMAm-Lac₁)

Cleavage of the TPS group of 4.5 mmol **11** (2.0 g) and subsequent purification by column chromatography (EtAc/Hex 25:1; $R_f = 0.33$) resulted in 700 mg of a colourless oil (**15**, 77 %). ¹H-NMR (CDCl₃) δ (ppm) 6.33 (s, 1H); 5.67 (s, 1H), 5.33 (s, 1H), 4.30 (m, 3H), 3.59 (q, $J = 5.6$ Hz, 2H), 1.93 (s, 3H), 1.39 (d, $J = 6.9$ Hz, 3H) ¹³C-NMR (CDCl₃) δ (ppm) 18.5 (CH₃), 20.3 (CH₃), 38.8 (CH₂), 64.1 (CH₂), 66.8 (CH), 118.4 (CH₂), 120.0 (CH₂), 139.6 (C), 168.7 (C_q), 175.6 (C_q); MS (ES) calculated [M + Na]⁺: 224.0899, measured [M + Na]⁺: 224.0787.

Compound 16: 1-methacrylamidopropan-2-yl 2-hydroxypropanoate (HPMAm-Lac₁)

The deprotection of **12** (530 mg, 1.2 mmol) was monitored with TLC and was completed after 30 hours. Column chromatography using EtAc/Hex (10:1, $R_f = 0.30$) yielded 215 mg of **16** as a mixture of diastereoisomers (85 %). ¹H-NMR (CDCl₃) δ (ppm) 6.21 (s, 1H), 5.66 (s, 1H), 5.33 (s, 1H), 5.11 (m, 1H), 4.25 (m, 1H), 3.48 (m, 2H), 2.76 (s, 1H), 1.93 (s, 3H), 1.38 (m, 2H), 1.27 (m, 2H); ¹³C-NMR (CDCl₃) δ (ppm) 17.6 (CH₃), 18.2 (CH₃), 20.0 (CH₃), 42.1 (CH₂), 66.3 (CH), 71.7 (CH), 118.4 (CH₂), 119.9 (CH₂), 139.7 (C_q), 168.9 (C_q), 175.7 (C_q); MS (ES) calculated [M + Na]⁺: 238.1056, measured [M + Na]⁺: 238.0489.

Compound 17: 1-(2-methacrylamidoethoxy)-1-oxopropan-2-yl 2-(2-(2-(tert-butyl)dimethylsilyloxy)propanoyloxy)propanoyloxy)propanoate (HEMAm-Lac₄-TBS)

Attachment of **13** (4.5 g, 16 mmol) to **6** (9.1 g, 33 mmol) using DCC (8.5 g, 41 mmol) as coupling agent and subsequent purification ($R_f = 0.15$) yielded 7.4 g of a colourless viscous oil (**17**, 85 %). ¹H-NMR (CDCl₃): δ (ppm) 5.70 (s, 1H), 5.32 (s, 1H), 5.12 (dq, $J = 7.1$ Hz, 2H), 5.04 (q, $J = 7.1$ Hz, 1H), 4.37 (q, $J = 6.9$ Hz, 1H), 4.25 (m, 2H), 3.61 (m, 1H), 3.50 (m, 1H), 1.93 (s, 3H), 1.53 (m, 9H), 1.42 (d, $J = 6.6$ Hz, 3H), 0.89 (s, 9H), 0.07 (s, 6H); ¹³C-NMR (CDCl₃) δ (ppm) -5.5 (CH₃), 16.5 (CH₃), 18.1 (C_q), 18.4 (CH₃), 21.0 (CH₃), 25.5 (CH₃), 38.4 (CH₂), 64.2 (CH₂), 67.7 (CH), 68.3 (CH), 68.9 (CH), 69.6 (CH), 119.8 (CH₂), 139.5 (C_q), 168.3 (C_q), 169.9 (C_q) 170.0 (C_q), 173.4 (C_q); MS (ES) calculated [M + Na]⁺: 554.2398, measured [M + Na]⁺: 554.2256.

Compound 18: 1-methacrylamidopropan-2-yl 2-(2-(2-(tert-butyl)diphenylsilyloxy)propanoyloxy)propanoyloxy)propanoate (HPMAm-Lac₃-TPS)

Similar to the previous procedure for **11** and **12**, 7 mmol **5** (2.9 g) was catalytic hydrogenated in THF and coupled to **14**. Purification by column

chromatography ($R_f = 0.50$) yielded 1.9 g of a mixture of diastereoisomers of **18** (viscous colourless oil, 45 %). $^1\text{H-NMR}$ (CDCl_3) δ (ppm) 7.66 (m, 4H, Ph), 7.40 (m, 6H), 6.45 (s, 1H), 6.35 (s, 1H), 5.73 (s, 1H), 5.68 (s, 1H), 5.30 (s, 1H), 5.26 (s, 1H), 5.10-4.84 (m, 3H), 4.33 (q, $J = 6.9$ Hz, 1H), 3.76-3.17 (m, 2H), 1.92 (d, $J = 6.3$ Hz, 3H), 1.37 (d, $J = 6.9$ Hz, 3H), 1.42 (m, 6H), 1.24 (m, 3H), 1.08 (s, 9H); $^{13}\text{C-NMR}$ (CDCl_3) δ (ppm) 16.6 (CH_3), 17.4 (CH_3), 18.5 (CH_3), 19.2 (C_q), 21.1 (CH_3), 26.7 (CH_3), 43.7 (CH_2), 68.4 (CH), 69.8 (CH), 71.9 (CH), 118.4 (CH_2), 119.9 (CH_2), 127.6 (CH), 129.8 (CH), 133.1 (C_q), 135.8 (CH), 139.6 (C_q), 168.4 (C_q), 169.6 (C_q), 170.6 (C_q), 173.3 (C_q); MS (ES) calculated $[\text{M} + \text{Na}]^+$: 620.2656, measured $[\text{M} + \text{Na}]^+$: 620.3037.

Compound 19: 1-(2-methacrylamidoethoxy)-1-oxopropan-2-yl 2-(2-(2-hydroxypropanoyloxy)-propanoyloxy)propanoate (HEMAm-Lac₄)

Cleavage of the TBS group of compound **17** with subsequent purification ($R_f = 0.31$) resulted in 5.5 g of a colourless viscous oil (**19**, 98 %). $^1\text{H-NMR}$ (CDCl_3): δ (ppm) 5.70 (s, 1H), 5.32 (s, 1H), 5.17 (m, 2H), 5.05 (q, $J = 7.1$ Hz, 1H), 4.34 (q, $J = 6.9$ Hz, 1H), 4.26 (m, 2H), 3.57 (m, 2H), 1.93 (s, 3H), 1.56 (d, $J = 7.1$ Hz, 6H), 1.50 (d, $J = 7.1$ Hz, 3H), 1.45 (d, $J = 6.9$ Hz, 3H); $^{13}\text{C-NMR}$ (CDCl_3) δ (ppm) 16.5 (CH_3), 18.3 (CH_3), 20.2 (CH_3), 38.5 (CH_2), 64.1 (CH_2), 66.4 (CH), 68.6 (CH), 69.0 (CH), 69.5 (CH), 120.0 (CH_2), 139.3 (C), 168.5 (C_q), 169.6 (C_q), 169.8 (C_q), 170.0 (C_q), 174.8 (C_q); MS (ES) calculated $[\text{M} + \text{Na}]^+$: 440.1533, measured $[\text{M} + \text{Na}]^+$: 440.1389.

Compound 20: 1-methacrylamidopropan-2-yl 2-(2-(2-hydroxypropanoyloxy)-propanoyloxy)-propanoate (HPMAm-Lac₃)

The deprotection of **18** (2.5 mmol) was completed after 40 hours. Column chromatography using EtAc/Hex (20:1, $R_f = 0.54$) yielded **20** as a mixture of diastereoisomers (viscous colourless oil, 2.2 mmol, 86 %). $^1\text{H-NMR}$ (CDCl_3) δ (ppm) 6.30 (s, 1H), 5.71 (s, 1H), 5.68 (s, 1H), 5.32 (m, 1H), 5.21-4.97 (m, 3H), 4.36 (m, 1H), 3.70-3.21 (m, 1H), 1.93 (s, 3H), 1.58 (m, 3H), 1.48 (m, 6H), 1.26 (d, $J = 6.6$ Hz, 3H); $^{13}\text{C-NMR}$ (CDCl_3) δ (ppm) 16.7 (CH_3), 17.4 (CH_3), 18.5 (CH_3), 20.4 (CH_3), 43.8 (CH_2), 66.6 (CH), 69.9 (CH), 71.6 (CH), 119.8 (CH_2), 139.6 (C_q), 168.6 (C_q), 169.8 (C_q), 170.1 (C_q), 175.0 (C_q); MS (ES) calculated $[\text{M} + \text{Na}]^+$: 382.1478, measured $[\text{M} + \text{Na}]^+$: 382.1404.

Results & discussion

Upon treatment of L-lactide with a tenfold excess of *sec*-phenethyl alcohol, the lactide is ring-opened and quantitatively converted into the linear dimer of lactic acid, yielding **1** (Scheme 1) ¹⁰. This linear dimer was used as building block for even oligolactate derivatives of primary or secondary hydroxyl methacrylamides. Vacuum distillation was performed to separate **1** from the excess *sec*-phenethyl alcohol. Complete removal of DMAP by flash chromatography was crucial before distillation since trace amounts of this compound caused transesterification reactions and concomitantly a mixture of oligomers was obtained. The terminal alcohol of **1** was protected with a tert-butyl dimethyl silyl (TBS) protecting group (**3**, 96 %) ⁶. Subsequent catalytic hydrogenation in a Parr machine (40 PSI hydrogen pressure) resulted in **6** (quantitative yield). This compound was coupled to HEMAm using DCC as coupling agent giving 85 % of **9**. Removal of the protective silyl group was achieved with a twofold excess of TBAF in THF ⁶. As TBAF is a weak nucleophile with alkaline properties, the TBAF solution (dried over molecular sieves) was neutralised prior to use by addition of three equivalents of glacial acetic acid ³. Cleavage of the TBS group from **9** was done under dry and aprotic conditions to prevent hydrolysis of the ester groups and yielded 70 % HEMAm-Lac₂ (**13**).

The general applicability of the reaction sequence was demonstrated not only with a methacrylamide containing a primary alcohol (HEMAm), but also with a compound containing a secondary alcohol (HPMAm). Indeed, it was shown that HPMAm could be successfully coupled to Lac₂-TBS to give **10** as a mixture of diastereoisomers. Upon deprotection, **14** was obtained with an overall yield of both reactions of 85 %. Elongation of the even lactic acid chain was achieved by the subsequent coupling of **13** to **6**. Subsequent removal of the protecting group yielded HEMAm-Lac₄ (**19**, 98 %) after deprotection.

For the synthesis of odd oligolactates, the above described method was applied with benzyl monolactate as starting compound; quantitative transesterification of the ring-opened diester product (benzyl dilactate) by an excess benzylalcohol in the presence of DMAP afforded selectively the monolactate **2**. In a subsequent reaction, the alcohol function was protected with TBS to give **4**. Applying the same catalytic hydrogenation conditions as used for **3**, compound **4** was not only converted into the desired monolactate-TBS (**7**) but also resulted in the formation of approximately 50 % 2-hydroxypropanoic acid (and thus removal of both protecting groups). Recently,

it was shown that the selective deprotection of silyl ethers and benzyl esters is highly dependent on the type of solvent used; the TBS group is selectively cleaved off when using 10 % Pd/C in methanol ⁷. In contrast, chemoselective hydrogenation of the benzyl ester group can be achieved when the reaction is performed in ethyl acetate or acetonitrile. However, in our case, the self-catalysed cleavage of **7** occurred irrespective of the solvent (either protic (e.g. EtOH) or aprotic (e.g. THF, ethyl acetate)), hydrogenation time, grade of palladium (normal Pd/C or Pd black), concentration (0.05M - 1M), hydrogen pressure (balloon or Parr equipment (40 PSI) or workup procedure. Likely, the proton of the carboxylic acid in **7** will attack the oxygen of the silyl ether, thereby forming a five-ring (Figure 2). Nevertheless, this ring is not stable and 2-hydroxypropanoic acid is generated as reaction product. In case of dilactate-PG, this cleavage is expected not to take place as ring formation is geometrically not feasible. To circumvent this cleavage, the less acid-sensitive *tert*-butyl-diphenylsilyl (TPS) was chosen as protective group ⁶. After workup of hydrogenated compound **8**, the fully deprotected 2-hydroxypropanoic acid was present again (approximately 20 %). Therefore, the procedure was adapted such that the coupling reaction proceeded immediately after removal of the Pd black. Using DCC as coupling agent, the carboxyl group of monolactate-TPS was esterified with either HEMAm (to give **11**) or HPMAm (to give **12**) and yielded after TPS cleavage **15** (80 %) or **16** (85 %), respectively.

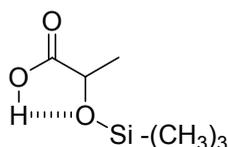


Figure 2 Formation of five-ring from monolactate-TBS (**7**)

Chain extension from even to odd oligolactates was also achieved via this route. After catalytic hydrogenation of **5** and removal of Pd black, the carboxylic acid was coupled to HPMAm-Lac₂ (**14**) via the DCC agent to give compound **18**. Subsequent removal of the protective group resulted in HPMAm-Lac₃ (**20**, 86 %). An overview of all synthesised compounds is given in Table 1. The purity of all end products was assessed by HPLC and appeared to be more than 99 %. It can be anticipated that the successful route presented here can be extended to obtain even larger oligolactates with different primary or secondary alcohols as end group.

nr	R ₂	PG	n	m	name
9	H	TBS	2	-	HEMAm-Lac ₂ -TBS
10	CH ₃	TBS	2	-	HPMAm-Lac ₂ -TBS
11	H	TPS	1	-	HEMAm-Lac ₁ -TPS
12	CH ₃	TPS	1	-	HPMAm-Lac ₁ -TPS
13	H	-	2	-	HEMAm-Lac ₂
14	CH ₃	-	2	-	HPMAm-Lac ₂
15	H	-	1	-	HEMAm-Lac ₁
16	CH ₃	-	1	-	HPMAm-Lac ₁
17	H	TBS	2	2	HEMAm-Lac ₄ -TBS
18	CH ₃	TPS	2	1	HPMAm-Lac ₃ -TPS
19	H	-	2	2	HEMAm-Lac ₄
20	CH ₃	-	2	1	HPMAm-Lac ₃

Table 1 Overview of synthesised monodisperse methacrylamidoalkyl-oligolactates (PG = protecting group)

Conclusion

The described step-by-step synthesis is a direct and facile route to obtain monodisperse methacrylamidoalkyl-oligolactate monomers that can be used as building blocks for biodegradable (thermosensitive) polymeric materials. A lactic acid chain of defined length is grafted onto primary as well as secondary alcohol derivatives of methacrylamides. The ring-opened dimer of lactic acid was protected with TBS, but the monoester required a different protecting group (TPS) as the TBS group was prone to self-catalysed hydrolysis during subsequent catalytic hydrogenation. Overall, every step was carried out on a multi-gram scale with fairly high yields.

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chapter 3

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chapter 4

Novel fast degradable thermosensitive polymeric micelles based on mPEG-*block-poly(N-(2-hydroxyethyl)-methacrylamide-oligolactates)*

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Abstract

The aim of this study was to design a thermosensitive polymeric micelle system with a relatively fast degradation time of around one day. These micelles are of interest for the (targeted) delivery of biologically active molecules. Therefore, *N*-(2-hydroxyethyl)methacrylamide-oligolactates (HEMAm-Lac_n) were synthesised and used as building blocks for biodegradable (block co)polymers. p(HEMAm-Lac₂) is a thermosensitive polymer with a cloud point (CP) of 22 °C which could be lowered by copolymerisation with HEMAm-Lac₄. The block copolymer mPEG-*b*-p((80%HEMAm-Lac₂)-*co*-(20%HEMAm-Lac₄)) self-assembled into compact spherical micelles with an average size of 80 nm above the CP of the thermosensitive block (6 °C). Under physiological conditions (pH 7.4; 37 °C), the micelles started to swell after four hours and were fully destabilised within eight hours due to hydrolysis of the lactate side chains. Rapidly degrading thermosensitive polymeric micelles based on mPEG-*b*-p((80%HEMAm-Lac₂)-*co*-(20%HEMAm-Lac₄)) have attractive features as a (targeted) drug carrier system for therapeutic applications.

Introduction

Amphiphilic block copolymers can self-assemble in aqueous solutions into micellar structures consisting of a hydrophilic shell and a hydrophobic core. Generally, micelles have a small size while their hydrophobic core can accommodate hydrophobic drugs such as phthalocyanines and paclitaxel^{20, 26}. These properties make polymeric micelles excellent drug delivery vehicles. Carriers that are currently under investigation for drug delivery purposes are based on block copolymers of the hydrophilic poly(ethylene glycol) (PEG) and a variety of hydrophobic blocks including polylactide, polyglycolide, poly(lactide-co-glycolide), poly(propylene oxide) (Pluronic®), poly(caprolactone) or poly(benzyl aspartate)⁶.

The ideal pharmaceutical nanoparticle displays a high drug encapsulation efficiency, protects the incorporated drug against chemical or enzymatic degradation and is able to release its content in a controlled way, preferable upon arrival at its target site^{5, 28}. Besides 'simple' solubilisation of the drug in the hydrophobic core, covalent binding of a drug (e.g. doxorubicin) onto the hydrophobic polymer segment via an enzymatically cleavable spacer¹ and via acid sensitive linkers⁶ has also been studied to improve and control the drug loading and release properties of polymeric micelles. The hydrophilic block needs to be long enough to provide steric stabilisation of the particles and at the same time to give the particle 'stealth' properties^{7, 25}. After intravenous (i.v.) administration, these long circulating PEGylated colloidal particles (< 200 nm) will extravasate and accumulate in tumours and other pathological areas referred to as the 'enhanced permeability and retention' (EPR) effect⁸.

Special classes of polymers are those that exhibit thermosensitive behaviour. Block copolymers composed of a hydrophilic block (PEG) and a thermosensitive block can form polymeric micelles above the cloud point temperature (CP) of the thermosensitive block. This has been demonstrated for PEG-PPO-PEG (Pluronic®)^{14, 18} and for mPEG-*b*-poly(*N*-isopropylacrylamide) (mPEG-*b*-pNIPAAm)²⁷. Recently, biodegradable thermosensitive polymeric micelles based on mPEG-*b*-(pNIPAAm-co-(*N*-(2-hydroxypropyl)methacrylamide-dilactate) (HPMAm-Lac₂)) and mPEG-*b*-p(HPMAm-Lac₂) were described^{12, 22}. These block copolymers formed small (\pm 55 nm) uniform micelles above the CP of the thermosensitive block. Under physiological conditions, the hydrophobic lactate side chains of the pHPMAm-Lac₂ in the micelles are hydrolysed. Concomitantly, the CP gradually increases and passes 37 °C, which is associated

with destabilisation of the micelles. This process is characterised by an initial swelling of the micelles during the first 60 hours due to an increasing hydrophilicity of the core, followed by dissolution of the micelles after approximately one week of incubation²².

The circulation time of nanoparticles in blood, even in the case of PEGylated systems, is restricted in time. With a few exceptions, all long-circulating polymeric nanoparticles are cleared from the systemic circulation of experimental animals within the first 8 to 10 hours after i.v. administration^{7,9}. This implicates that the ideal carrier is stable for this time and destabilises upon arrival at its site of action with a concomitant release of the loaded drug. Consequently, there is a need for fast degrading systems, which completely destabilise within approximately 24 hours.

The aim of this study was to design thermosensitive block copolymers that form stable micelles at 37 °C but which will destabilise within one day under physiological conditions. From previous research, it is known that the hydrolysis kinetics of (2-hydroxyethyl)methacrylate-dilactate (HEMA-Lac₂) is five times faster than HPMAM-Lac₂¹³. This kinetic difference is explained by the faster hydrolysis of an ester of a primary alcohol in HEMA-Lac₂ versus a secondary alcohol in the HPMAM-Lac₂. However, it was shown that HEMA-Lac₂ was inappropriate as a (co)monomer. The corresponding degraded polymer (HEMA (co)polymer) was not hydrophilic enough to have a CP above body temperature¹⁰, which is a prerequisite for the present application. It was therefore anticipated that a methacrylamide with a primary alcohol function, viz. *N*-(2-hydroxyethyl)methacrylamide (HEMAM), could be more suitable. This paper describes the synthesis and degradation kinetics of *N*-(2-hydroxyethyl)methacrylamide (HEMAM)-lactate derivatives as well as the synthesis and characterisation of (co)polymers of these derivatives. Furthermore, amphiphilic and temperature sensitive block copolymers with poly(ethylene glycol) were synthesised. The micelle forming properties of these block copolymers were studied and subsequently the degradation kinetics of the micelles.

Materials & methods

Materials

Methacryloylchloride, α,α -azoisobutyronitrile (AIBN), 4-methoxyphenol (99 %), lithium chloride (LiCl), monomethylether of poly(ethylene glycol), M_w is 5000 g/mol (mPEG₅₀₀₀) and pyrene were obtained from Fluka Chemie AG (Buchs, Switzerland). L-lactide ((3*S*-*cis*)-3,6-dimethyl-1,4-dioxane-2,5-dione, > 99.5 %) was obtained from Purac Biochem BV (Gorinchem, The Netherlands). Ethanolamine (> 99 %), stannous 2-ethyl hexanoate (~ 95 %) and deuterated dimethyl sulphoxide, (99.9 %, DMSO-*d*₆) were obtained from Acros (Zwijndrecht, The Netherlands). The mPEG₂-macroinitiator with mPEG₅₀₀₀ was prepared with 4,4-azobis(4-cyanopentanoic acid) (ABCPA) as described previously¹². Diethyl ether (analytical grade) and triethylamine were obtained from Merck (Darmstadt, Germany). Acetonitrile (HPLC-S, gradient grade), dichloromethane, dimethylformamide (DMF) and 1,4-dioxane were purchased from Biosolve Ltd. (Valkenswaard, The Netherlands). 1,4-Dioxane was purified by distillation prior to use. All buffers were filtered through a 0.2 μ m filter (Schleicher & Schuell MicroScience GmbH, Dassel, Germany) prior to use. The other chemicals were used as received.

Synthesis of HEMAm & HEMAm-Lac_n

N-(2-hydroxyethyl)methacrylamide (HEMAm) was synthesised essentially as described by Song *et al*²³. In brief, freshly distilled methacryloylchloride (130 mL, 1.34 mol) dissolved in 250 mL dichloromethane was added dropwise to an ice-cold solution of 2-aminoethanol (162 mL, 2.68 mol) and triethylamine (109 mL, 1.47 mol) in 250 mL dichloromethane in 3 hours under a nitrogen atmosphere. Subsequently, the mixture was stirred overnight at room temperature under a nitrogen atmosphere. The crude product was filtered and purified by flash chromatography using dichloromethane/methanol (85/15) as the eluent (R_f = 0.45).

The oligolactate derivatives of HEMAm were synthesised via the procedure as reported by Van Dijk-Wolthuis *et al*²⁹. Briefly, L-lactide (33.5 g, 0.233 mol) and HEMAm (20 g, 0.155 mol) were stirred at 110 °C until the lactide had melted. 4-Methoxyphenol (~ 0.1 mol % relative to HEMAm) was added as radical scavenger. Subsequently, a catalytic amount of stannous 2-ethyl hexanoate (630 mg, 1 mol % relative to HEMAm) was added. The resulting

mixture was stirred for 2 hours and allowed to cool to room temperature. After dissolution of the product in 250 mL water-acetonitrile (50:50), the HEMAm-oligolactates were fractionated with preparative chromatography essentially as described by Neradovic *et al*¹³. In detail, the HPLC system (Waters Associates Inc., Milford, MA, USA) consisted of a pump Model 600, a variable wavelength absorbance detector Model 2487, a sample manager Model 2700 and a Xterra Prep MS C18 (10 μ m, 19 x 250 mm i.d.) with a Xterra Prep MS C18 guard column (19 x 10 mm) was used. The injection volume was 5 mL and the detection wavelength was 254 and 280 nm. A gradient was run using water/acetonitrile = 95:5 (w/w) (eluent A) and acetonitrile/water = 95:5 (w/w) (eluent B) from 0 % B to 50 % B in 35 minutes with a flow rate of 10.0 mL/min. Four fractions were collected and after evaporation of the solvents, the identity of the obtained products, HEMAm mono-, di-, tri-, and tetralactate (further abbreviated as HEMAm-Lac₁, HEMAm-Lac₂, HEMAm-Lac₃ and HEMAm-Lac₄; see Figure 1), was established by ¹H-NMR; the purity was assessed by HPLC (system as described below).

¹H-NMR spectroscopy

¹H-NMR spectra were recorded with a Gemini 300 MHz spectrometer (Varian Associates Inc., NMR Instruments, Palo Alto, CA, USA). Spectra were obtained in DMSO-*d*₆. The central line of DMSO at 2.49 ppm was used as reference line.

Hydrolysis kinetics of HEMAm-oligolactates

The degradation studies of HEMAm-oligolactates were conducted according to the procedure as described by Neradovic *et al*¹³. Briefly, a 10 mM solution of HEMAm-oligolactate in DMSO was diluted ten times with phosphate buffer pH 7.2 (100 mM) in a glass vial and the pH was adjusted to pH 7.4 with 4 M HCl. The resulting solutions of HEMAm-Lac₁, HEMAm-Lac₂, HEMAm-Lac₃ and HEMAm-Lac₄ were incubated in a water bath at 37 °C. At regular time intervals, samples of 300 μ L were withdrawn and 700 μ L of 1 M sodium acetate buffer (pH 3.8) was added to prevent further hydrolysis. The samples were stored at 4 °C prior to HPLC analysis. The hydrolysis of HEMAm-Lac₃ and HEMAm-Lac₄ was also investigated in an acetonitrile-phosphate buffer pH 7.2 (100mM) mixture (50:50 w/w) to compare the kinetic data with previous results³⁰. HPLC analysis was carried out on a Waters system (Waters Associates Inc., Milford, MA, USA) consisting of a pump Model 600, an auto-injector Model 717, a variable

wavelength absorbance detector Model 996 and an analytical reversed phase column LiChrosphere 100 RP-18 (5 μm, 125X4 mm i.d.) with a RP-18 guard column (4x4 mm) (Merck). The injection volume was 50 μL and the detection wavelength was 254 nm. After 5 minutes isocratic flow of eluent A, a gradient was run using 100 % eluent A to eluent B in 30 minutes with a flow rate of 1.0 mL/min. The chromatograms were analysed with Empower Software Version 1154 (Waters Associates Inc.). Calibration curves were generated for each monomer and for HEMAm with freshly prepared standard solutions in DMSO/phosphate buffer (pH 7.2, 100 mM)/sodium acetate buffer (pH 3.8, 1 M) (3:27:70) and were linear between 0.07 and 15 μM.

Synthesis of HEMAm-oligolactates (co)-polymers

Homopolymers (HEMAm, HEMAm-Lac_n) and copolymers (mixtures of HEMAm-Lac₂ and HEMAm-Lac₄) were synthesised by free radical polymerisation in airtight screw-cap glass vials. AIBN dissolved in 1,4-dioxane (ratio of monomers/initiator varies between 75 and 150 mol/mol) was added to 200 mg monomer dissolved in 1 mL of dioxane. After addition of the initiator, the solution was flushed with nitrogen for at least 10 minutes. Next, the solution was heated to 70 °C and stirred for 24 hours. The polymers were precipitated by dropwise addition of the solution to an excess of diethyl ether. After centrifugation, the residue was dried overnight in a vacuum oven at 40 °C. ¹H-NMR (DMSO, d₆): δ = 7.5 (b, CO-NH-CH₂), 5.5 (b, CH-OH), 5.0 (b, CO-CH(CH₃)-O), 4.1 (b, CO-CH-(CH₃)-OH), 4.0 (b, CH₂-CH₂-O), 3.4 (b, NH-CH₂-CH₂), 1.4, (b, CO-CH-CH₃), 1.3 (b, HO-CH-CH₃), 1.0-0.6 (pHEMAm-Lac_n main chain protons). The HEMAm-Lac₂/HEMAm-Lac₄ monomer ratio (mol/mol) in the copolymer was determined by ¹H-NMR from the ratio of the integral of the methine protons (H_h; δ= 5.0 ppm) to the alcoholic proton (H_{oh} δ= 5.5 ppm). The following equation was used:

$$\%HEMAm - Lac_4 = \frac{I_{Hh} - I_{Hoh}}{2} * 100\% \quad (1)$$

Synthesis of mPEG₅₀₀₀-b-poly(HEMAm-Lac_n)

Block copolymers with HEMAm-Lac_n as thermosensitive block and mPEG as hydrophilic block were prepared by radical polymerisation using mPEG₂-ABCPA as macroinitiator (ratio of monomer/initiator = 150:1 mol/mol) according to the procedure as previously described for the synthesis of related block copolymers^{12, 22}. The concentration of the starting materials (monomer

plus macroinitiator) was 300 mg/mL in acetonitrile in airtight glass vials. The solution was flushed with nitrogen for at least 10 minutes, heated to 70 °C and stirred for 24 hours. Next, by dropwise addition of the solution to an excess of diethyl ether, the polymers were precipitated. After centrifugation, the residue was dried overnight in a vacuum oven at 40 °C. ¹H-NMR (DMSO, *d*₆): δ = 7.5 (b, CO-NH-CH₂), 5.5 (b, CH-OH), 5.0 (b, CO-CH(CH₃)-O), 4.1 (b, CO-CH-(CH₃)-OH), 4.0 (b, CH₂-CH₂-O), 3.6 (b, PEG methylene protons, O-CH₂-CH₂), 3.4 (b, NH-CH₂-CH₂), 1.4, (b, CO-CH-CH₃), 1.3 (b, HO-CH-CH₃), 1.0-0.6 (pHEMAm-Lac_n main chain protons). The number average molecular weight (*M*_n) of the thermosensitive block was determined by ¹H-NMR as follows (in the situation of copolymers, an average molecular weight of the monomers was used):

$$M_n = \frac{M_{W_{ave}} (HEMAM - Lac_n) \cdot I_{HEMAM-Lac_n}}{I_{PEG} / 454} \quad (2)$$

*I*_{HEMAM-Lac_n} is the value of the integral of the alcoholic proton of the HEMAm-Lac_n (H_{oh} δ= 5.5 ppm); *I*_{PEG/454} is the value of the integral of the PEG protons divided by average number of protons per PEG₅₀₀₀ chain (=454).

Gel Permeation Chromatography (GPC)

The molecular weights and their distribution of the different polymers were determined by GPC. Two serial Plgel 3 μm MIXED-D columns (Polymer Laboratories) were attached to a Waters System (Waters Associates Inc., Milford, MA, USA) with a differential refractometer Model 410. Poly(ethylene glycol)s of defined molecular weights were used as standards. The eluent was DMF containing 10 mM LiCl²¹. The samples were dissolved overnight at a concentration of 5 mg/mL in the eluent and filtered through a 0.45 μm filter prior to analysis. The flow rate was 0.7 mL/min and the column temperature was 40 °C. Peak areas were determined with Empower Software Version 1154 (Waters Associates Inc.).

Determination of the Cloud Point (CP) of (block co) polymers

The CP of the synthesised (block co)polymers in aqueous solutions was determined with static light scattering using a Horiba Fluorolog fluorometer (650 nm, 90 ° angle)^{4, 12, 22}. The polymers were dissolved overnight at 4 °C in ammonium acetate buffer (pH 5.0, 120 mM) at a concentration of 1 and 2 mg/mL for the copolymers and the block copolymers, respectively. The scattering intensity was measured every 0.2 °C during heating (heating rate

approximately 1 °C/min) thereby stirring the solution in the cuvette. The onset on X-axis, obtained by extrapolation of the intensity-temperatures curves to intensity zero, was considered as the CP. The CP determinations were done at least two times and the deviations were smaller than 1 °C.

Micelle formation

Micelles were formed via the quick heating procedure as described previously²². In brief, the block copolymers were dissolved overnight at 4 °C at various concentrations in ammonium acetate buffer (pH 5.0, 120 mM). The solutions were incubated at 0 °C for at least 15 minutes in glass vials. Subsequently, the vials were rapidly heated from 0 to 50 °C by putting them into a water bath under vigorous stirring to form micelles. After 1 minute of incubation at 50 °C, the mixtures were slowly cooled down to room temperature and filtrated through a 0.22 µm filter.

Dynamic Light Scattering (DLS) & Static Light Scattering (SLS)

Dynamic and static light scattering (DLS and SLS, respectively) were used to characterise the polymeric micelles. The equipment consisted of a Malvern CGS-3 multi-angle goniometer (Malvern Ltd., Malvern, U.K.) with He-Ne JDS Uniphase laser ($\lambda = 632.8$ nm, 22 mW output power), an optical fiber based detector, a digital LV/LSE-5003 correlator and a temperature controller (Julabo Waterbath). Time correlation functions were analysed using the ALV-60X0 Software V.3.X provided by Malvern. DLS provides the hydrodynamic radius using the Stokes-Einstein equation:

$$R_h = \frac{k_b \cdot T \cdot q^2}{6\pi \cdot \eta \cdot \Gamma} \quad (3)$$

where k_b is the Boltzmann constant, q is the scattering vector, ($q=(4\pi \cdot n \cdot \sin(\theta/2))/\lambda$, where n is the refractive index of the solution, θ is the scattering angle and λ is the wavelength of the incident laser light), η is the solvent viscosity, and Γ is the decay rate. Scattering of the micellar solutions was measured at an angle of 90° at 25 °C in an optical quality 8 mL borosilicate cell giving rise to the hydrodynamic diameter ($Z_{ave} = 2 \cdot R_h$) of the micelles and their polydispersity (PD). The destabilisation behaviour of the polymeric micelles was studied at 2 mg/mL in phosphate buffer (pH 7.4, 100 mM). A cell with approximately 1 mL micellar solution was incubated in the DLS machine at 37 °C and measured at regular time intervals. The radius of gyration (R_g) and

the aggregation number (N_{agg}) are two important physical parameters commonly used to describe the characteristics of (polymeric) micelles. The scattered intensities of the polymer solutions at different angles (between 30° and 150°) and concentrations were analysed by the graphical method firstly reported by Zimm³². This method involves simultaneously the extrapolation of the scattering data to both zero angle and zero concentrations. The relationship between the concentration and the intensity of the scattered light is given by following equation:

$$\frac{K.c}{R(q)} = \frac{1}{M_w} \left(1 + \frac{1}{3} R_g^2 q^2 \right) + 2A_2c \quad (4)$$

where c is the concentration, M_w is the weight average molecular weight of the micelles, R_g is the radius of gyration, A_2 is the second virial coefficient, and $R(q)$ is the excess Rayleigh ratio of the solute (excess intensity of scattered light at scattering vector q). The optical constant K is defined as:

$$K = \frac{4\pi^2}{N_A \lambda^4} n_T^2 \left(\frac{dn}{dc} \right)^2 \quad (5)$$

where n_{tol} the refractive index of toluene (1.494), (dn/dc) the specific refractive index increment of the micellar solution, N_A the Avogadro's constant. The parameter dn/dc was here estimated to be 0.1 based on previous experiments on mPEG-*b*-(HPMAM-Lac₂)²². The absolute excess time-averaged scattered intensity, i.e. Rayleigh ratio $R(q)$ is expressed by the equation:

$$R(q) = R_{tol,90} \left(\frac{n}{n_{tol}} \right)^2 \frac{I - I_0}{I_{tol}} \sin(\theta) \quad (6)$$

where $R_{tol,90}$ is the Rayleigh ratio of toluene at an angle of 90° , n is the refractive index of the solvent, I , I_0 and I_{tol} are the scattered intensities of the solution, solvent and toluene, respectively, and θ is the measurement angle.

Critical Micelle Concentration (CMC)

The CMC of the block copolymers was determined using pyrene as a fluorescent probe³¹. Micelles of the different block copolymers were formed as described above in 120 mM ammonium acetate buffer (pH 5.0) at a concentration of 2 mg/mL. The micellar solutions were cooled to room temperature and subsequently diluted with the same buffer yielding different

polymer concentrations ranging from $1 \cdot 10^{-5}$ to 1 mg/mL. Next, 15 μ L of pyrene dissolved in acetone (concentration $1.8 \cdot 10^{-4}$ M) was added to 4.5 mL polymer solution (final pyrene concentration $6 \cdot 10^{-7}$ M). Samples were incubated for 15 hours at room temperature in the dark to allow equilibration. Prior to the measurements, the solutions were incubated at 37 °C for at least 15 minutes. Fluorescence excitation spectra of pyrene were obtained using a Horiba Fluorolog fluorometer (90 ° angle). The excitation spectra were recorded at 37 °C from 300 to 360 nm with the emission wavelength at 390 nm. The excitation and emission band slits were 4 and 2 nm, respectively. The intensity ratio of I_{338}/I_{333} was plotted against the polymer concentration to determine the CMC.

Cryogenic Transmission Electron Microscopy (Cryo-TEM)

Cryo-TEM imaging was performed on 10 mg/mL micellar solutions in 120 mM ammonium acetate buffer (pH 5.0). The sample preparation was done in a temperature and humidity controlled chamber using a “Vitrobot”. A thin aqueous film of micellar solution was formed by blotting a 200 mesh copper grid covered with Quantifoil holey carbon foil (Micro Tools GmbH, Germany) at 22 °C and at 100 % relative humidity (glow discharged grid; 1 blot during 0.5 sec). The thin film was vitrified by quickly plunging the grid into liquid ethane. The grids with the vitrified thin films were transferred into the microscope chamber using a GATAN 626 cryo-holder system. A Tecnai12 transmission electron microscope (Philips) operating at 120kV was used with the specimen at -180 °C and using low-dose imaging conditions to avoid melting of the vitrified film. Images were recorded on a SIS-CCD camera and processed with AnalySIS software. Images were taken of micelles captured in the thin vitrified liquid film in the holes of the carbon foil to image them as undisturbed as possible.

Results & discussion

Synthesis of HEMAm & HEMAm-Lac_n

N-(2-hydroxyethyl)methacrylamide (HEMAm) was synthesised by the reaction of ethanolamine with methacryloylchloride ²³. After column chromatography, the product was obtained in a high yield (85 %) as a light-yellow oil and a high purity (> 95 %, ¹H-NMR). Oligolactate esters of *N*-(2-hydroxyethyl)methacrylamide (HEMAm-oligolactate) were obtained by ring-opening oligomerisation of L-lactide, using HEMAm as the initiator and stannous 2-ethyl hexanoate as a catalyst ¹⁰. The monomers HEMAm-Lac₁ to HEMAm-Lac₄ (Figure 1) were obtained with high purity and good yield (~ 50 %) after fractionation with a preparative HPLC system.

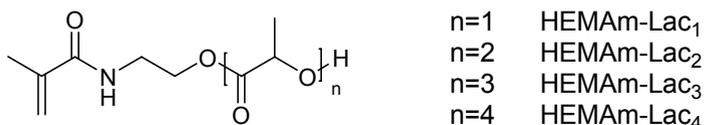


Figure 1 *N*-(2-hydroxyethyl)methacrylamide (HEMAm)-oligolactates

Hydrolysis kinetics of HEMAm-oligolactates

The monodisperse HEMAm-oligolactates (Figure 1) hydrolysed when incubated at pH 7.4 and at 37 °C and followed pseudo-first order kinetics with HEMAm and lactic acid as final degradation products. The concentrations of HEMAm-Lac₁ to HEMAm-Lac₄ were determined by HPLC. From the concentration versus time plots, the half-lives ($t_{1/2}$) were determined (Table 1). The buffer solution used for the degradation studies also contained DMSO (10 %) to solubilise the oligolactates. Therefore, the half-lives in pure water are expected to be less, approximately half of the reported values, as discussed by Neradovic ¹³. Since the formation of methacrylic acid was not detected, it is concluded that the amide bond in HEMAm (oligolactates) is highly stable under the selected conditions. Table 1 shows that the half-lives of HEMAm-Lac₁ and HEMAm-Lac₂ are 58 and 5.6 hours, respectively. The about ten times faster degradation rate of the dilactate as compared to the monolactate was also observed for HEMA-lactates ¹³ and can be explained by intramolecular transesterification, also known as ‘back-biting’ ³. The possibility of two lactic acid units to form an

intermediate 6-membered ring structure increases the hydrolysis rate. HEMAm-Lac₁ is not able to form a ring intermediate and is accordingly hydrolysed with a slower rate. At similar conditions (pH 7.5, 10 % DMSO), the half-lives of the methacrylate analogues of HEMAm-lactates, i.e. (2-hydroxyethyl)methacrylate (HEMA) mono- and dilactate, were 31 hours and 3 hours, respectively¹³ indicating that HEMAm-Lac₁ and HEMAm-Lac₂ are slightly more stable (half lives 58 and 5.6 hours, respectively). In contrast, the half-lives of HPMAm-Lac₁ and HPMAm-Lac₂ are 87 and 15 hours, respectively. This higher stability of esters of secondary alcohols (HPMAm-Lac_n) compared to esters of primary alcohols (HEMA-Lac_n and HEMAm-Lac_n) can be ascribed to steric effects. It has been reported that the presence of increasingly branched aliphatic groups has a retarding influence on the alkaline catalysed hydrolysis of esters¹⁶.

monomer	pH	cosolvent (%)	primary hydrolysis product	t _{1/2} (h) ^a	reference
HEMAm-Lac ₁	7.4	10 % DMSO	HEMAm	58	this work
HEMAm-Lac ₂	7.4	10 % DMSO	HEMAm	5.6	this work
HEMAm-Lac ₃	7.4	10 % DMSO	HEMAm-Lac ₁	0.63	this work
HEMAm-Lac ₄	7.4	10 % DMSO	HEMAm-Lac ₂	0.49	this work
HEMAm-Lac ₃	7.4	50 % ACN	HEMAm-Lac ₁	4.2	this work
HEMAm-Lac ₄	7.4	50 % ACN	HEMAm-Lac ₂	3.4	this work
HEMA-Lac ₁	7.5	10 % DMSO	HEMA	31	¹³
HEMA-Lac ₂	7.5	10 % DMSO	HEMA	3	¹³
HPMAm-Lac ₁	7.5	10 % DMSO	HPMAm	87	¹³
HPMAm-Lac ₂	7.5	10 % DMSO	HPMAm	15	¹³
HPMAm-Lac ₇	7.4	50 % ACN	HPMAm-Lac ₅	3.1	³⁰
HPMAm-Lac ₁₂	7.4	50 % ACN	HPMAm-Lac ₁₀	3.1	³⁰

Table 1 Hydrolysis kinetics of HEMAm-oligolactates and primary structures at 37 °C (^a Experimental error is ± 5 %)

HEMAm derivatives with three and four lactic acid units (HEMAm-Lac₃ and HEMAm-Lac₄) had faster hydrolysis kinetics than HEMAm-Lac_{1,2}. Again, this fast degradation is caused by the back biting mechanism. The HPLC analysis

clearly showed that HEMAm-Lac_n is primarily converted into HEMAm-Lac_{n-2} as an intermediate product that will ultimately be converted into HEMAm. In order to compare our results with the half-lives obtained for the previously reported HPMAM-Lac₇ and HPMAM-Lac₁₂³⁰, the degradation experiments were also carried out with ACN as cosolvent. The half-lives of both HPMAM-Lac₇ and HPMAM-Lac₁₂ were 3.1 hours, which are close to those of HEMAm-Lac₃ and HEMAm-Lac₄ (4.2 and 3.4 hours respectively). Taken together, the degradation data of the monomers showed that, as anticipated, the hydrolysis of HEMAm-Lac_n into HEMAm is faster than the timescale wherein HPMAM-Lac_n is converted into HPMAM.

Homopolymers of HEMAm-Lac_n: synthesis & characterisation

monomer	ratio M/I	yield (%)	M _w (GPC)	M _w /M _n	CP (°C)
HEMAM	150:1	92	24000	3.7	>75
HEMAM-Lac ₁	150:1	71	53000	3.1	>75
HEMAM-Lac ₂	100:1	81	68000	3.0	22
HEMAM-Lac ₃	75:1	83	24000	3.3	< 0
HEMAM-Lac ₄	100:1	76	59000	2.6	< 0

Table 2 Characteristics of the homopolymers of HEMAm-Lac_n

Free radical polymerisation of HEMAm-Lac_n in 1,4-dioxane yielded the corresponding polymers. Table 2 summarises their properties. pHEMAM and the pHEMAM-oligolactates were obtained in high yields (around 80 %) under the selected polymerisation conditions. The molecular weights were between 24000 and 68000 g/mol with a polydispersity (M_w/M_n) of ± 3, which is standard for polymers synthesised by free radical polymerisation. The thermosensitive properties of the polymers were investigated by static light scattering. To prevent hydrolysis of the lactic acid side chains, the polymers were dissolved in a pH 5.0 buffer at a concentration of 1 mg/mL. p(HEMAM-Lac₃) and p(HEMAM-Lac₄) did not dissolve at 0 °C, suggesting that the cloud point of these polymers is below this temperature. The scattering behaviour of p(HEMAM), p(HEMAM-Lac₁) and p(HEMAM-Lac₂) solutions is displayed in Figure 2.

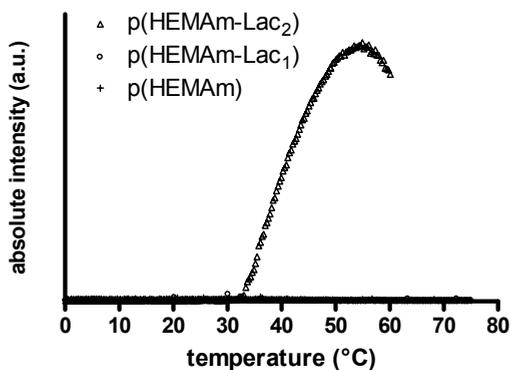


Figure 2 Scattering behaviour of p(HEMAm) (+), p(HEMAm-Lac₁) (O) and p(HEMAm-Lac₂) (Δ)

p(HEMAm) and p(HEMAm-Lac₁) displayed a good watersolubility and did not show any scattering up to 75 °C. An aqueous solution of p(HEMAm-Lac₂) showed a sharp increase in scattering intensity when the temperature was increased above 22 °C, demonstrating that this polymer has temperature sensitive properties. Figure 2 shows that the scattering intensity decreases above 36 °C. The polymer chains start to become insoluble due to dehydration at the CP and collapse into loose aggregates. This is visualised by the fast increase in scattering intensity. The further increase in temperature results in full dehydration with concomitant strong intra- and intermolecular interactions and the formation of more compact aggregates that scatter less than the loose aggregates. Recently, it was found that the related polymers p(HPMAm-Lac₁) and p(HPMAm-Lac₂) had a CP of 63 °C and 10 °C, respectively ²¹. HPMAm contains an extra methyl group, which makes it slightly more hydrophobic than HEMAm. In line herewith, p(HPMAm-Lac₂) exhibits a lower CP than p(HEMAm-Lac₂).

p((HEMAm-Lac₂)-co-(HEMAm-Lac₄)) copolymers

For practical reasons, it was aimed to obtain a polymer with a CP below room temperature. As reported above, the homopolymer of HEMAm-Lac₂ displayed its CP at 22 °C. To lower the CP of p(HEMAm-Lac₂), copolymers of HEMAm-Lac₂ with the more hydrophobic monomer HEMAm-Lac₄ were synthesised. Table 3 shows that the yields and molecular weights were comparable with those of the homopolymers (Table 2).

% HEMAm-Lac ₄ in the feed	yield (%)	% HEMAm-Lac ₄ incorporated ^a	M _w (GPC)	M _w /M _n (GPC)	CP (°C)
6	79	8	71000	3.0	14.5
12	76	11	62000	2.8	9.5
15	88	15	71000	2.6	7.0
18	77	18	68000	3.0	5.0
24	76	23	61000	2.8	< 0

Table 3 Characteristics of p((HEMAm-Lac₂)-co-(HEMAm-Lac₄)) copolymers (^a determined by ¹H-NMR)

The copolymer composition corresponded with the feed ratio. Light scattering measurements of aqueous solutions of these copolymers indeed showed that the CP of the copolymers linearly decreased with the amount of hydrophobic HEMAm-Lac₄ that was incorporated (Figure 3). From this Figure, it can be extrapolated that copolymers with more than 22 % of HEMAm-Lac₄ are not soluble in water at 0 °C. Indeed, the copolymer with 24 % HEMAm-Lac₄ did not dissolve in water at 0 °C (Table 3).

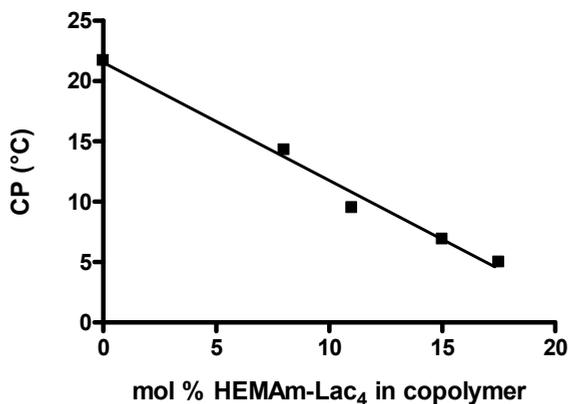


Figure 3 Cloud point (CP, °C) of p((HEMAm-Lac₂)-co-(HEMAm-Lac₄)) copolymers as a function of the mol percentage of HEMAm-Lac₄

Synthesis of mPEG-*b*-poly(HEMAm-oligolactates)

An mPEG₅₀₀₀-substituted azomacroinitiator initiated the free radical polymerisation of HEMAm-Lac_n (Figure 4) as reported previously for the synthesis of related block copolymers^{12, 22}. Amphiphilic polymer comprising either a HEMAm-Lac₂ or a p((20%HEMAm-Lac₄)-*co*-(80%HEMAm-Lac₂)) as thermosensitive block were synthesised (Table 4).

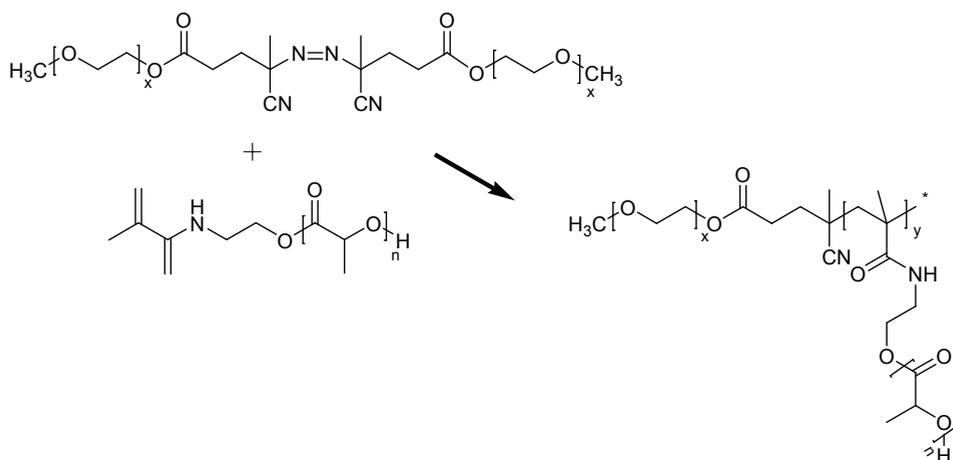


Figure 4 Synthesis of mPEG-*b*-p(HEMAm-Lac_n) block copolymers via an (mPEG₅₀₀₀)₂-azomacroinitiator

block copolymer	yield (%)	M _n	M _w /M _n	M _n Lac _n block	CMT (°C) ^a	CMC (mg/mL)	Z _{Ave} ^a	PD ^a
mPEG- <i>b</i> -(HEMAm-Lac ₂)	69	28000	1.5	10800	22	0.4	124	0.2
mPEG- <i>b</i> -((80%HEMAm-Lac ₂)-(20%HEMAm-Lac ₄))	85	24000	1.6	8700	6	0.08	80	0.1

Table 4 Characteristics of mPEG-*b*-p(HEMAm-Lac_n) block copolymers (^a 2 mg/mL solution, the M_n is given as g/mol, M_n and M_w/M_n are determined by GPC, the M_n of the Lac_n block is determined by ¹H-NMR)

Block copolymers were obtained in a high yield. $^1\text{H-NMR}$ showed that the amount of HEMAm-Lac₄ incorporated in mPEG-*b*-p((80%HEMAm-Lac₂)-*co*-(20%HEMAm-Lac₄)) was equal to the feed ratio (21 %). Micelles were formed during heating of an ice-cold polymer solution at the CP of the thermosensitive part of the block copolymer, which is 6 °C for mPEG-*b*-p((80%HEMAm-Lac₂)-*co*-(20%HEMAm-Lac₄)). Previous research has shown that the smallest and relatively monodisperse micelles are formed by applying the so-called fast heating method ²². The rapid heating from 0 till 50 °C results in full dehydration of the thermosensitive blocks and promotes the formation of a compact hydrophobic micellar core. The particle size and particle size distributions are displayed in Table 4. The incorporation of 20 % HEMAm-Lac₄ in the thermosensitive block resulted in a decrease of the CMT from 22 to 6 °C, but also in a significant decrease in particle size (from 124 to 80 nm). Obviously, longer lactic acid side chains increased the hydrophobic interactions and created a more compact micellar core. This observation is consistent with previous results from Soga *et al* ²². The morphology of mPEG-*b*-p((80%HEMAm-Lac₂)-*co*-(20%HEMAm-Lac₄))-micelles was studied with Cryo-TEM. A representative image (Figure 5) shows the spherical shape of the micelles as well as their narrow particle size distribution. Only the micellar core is visible since the density of mPEG is too low.

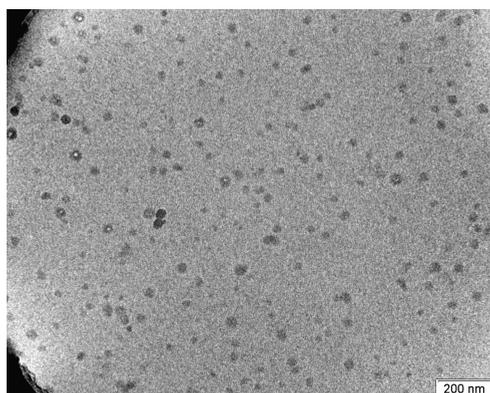


Figure 5 Cryo-TEM image of a mPEG-*b*-p((80%HEMAm-Lac₂)-*co*-(20%HEMAm-Lac₄)) micellar dispersion (polymer concentration 10 mg/mL in buffer pH 5.0)

The Cryo-TEM image shows that the radius of the hydrophobic core (R) of the micelles was between 10 and 23 nm, which corresponds to radius of gyration (R_g) ranging between 8 and 18 nm (using equation $R = \sqrt{(5/3) \cdot R_g}$).

Neutron scattering experiments for related block copolymers (mPEG₅₀₀₀-*b*-(HPMAm-Lac₂)) showed that the R_g of mPEG₅₀₀₀ is approximately 10 nm¹⁷, making the values of R_g of the mPEG-*b*-p((80%HEMAm-Lac₂)-*co*-(20%HEMAm-Lac₄)) micelles between 18 and 28 nm, which are in the range of the R_g measured by SLS (R_g = 31.4 nm; see section below). The critical micelle concentration was assessed with pyrene as a fluorescent probe³¹. The CMC was determined from the plot of the excitation intensity ratio I_{338}/I_{333} as a function of the concentration of the block copolymer (Figure 6).

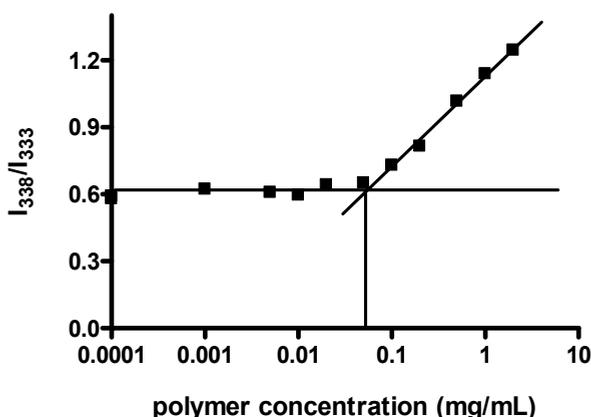


Figure 6 I_{338}/I_{333} ratio for pyrene as a function of the concentration of mPEG-*b*-p((80%HEMAm-Lac₂)-*co*-(20%HEMAm-Lac₄)) in acetate buffer (pH 5.0, 120 mM). The CMC was taken from the intersections of the horizontal line at low polymer concentrations with the tangens of the curve at high polymer concentrations.

mPEG-*b*-p(HEMAm-Lac₂) had a CMC of 0.4 mg/mL (Table 4) while mPEG-*b*-p((80%HEMAm-Lac₂)-*co*-(20%HEMAm-Lac₄)) formed micelles above a concentration of 0.08 mg/mL (Figure 6). This fivefold lower CMC for the latter system is likely because of stronger hydrophobic interactions in the core. In comparison, mPEG-*b*-(HPMAm-Lac₂) had a CMC of 0.03 mg/mL²². Again, due to the extra methyl group, HPMAm-Lac₂ is slightly more hydrophobic than HEMAm-Lac₂, which results in a lower CMC. Using the fast heating procedure, micelles were prepared from various polymer concentrations above the CMC (0.2 - 20 mg/mL) (Figure 7). Previously, it was described that rapid dehydration of the thermosensitive segments takes place and thereby well-defined core-shell structures were formed via the fast heating procedure²². Figure 7 shows that relatively large and polydisperse micelles are formed at concentrations below

0.5 mg/mL, which is close to the CMC. In the concentration range 0.5 - 10 mg/mL, the particle sizes of the micelles were relatively small (80 nm) and their polydispersity low (0.1 - 0.2). Above this concentration, the size and PD of the micelles slightly increases, which can be ascribed to a higher probability of interpolymer aggregation at higher polymer concentrations ¹¹.

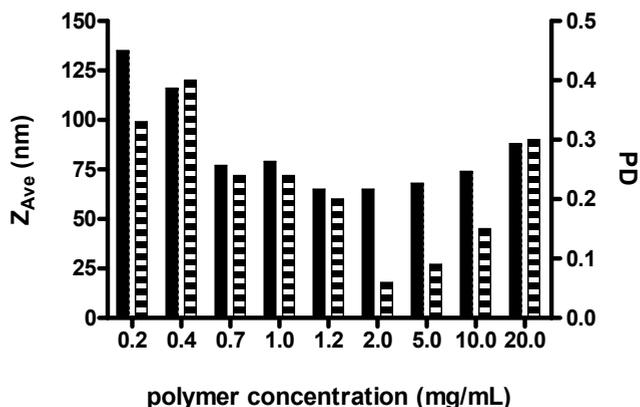


Figure 7 Size (filled bars) and polydispersity (dashed bars) of mPEG-*b*-p((80%HEMAM-Lac₂)-*co*-(20%HEMAM-Lac₄)) micelles solutions versus the block copolymer concentration ($n = 2$, experimental error < 10 %)

SLS analysis of mPEG-*b*-p((80%HEMAM-Lac₂)-*co*-(20%HEMAM-Lac₄)) micelles

Three micellar solutions of mPEG-*b*-p((80%HEMAM-Lac₂)-*co*-(20%HEMAM-Lac₄)) copolymers with concentrations 0.6, 0.8 and 1 mg/mL in pH 5.0 buffer were measured by SLS. By extrapolating the scattered intensities to zero concentration and by plotting $(K \cdot c)/R(q)$ against q^2 , both M_w and R_g were obtained from the “y” intercept to zero scattering angle and from the slope, respectively ³². For mPEG-*b*-p((80%HEMAM-Lac₂)-*co*-(20%HEMAM-Lac₄)) micelles, the M_w was $(27.0 \pm 1.7) \times 10^6$ g/mol and R_g was equal to 31.4 ± 5.0 nm. The ratio R_g/R_h can be used to examine the morphology of the self-assembled micelles. With an R_h of 40 ± 2 nm (Table 4), an R_g/R_h of 0.785 ± 0.131 was calculated. Recently, an R_g/R_h ratio of 0.855 was reported for a core-shell structure ²; for another core-shell structure a much lower value of R_g/R_h (0.66) was found and ascribed to a thick hydrated mPEG shell ¹⁵. The R_g/R_h value for

mPEG-*b*-p((80%HEMAm-Lac₂)-*co*-(20%HEMAm-Lac₄)) micelles is between the values mentioned above, suggesting a core-shell structure for this system. The aggregation number of the micelle was calculated using the equation $N_{agg} = M_w/M_{n,o}$, in which $M_{n,o}$ is the molar mass of the single copolymer chain obtained from ¹H-NMR measurements (e.g. the M_n for mPEG-*b*-p((80%HEMAm-Lac₂)-(20%HEMAm-Lac₄)) is 13700) and M_w is the weight average molecular weight of the micelle obtained from SLS. For the mPEG-*b*-((80%HEMAm-Lac₂)-*co*-(20%HEMAm-Lac₄)) micelles, N_{agg} is 1970. The density of the micelle was calculated: $\rho_{mic} = M_{w(mic)}/N_A \cdot V$, where N_A is Avogadro's number and V is the average volume of micelles (V was calculated via R_h). The surface area of the micellar shell available per mPEG chain (S/N_{agg}) was calculated by dividing S (surface area of the shell of micelles calculated based on R_h) by N_{agg} . For the mPEG-*b*-p((80%HEMAm-Lac₂)-*co*-(20%HEMAm-Lac₄)) micelles, the ρ_{mic} and S/N_{agg} are 0.167 g/cm³ and 10.2 nm², respectively. mPEG₅₀₀₀-*b*-p(HPMAm-Lac₂) (M_n is 11900) micelles gave comparable results with a ρ_{mic} of 0.16 g/cm³ and S/N_{agg} 12.7 nm². The small surface area per mPEG chain (i.e. higher grafting density) indicates that the micelles have a compact structure when compared to other micellar systems e.g. mPEG-*b*-PLA copolymers¹⁹. The distance between mPEG chains on the surface of nanoparticles (d) is critical to avoid adsorption of plasma proteins. For instance, it has been reported that a decrease in the distance between mPEG chains on the surface of polystyrene from 6.2 to 5.1 nm drastically decreases the adsorption of apolipoproteins up to 90 %²⁴. The distance d can be calculated via $\sqrt{(4 \cdot S)/\pi}$. For the mPEG-*b*-p((80%HEMAm-Lac₂)-*co*-(20%HEMAm-Lac₄)) micelles, it was calculated that the distance between neighbouring mPEG chains is 3.6 nm which will likely prevent adsorption of serum proteins.

Destabilisation of the micelles

Micelles prepared from mPEG-*b*-p((80%HEMAm-Lac₂)-*co*-(20%HEMAm-Lac₄)) were incubated in a phosphate buffer of pH 7.4 at 37 °C while following their size in time by DLS. The micelles were also incubated at pH 5.0 to slow down hydrolysis. Figure 8 shows that the micelles were stable for at least 18 hours at pH 5.0. At pH 7.4 and 37 °C, the particle size hardly changed during the first four hours, followed by a swelling phase until 6 hours. After that period, the micelles started to dissolve as seen by the measured scattering intensity that first increased (during 4 till 6 hours of incubation) and then dropped until below detectable levels (after 8 hours). Previously, Soga *et al*

showed that mPEG-*b*-p(HPMAm-Lac₂) micelles dissolved after approximately one week incubation at the same (physiological) conditions²². It was shown above (Table 1) that HEMA_m-oligolactates hydrolysed faster than the corresponding HPMAm-oligolactate monomers. In line with these results, polymeric micelles based on mPEG-*b*-p((80%HEMA_m-Lac₂)-*co*-(20%HEMA_m-Lac₄)) are far more sensitive to hydrolysis than the related HPMAm system and fully destabilised within eight hours.

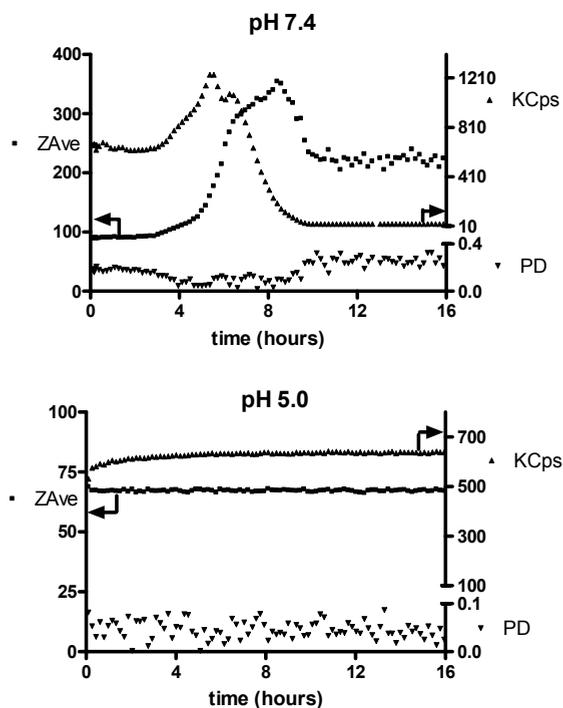


Figure 8 Z_{Ave} (■), PD (▼) and scattering intensity (▲) of a 2 mg/mL mPEG-*b*-p((80%HEMA_m-Lac₂)-*co*-(20%HEMA_m-Lac₄)) micellar solution versus time at pH 7.4 (left) and pH 5.0 (right) at 37 °C

Conclusion

This study shows that, as anticipated, *N*-(2-hydroxyethyl)methacrylamide-oligolactates (HEMAm-Lac_n) are more rapidly hydrolysed than *N*-(2-hydroxypropyl)methacrylamide-oligolactates (HPMAm-Lac_n). Thermosensitive (block co)polymers of HEMAm-oligolactates were synthesised in high yields by free radical polymerisation. It appeared that the CP was influenced by the composition of the HEMAm-oligolactates copolymer. Furthermore, the hydrophobicity of the thermosensitive block did not only influence the CMT, but also the CMC and the micellar particle size. Incorporation of 20 % of HEMAm-Lac₄ in the thermosensitive block resulted in a low CMT (6 °C), a low CMC (0.08 mg/mL) and small micelles (80 nm). Static light scattering measurements and Cryo-TEM images showed that the micelles have a compact core with a concomitant high density of mPEG chains on the surface that are a prerequisite to prevent the adsorption of serum proteins. Under physiological pH and temperature, micelles based on mPEG-*b*-p((80%HEMAm-Lac₂)-*co*-(20%HEMAm-Lac₄)) started to swell after 4 hours and were fully destabilised after 8 hours. This unique destabilisation profile might be advantageous for *in vivo* use because the observed induction period is just long enough to allow accumulation of the micelles after intravenous administration at their site of action, e.g. a tumour via the EPR effect. The drug loading capacity and release behaviour of these novel thermosensitive fast degrading polymeric micelles are currently under investigation.

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chapter 5

Photosensitiser-loaded biodegradable polymeric micelles: preparation, characterisation & *in vitro* PDT efficacy

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Abstract

The application of photosensitisers (PSs) in photodynamic therapy (PDT) is often hampered by their hydrophobicity, as this complicates their formulation and results in an unfavourable biodistribution like skin accumulation whereas tumour uptake is desirable. Thermosensitive mPEG-*b*-p(HPMAm-Lac₂) micelles were evaluated as carrier system for a very hydrophobic solketal-substituted phthalocyanine (Si(sol)₂Pc). Loaded micelles were prepared by rapid heating an aqueous polymer solution supplemented with 0.1 volume equivalent of Si(sol)₂Pc in THF to above the critical micelle temperature (CMT). DLS and Cryo-TEM measurements revealed that the self-assembled micelles had a size of approximately 75 nm with an encapsulation efficiency between 60 and 100 %. The highest concentration of Si(sol)₂Pc obtained in the micelles dispersion was 0.158 mg/mL (196 μM, 9 mg/mL polymer). Membrane filtration was used to concentrate the micellar dispersion to yield a Si(sol)₂Pc concentration of ~ 2 mg/mL. At low concentrations (≤ 0.05 μM, 0.45 mg/mL polymer), the PS was molecularly dissolved in the micellar core whereas, Si(sol)₂Pc was present in an aggregated form at higher concentrations (UV-vis spectrophotometry and fluorometry). In B16F10 and 14C cells, the photodynamic efficacy of Si(sol)₂Pc-loaded micelles (PS < 0.05 μM) was similar to free PS, for example the IC₅₀ was 3.0 ± 0.2 nM in the presence of 10 % serum in 14C cells. Remarkably, at higher serum concentrations, the photocytotoxicity of both micellar and free PS decreased similarly, e.g. the IC₅₀ of free Si(sol)₂Pc in the presence of 50 % serum was 14.0 ± 2.0 nM. Apparently, the molecularly dissolved PS in the micellar core easily diffuses into the medium but subsequent complexation with serum components inhibits the cellular uptake of the Si(sol)₂Pc. Cellular uptake experiments with PS-loaded micelles could not be performed under the same conditions of the photocytotoxicity studies, but required a higher PS concentration. Therefore, high-loaded micelles (10 μM Si(sol)₂Pc) were incubated with cells. The results demonstrated that the cellular uptake was low and independent of the serum concentration. Furthermore, the encapsulated aggregates of Si(sol)₂Pc in the micellar core were only released upon hydrolysis-induced micellar dissociation which was observed after 5.5 hours at pH 8.7 at 37 °C. The stability of the high-loaded micellar Si(sol)₂Pc formulation also in the presence of serum, the controlled release upon micellar disintegration and the high photocytotoxicity of the Si(sol)₂Pc make these micelles interesting for future *in vivo* studies.

Introduction

Photodynamic therapy (PDT) is a modality to treat localised tumours, e.g. neck/head cancer, oesophagus and skin malignities ^{1, 3, 12}. Upon illumination, a so-called photosensitiser (PS) converts oxygen into cytotoxic reactive oxygen species (ROS). These ROS do not only exert a direct toxic effect on cells ultimately resulting in necrosis or apoptosis ¹³, but also induce vascular damage leading to thrombosis and haemorrhages and finally induce an acute inflammation with resulting anti-tumour-immunity ^{4, 6, 7, 30}. Important advantages of PDT over other therapies and treatments are that it is less invasive than surgery, is precisely targeted through selective illumination and can be repeated several times at the same site if necessary.

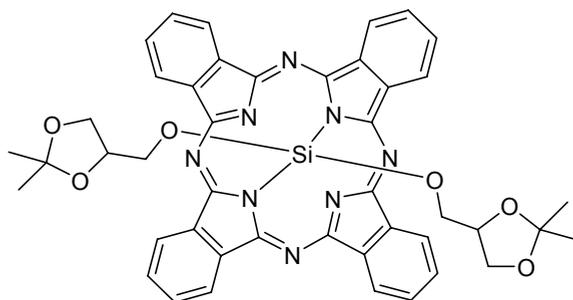


Figure 1 Chemical structure of the axially solketal-substituted silicon phthalocyanine $\text{Si(sol)}_2\text{Pc}$

For successful PDT, the availability of a suitable PS and a proper formulation is of crucial importance. The ideal PS has minimal dark toxicity, preferably accumulates in the target tissue, and is rapidly excreted from the body after illumination to prevent daylight induced side effects. Besides, it absorbs light of wavelengths higher than 600 nm to ensure the deepest tissue penetration and finally it has a high singlet oxygen quantum yield ^{2, 11}. However, the current generation of PSs evaluated in clinical trials and already on the market (e.g. Photofrin[®], Foscan[®], Visudyne^{® 2}) display several serious drawbacks, mainly caused by their hydrophobicity and non-selectivity. Their very hydrophobic character and thus poor water-solubility make them difficult to formulate while their aggregation tendency decreases the ROS formation ⁸. After intravenous (i.v.) administration of a PS formulated in ethanol - polypropylene glycol (Foscan[®]) or lipidic mixture (Visudyne[®]), the PSs are strongly bound to and transported by lipoproteins ^{15, 45}. Their biodistribution is

unfavourable and severe skin toxicity is observed¹. In a previous study, we demonstrated that the novel axially solketal-substituted silicon phthalocyanine Si(sol)₂Pc (Figure 1) had an very high photodynamic efficacy in both 14C and B16F10 cell lines¹⁷. However, Si(sol)₂Pc has a very low aqueous solubility and consequently a suitable formulation needs to be developed, which is the subject of the present paper.

The targeted delivery of PSs is investigated by their incorporation into various drug carriers like liposomes^{5, 10, 15, 28, 32}, dendrimers^{18, 22}, polymeric nanoparticles^{14, 23, 34, 43, 47, 48}, and polymeric micelles^{9, 24, 25, 29, 38, 39, 46}. Polymeric micelles are attractive delivery vehicles because their core can accommodate relatively large amounts of hydrophobic drugs²⁰, whereas the hydrophilic surface coating (mainly polyethylene glycol, PEG) prevents opsonisation and subsequent recognition by the mononuclear phagocyte system (MPS). Moreover, the small micellar size (~ 10 - 100 nm) enables tumour accumulation after i.v. administration due to the enhanced permeation and retention (EPR) effect¹⁹. Furthermore, micelles have been developed that are sensitive to environmental triggers³⁵ by which their contents are released 'on-demand'.

Biodegradable thermosensitive polymeric micelles are potentially suitable for the selective and specific delivery of hydrophobic drugs^{36, 41, 42}. For example, the thermosensitive block copolymer ω-methoxy poly(ethyleneglycol)-*block*-poly(*N*-(2-hydroxypropyl)methacrylamide-dilactate) (mPEG-*b*-p(HPMAm-Lac₂)) self-assembled in water into small micelles (~ 60 nm) above their critical micelle temperature (CMT). Hydrolysis of the hydrophobic lactate side chains at physiological conditions gradually increased the CMT and, as a consequence, after approximately one week the micelles fell apart. The cytostatic drug paclitaxel was almost quantitatively encapsulated by the micelles up to a loading of 22 % (w/w) without affecting the micellar size⁴¹. The final degradation products of the block copolymers (PEG, pHPMAm and lactic acid) are known to be safe and non-toxic, which is highly favourable in terms of biocompatibility. These biodegradable micelles may also be very attractive to formulate and selectively deliver hydrophobic PSs to tumour tissue. Therefore, the aim of this study was to encapsulate Si(sol)₂Pc in mPEG-*b*-p(HPMAm-Lac₂) micelles and to study their release behaviour. Next, the *in vitro* uptake and photocytotoxicity of the micellar formulated Si(sol)₂Pc were investigated in B16F10 and 14C cancer cell lines and compared to that of free PS.

Materials & methods

Materials

Bovine serum albumin (BSA) was from Sigma Aldrich (Zwijndrecht, The Netherlands). Dimethylformamide (DMF) and tetrahydrofuran (THF) were purchased from Biosolve Ltd. (Valkenswaard, The Netherlands). All buffers were filtered through 0.2 μm filters (Schleicher & Schuell MicroScience GmbH, Dassel, Germany) prior to use. The other chemicals were used as received. $\text{Si}(\text{sol})_2\text{Pc}$ and the block copolymer $\text{mPEG}_{5000}\text{-}b\text{-p}(\text{HPMam-Lac}_2)$ were synthesised and characterised as described before; the number average molecular weight of the polymer was 21000 g/mol as determined by GPC (M_w/M_n was 1.6)^{17, 42}. The murine B16F10 cell line was kindly provided by Prof. Dr. Ernst Wagner (Pharmaceutical Biology-Biotechnology, Department of Pharmacy, Ludwig-Maximilians-Universität, Munich, Germany) and was originally obtained from I.J. Fidler (Texas Medical Center, Houston, USA). B16F10 cells were cultured in DMEM 1 (Dulbecco's modification of Eagle's Medium, with 3.7 g/L sodium bicarbonate, 1 g/L glucose, Gibco BRL, Breda, The Netherlands), completed with antibiotics/antimycotics (100 IU penicillin G sodium/mL, 100 μg streptomycin sulphate/mL and 0.25 μg amphotericin B/mL, Gibco BRL, Breda, The Netherlands), L-glutamine (2 mM, Gibco BRL, Breda, The Netherlands), and 10 % foetal bovine serum (FBS, Integro, Zaandam, The Netherlands). The human head and neck squamous carcinoma cell line UM-SCC-14C (developed by Dr. T.E. Carey, Ann Arbor, MI, and further abbreviated as 14C) was kindly provided by Prof. Dr. G.A.M.S. van Dongen (Department of Otolaryngology/Head and Neck Surgery, VU University Medical Center, Amsterdam, The Netherlands). This cell line was cultured in DMEM 2 (Dulbecco's modification of Eagle's Medium, with 3.7 g/L sodium bicarbonate, 4.5 g/L glucose, Gibco BRL, Breda, The Netherlands), completed with antibiotics/antimycotics and 5 % FBS.

Micelle formation & loading with $\text{Si}(\text{sol})_2\text{Pc}$

Micelles were formed via the 'rapid heating' procedure as described previously^{36, 41}. In brief, the block copolymer was dissolved (2 - 10 mg/mL) overnight at 4 °C in ammonium acetate buffer (pH 5, 120 mM) or in phosphate buffer (pH 7.4, 100 mM). The solutions (1 - 4 mL) were incubated at 0 °C for at least 15 min in glass vials. Subsequently, the vials were rapidly heated by putting them into a water bath kept at 50 °C while vigorously stirring to form micelles. $\text{Si}(\text{sol})_2\text{Pc}$ -loaded micelles were formed by addition of a 0.1 volume

equivalent of Si(sol)₂Pc solution in THF to the polymer solution immediately before heating. After 1 min of incubation at 50 °C, the mixtures were slowly cooled down to room temperature. To remove non-encapsulated Si(sol)₂Pc, the micelles were filtrated through a 0.45 µm filter. The micellar dispersions were concentrated by membrane filtration using Vivaspin® vials with a MWCO of 5000 (Sartorius AG, Germany), while assuring that the temperature remained above 20 °C. The starting volume was ~ 3 mL and after centrifugation (3000 rpm) for 10 - 180 minutes, the volume reduction was 1.5 to 15 times.

Dynamic Light Scattering (DLS)

Dynamic light scattering (DLS) was used to determine the size and size distribution of the polymeric micelles. The equipment consisted of a Malvern CGS-3 multi-angle goniometer (Malvern Ltd., UK) with He-Ne JDS Uniphase laser ($\lambda = 632.8$ nm, 22 mW output power), an optical fibre based detector, a digital LV/LSE- 5003 correlator, and a temperature controller (Julabo water bath). Time correlation functions were analysed using the ALV-60.0 software V.3.X provided by Malvern. Scattering of the micellar solutions was measured at an angle of 90 ° and at 25 °C in an optical quality 8 mL borosilicate cell thereby giving the average hydrodynamic diameter of the micelles (Z_{Ave}) and their polydispersity (PD).

Determination of the Si(sol)₂Pc concentration

The concentration of Si(sol)₂Pc in the micellar dispersions was determined by diluting the micelles with DMF and measuring the absorbance at 674 nm using a Perkin-Elmer Lambda 2 UV-vis spectrophotometer. A calibration curve of Si(sol)₂Pc in DMF was linear between 0.05 and 3.1 µg/mL. The encapsulation efficiency (EE) and loading capacity (LC) were calculated as follows:

$$EE \% = \frac{\text{concentration Si(sol)}_2\text{Pc measured}}{\text{concentration Si(sol)}_2\text{Pc added}} * 100 \%$$

$$LC \% (w/w) = \frac{\text{concentration Si(sol)}_2\text{Pc measured}}{\text{concentration polymer added}} * 100 \%$$

Cryogenic Transmission Electron Microscopy (Cryo-TEM)

Cryo-TEM measurements were performed on micellar solutions in ammonium acetate buffer (pH 5.0, 120 mM), i.e. empty micelles (10 mg/mL

polymer), loaded micelles (0.15 mg/mL Si(sol)₂Pc, 9 mg/mL polymer) and concentrated dispersions (approximately 20 mg/mL polymer, 1.25 mg/mL Si(sol)₂Pc) of empty and loaded micelles. The sample preparation was done in a temperature and humidity controlled chamber using a “Vitrobot”. A thin aqueous film of micellar dispersion was formed by blotting a 200 mesh copper grid covered with Quantifoil holey carbon foil (Micro Tools GmbH, Germany) at 22 °C and at 100 % relative humidity (glow discharged grid; 1 blot during 0.5 sec). The thin film was rapidly vitrified by quickly plunging the grid into liquid ethane. The grids with the vitrified thin films were transferred into the microscope chamber using a GATAN 626 cryo-holder system. A Tecnai12 transmission electron microscope (Philips) operating at 120kV was used with the specimen at -180 °C and using low-dose imaging conditions to avoid melting of the vitrified film. Images were recorded on a SIS-CCD camera and processed with AnalySIS software.

Physical state of Si(sol)₂Pc encapsulated in the micelles

Micelles with various Si(sol)₂Pc loadings were prepared as described above and diluted 1:20 with ammonium acetate buffer (pH 5, 120 mM) to obtain final concentrations of Si(sol)₂Pc between 0.001 and 12.56 μM while the polymer concentration was kept at 0.45 mg/mL. The UV-vis spectrum (300 - 800 nm) and fluorescence ($\lambda_{\text{ex}} = 674 \text{ nm}$, $\lambda_{\text{em}} = 679 \text{ nm}$; Horiba Fluorolog fluorometer) of these micellar dispersions were recorded.

Photocytotoxicity

For the illumination of well plates, in which cells were seeded, a home made device consisting of 96 LED lamps ($670 \pm 10 \text{ nm}$, 1 LED/well) was used and connected to a water bath thermostated at 37 °C. The well plate was placed on top of the LED-device with a water layer in between to control the temperature (preventing excessive heating) and to give a homogeneous illumination area. Illumination time and intensity of the LED lamps could be adjusted by a controller. The intensity of the emitted light was measured at the height of the well plate by an Orion Laser power/energy monitor (Ophir Optronics LTD, Jerusalem, Israel). Cells were seeded in 96 well tissue culture plates (Greiner) at 2×10^4 cells per well (100 μL cell suspension) and cultured overnight at 37 °C under a 5 % CO₂ atmosphere. Freshly prepared micellar solutions or stock solutions of Si(sol)₂Pc in THF were diluted in medium containing different amounts of FBS. The concentration of the organic solvent was less than 0.5 %

(v/v); a concentration at which no effect on the cell viability was observed. The medium was removed from the wells, 100 μL of fresh medium with different concentrations of $\text{Si}(\text{sol})_2\text{Pc}$ was added and the plates were incubated at 37 °C under a 5 % CO_2 atmosphere for 6 hours. For every plate another identical plate was prepared, which was kept in the dark as a control. After incubation, the medium was removed and the cells were washed twice with 100 μL PBS and 100 μL of fresh medium was added. The plates were illuminated for 10 min with 3.5 mW/cm^2 light intensity, after which the cells were incubated overnight at 37 °C under a 5 % CO_2 atmosphere. Next, the cell survival, relative to cells without added photosensitiser, was determined by a colorimetric assay, using the tetrazolium salt XTT (sodium 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide ³⁷ (Sigma-Aldrich), according to the manufacturers instructions.

Cellular uptake

B16F10 and 14C cells were seeded in a 24 well plate at 15×10^4 cells per well (1000 μL cell suspension) and the plates were incubated overnight at 37 °C under a 5 % CO_2 atmosphere. The medium was removed and 500 μL fresh medium, containing 10 μM of $\text{Si}(\text{sol})_2\text{Pc}$ (either a 200-fold dilution in medium of a $\text{Si}(\text{sol})_2\text{Pc}$ stock solution or a 20-fold dilution in medium of $\text{Si}(\text{sol})_2\text{Pc}$ -loaded micelles (final polymer concentration constantly 0.45 mg/mL)), was added and the cells were incubated for 6 h at 37 °C under a 5 % CO_2 atmosphere. The medium was removed and the cells were washed twice with 500 μL PBS. To lyse the cells, 500 μL of lysis buffer (50 mM Tris/HCl (pH 8), 150 mM NaCl and 1 % Triton X-100) was added followed by incubation on ice for 20 min. The concentration of photosensitiser in the cell lysate was determined by dilution of 100 μL of the cell lysate with 900 μL DMF and measuring the fluorescence of the samples ($\lambda_{\text{ex}} = 674 \text{ nm}$, $\lambda_{\text{em}} = 679 \text{ nm}$). Calibration curves were prepared by dilution of photosensitiser stock solutions in DMF with cell lysate to a final lysate concentration of 10 % (v/v). An aliquot (20 μL) of the cell lysates was used for determination of the cellular protein content, with the Micro BCATM protein assay ⁴⁰ (Pierce, Rockford, USA), according to the instructions of the supplier. The uptake of photosensitiser was calculated as nmol $\text{Si}(\text{sol})_2\text{Pc}$ per mg of cellular protein.

Intracellular photosensitiser localisation

To promote cell adhesion on the plates, 35 mm glass bottom microwell dishes (MatTek Corporation, Ashland, USA) were pre-coated with gelatin (10 %, Sigma) by incubation for 4 hours at 37 °C. Cell suspensions of 14C or B16F10 cells (2×10^5 cells in 1.5 mL) were added to the plates and they were incubated overnight at 37 °C under a 5 % CO₂ atmosphere. The medium was removed and the sample solution (1.5 mL), diluted in medium and supplemented with FBS, was added. The cells were incubated for 6 hours at 37 °C. To visualise different organelles, cells pre-incubated for 6h with or without photosensitiser were incubated for 5 minutes at 37 °C with two different tracker dyes, i.e. lysotracker green (Invitrogen, 130 nM, from 0.1 mM stock in DMSO) and Nile red (Invitrogen, 160 nM, from 1 mg/mL stock in DMSO). The cells were examined with a laser scanning confocal fluorescence microscope (Nikon Eclipse TE2000-U microscope), equipped with a C1 scanning unit, three excitation lasers, i.e. Argon-Ion laser (488 nm), He-Ne laser (543 nm), and He-Ne laser (633 nm), two bandpass emission filters, i.e. 515 ± 15 nm and 585 ± 15 nm, and one longpass emission filter (665 nm).

pH-dependent release of Si(sol)₂Pc

Approximately 2 mL of Si(sol)₂Pc loaded micelles (250 µM PS, 9 mg/mL polymer) were transferred into dialysis cassettes (MWCO 3.5 kDa, 0.5 - 3 mL, Pierce, Perbio Sciences, Etten-Leur, Netherlands) and dialysed against a 300-fold excess of either ammonium acetate buffer (pH 5, 120 mM) or Tris buffer (pH 8.5, 100 mM) at 37 °C. In another dialysis experiment, the buffer solution was supplemented with 1 % BSA to explore a possible effect of this serum protein on the release of the PS from the loaded micelles. At regular time intervals, a sample was taken from the content of the dialysis bag and the dialysate and the concentration Si(sol)₂Pc was determined spectrophotometrically.

Effect of serum on the integrity of empty & loaded micelles

Si(sol)₂Pc-loaded micelles were tenfold diluted with sodium borate buffer (pH 9, 100 mM) and the fluorescence of 1 mL micellar dispersion was measured ($\lambda_{\text{ex}} = 674$ nm, $\lambda_{\text{em}} = 679$ nm). After 3 minutes, 1 mL of a serum dilution in phosphate buffer (pH 7.4, 100 mM) was added to obtain final FBS concentrations of 0, 10, 25 and 50 %. The concentration Si(sol)₂Pc and polymer

were 12.5 μM and 0.45 mg/mL, respectively, and the pH was 8.7. The fluorescence of the solution was measured for at least 12 hours at 37 °C. Similarly, UV-spectra (550 - 750 nm) of an equal micellar dilution in buffer (final FBS concentration of 25 %) were also recorded in time at 37 °C. Besides, the fluorescence of free $\text{Si}(\text{sol})_2\text{Pc}$ ($\lambda_{\text{ex}} = 674 \text{ nm}$, $\lambda_{\text{em}} = 679 \text{ nm}$) in 10, 25 or 50 % FBS was measured as function of the PS concentration. Therefore, 5 μL $\text{Si}(\text{sol})_2\text{Pc}$ (various concentrations in THF) was added to 995 μL of a serum solution (comprising a dilution of FBS in PBS buffer (pH 7.4, 100 mM) and sodium borate buffer (pH 9, 100 mM), ratio 1:1) to yield final $\text{Si}(\text{sol})_2\text{Pc}$ concentrations of 0.015 to 3 μM .

Results & discussion

Encapsulation of Si(sol)₂Pc in micelles

As the first step in the development of photosensitiser-loaded biodegradable micelles for targeted delivery purposes, the encapsulation of Si(sol)₂Pc in mPEG-*b*-p(HPMAm-Lac₂) micelles was investigated and the resulting nanoparticles were characterised. Si(sol)₂Pc-loaded micelles were formed by addition of a 0.1 volume equivalent of Si(sol)₂Pc in THF to a 2 or 10 mg/mL polymer solution in buffer (pH 7.4) followed by rapid heating from 0 to 50 °C (i.e. to above the CMT of 8 °C⁴²). After micelle formation, the resulting solution was filtered (0.45 µm) to remove non-encapsulated drug present in the form of aggregates. The size (Z_{Ave}) and polydispersity (PD) of loaded micelles as well as the solubilised concentration of Si(sol)₂Pc, the encapsulation efficiency (EE %) and loading capacity (LC %) are given in Table 1. Empty mPEG-*b*-p(HPMAm-Lac₂) micelles had a diameter of 64 nm. Addition of a 0.1 volume equivalent of THF increased the particle size with 14 nm which is in agreement with previous data⁴¹. In presence of 10 % THF, the hydrophobic interactions between the core forming blocks are not as pronounced as in water, resulting in a slightly swollen core and an increased particle diameter. Encapsulation of a low amount (≤ 0.05 mg/mL, 62 µM) of Si(sol)₂Pc did not influence the particle size although the PD increased slightly, e.g. from 0.10 to 0.22 upon addition of 0.05 mg/mL PS. No reliable DLS measurements on micellar dispersions with Si(sol)₂Pc concentrations higher than 0.05 mg/mL could be done due to absorption of the laser light ($\lambda = 632$ nm) by the PS. Si(sol)₂Pc was encapsulated in the micelles (9 mg/mL) with an efficiency of 60 - 75 % for the higher concentrations (43 - 196 µM) and quantitatively for the lower concentrations (1 - 1000 nM) added. The highest concentration of Si(sol)₂Pc that could be achieved in the 9 mg/mL micelles was 0.158 ± 0.006 mg/mL with an LC of 1.75 % (w/w). The low solubility of Si(sol)₂Pc in THF (2.5 mg/mL) hampered to obtain a higher concentration of Si(sol)₂Pc in the micellar cores. A lower polymer concentration (1.8 mg/mL) resulted in equal encapsulation efficiency (70 - 80 %) and increased the LC up to 10 % (w/w). It can be concluded that the mPEG-*b*-p(HPMAm-Lac₂) micelles efficiently encapsulate the hydrophobic Si(sol)₂Pc. Other PS-micellar systems displayed similar EE, e.g. ~ 90 % of meso-tetraphenyl porphine in pluronic micelles (LC varied between 4 and 8 %) ³⁸ or 50 - 60 % of aluminium phthalocyanine in pH-responsive polymeric micelles (LC approximately 3 %) ⁴⁴.

Si(sol) ₂ Pc added mg/mL	Si(sol) ₂ Pc measured mg/mL	Si(sol) ₂ Pc measured μ M	EE %	polymer mg/mL	LC % (w/w)	Z _{Ave} (nm)	PD
0.25	0.158 ± 0.006	196	63 ± 2	9	1.75 ± 0.07	n.p.	n.p.
0.20	0.141 ± 0.039	176	70 ± 20	9	1.57 ± 0.44	n.p.	n.p.
0.15	0.093 ± 0.013	116	62 ± 9	9	1.04 ± 0.15	n.p.	n.p.
0.10	0.075 ± 0.011	94	75 ± 11	9	0.83 ± 0.12	n.p.	n.p.
0.05	0.035 ± 0.007	43	70 ± 13	9	0.39 ± 0.07	76 ± 10	0.22 ± 0.04
0.25	0.189 ± 0.024	235	74 ± 6	1.8	10.5 ± 1.4	n.p.	n.p.
0.05	0.039 ± 0.004	49	79 ± 9	1.8	2.2 ± 0.3	78 ± 13	0.37 ± 0.02
16 × 10 ⁻⁵	(17.1 ± 0.1) × 10 ⁻⁵	0.214	107 ± 1	9	(20.1 ± 0.1) × 10 ⁻⁴	n.d.	n.d.
14.1 × 10 ⁻⁶	(14.1 ± 0.1) × 10 ⁻⁶	0.017	98 ± 1	9	(20.1 ± 0.1) × 10 ⁻⁵	n.d.	n.d.
0	n.a.	n.a.	n.a.	9	n.a.	78 ± 3	0.10 ± 0.03
without THF	n.a.	n.a.	n.a.	10	n.a.	64 ± 1	0.03 ± 0.02

Table 1 Particle size (Z_{Ave}), polydispersity (PD), encapsulation efficiency (EE %) and loading capacity (LC %) of Si(sol)₂Pc-loaded mPEG-*b*-p(HPMAM-Lac₂) micelles (final polymer concentration was 1.8 or 9 mg/mL, n.a. = not applicable, n.p. = not possible, n.d. = not determined, these results are the average ± SD of three independently prepared micelle formulations)

Previously, mPEG-*b*-p(HPMAm-Lac₂) micelles were able to solubilise the hydrophobic cytostatic agent paclitaxel up to a final concentration of 2 mg/mL (9 mg/mL polymer)⁴¹. To increase the Si(sol)₂Pc concentration in the formulation, the loaded micelles (polymer concentration of 1.8 mg/mL) were concentrated by membrane filtration using Vivaspin tubes (MWCO 5000) at room temperature (i.e. above their CMT of 8 °C) (Table 2).

initial Si(sol) ₂ Pc (mg/mL)	centrifugation time (min)	Si(sol) ₂ Pc after centrifugation (mg/mL)
0.10	10	0.12
0.10	20	0.15
0.10	45	0.21
0.159	150	1.26
0.163	180	2.16

Table 2 Concentration of Si(sol)₂Pc encapsulated in micelles (1.8 mg/mL polymer) through membrane filtration (original volume ~ 3 mL; centrifugation speed 3000 rpm)

After 180 minutes centrifugation, a Si(sol)₂Pc concentration of 2.16 mg/mL was achieved which is comparable with the solubility in an organic solvent (e.g. THF). Thus, by incorporating the Si(sol)₂Pc in polymeric micelles, the water insolubility of the PS is easily circumvented and these aqueous solutions are concentrated enough to be administered at a typical therapeutic dose of 1 mg/kg in animals for *in vivo* PDT evaluations. To determine the possible effect of the concentration procedure on the size of the micelles, empty micelles (1.8 mg/mL polymer, size 70 nm) were centrifuged for 45 minutes. The resulting size (by DLS) was 66 nm with a PD of 0.05, indicating micellar integrity upon membrane filtration. As DLS measurements were not possible with loaded micelles containing > 0.05 mg/mL PS due to light absorption by the laser (vide supra), empty and Si(sol)₂Pc-loaded micelles were examined, before and after the concentration step, with Cryo-TEM (Figure 2). All micelles were observed as almost monodisperse spherical structures with a micellar core diameter of ~ 52 nm. The PEG₅₀₀₀ shell of the micelles (not visualised with Cryo-TEM) comprises approximately 7 nm³³ making the final micelle diameter 66 nm, which is in excellent agreement with the DLS measurements on empty micelles.

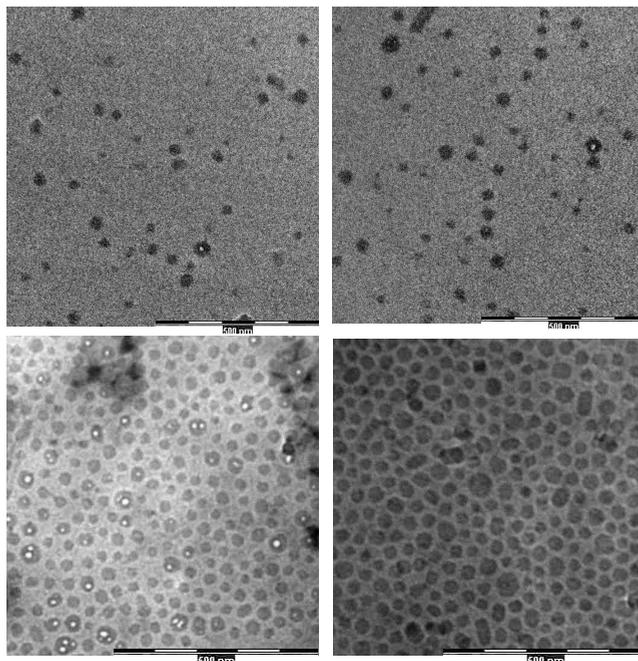


Figure 2 Cryo-TEM images of empty micelles (10 mg/mL, upper left), Si(sol)₂Pc-loaded micelles (0.15 mg/mL PS, 9 mg/mL polymer, upper right), concentrated empty micelles (~ 20 mg/mL, lower left) and concentrated Si(sol)₂Pc-loaded micelles (1.25 mg/mL PS, ~ 20 mg/mL polymer, lower right)

Physical state of Si(sol)₂Pc encapsulated in the micelles

The physical state of Si(sol)₂Pc in high-loaded micelles (6.2 μM PS, 0.45 mg/mL polymer, LC 0.9 %) was determined by UV-vis spectrophotometry (Figure 3). Moreover, absorption spectra of free Si(sol)₂Pc were also measured in DMF and in 10 % FBS (in both cases 6.2 μM PS). Compared to the molecularly dissolved phthalocyanine in DMF, the absorption of Si(sol)₂Pc loaded in the micelles was clearly lower (Figure 3). Besides, the Q-band displayed a broadening as well as bathochromic shift of 15 nm (from 674 nm to 689 nm). Also, the two small vibrational bands of the PS in DMF at 645 and 606 nm had merged to one broad peak with a maximum at 640 nm. Furthermore, the B- or Soret band at 350 nm was shifted to a higher wavelength (+ 9 nm). From this UV-vis spectrum, it can be concluded that Si(sol)₂Pc is present in an aggregated form in the micellar core^{16, 21}. Si(sol)₂Pc in 10 % serum also showed a broad and shifted Q-band (to 694 nm) which points to aggregation.

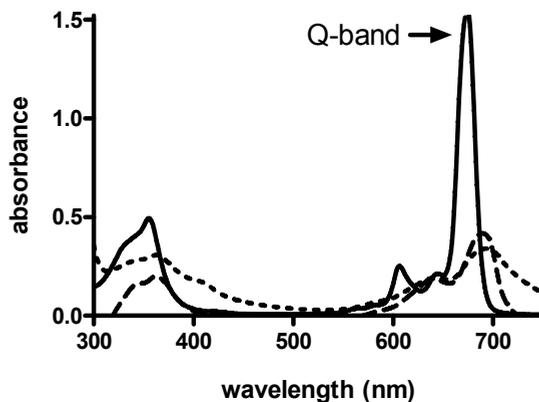


Figure 3 UV-vis spectra of (- -) $\text{Si}(\text{sol})_2\text{Pc}$ -loaded micelles in buffer ($6.2 \mu\text{M}$ $\text{Si}(\text{sol})_2\text{Pc}$, 0.45 mg/mL polymer), (...) $\text{Si}(\text{sol})_2\text{Pc}$ ($6.2 \mu\text{M}$) in 10 % FBS and (—) $\text{Si}(\text{sol})_2\text{Pc}$ ($6.2 \mu\text{M}$) in DMF

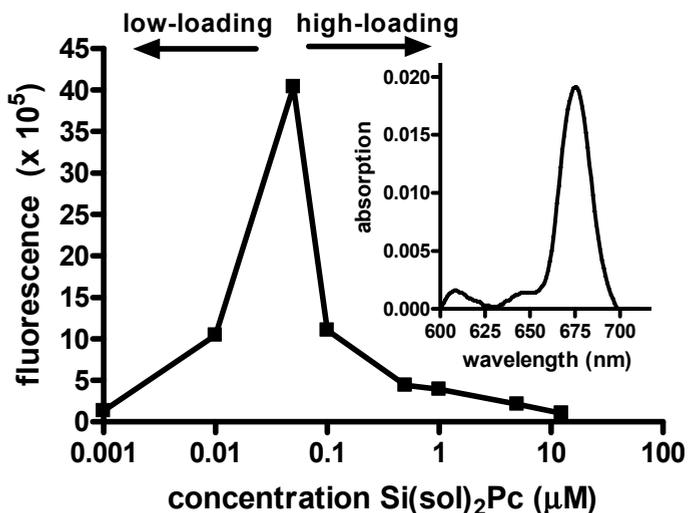


Figure 4 Fluorescence of $\text{Si}(\text{sol})_2\text{Pc}$ -loaded micelles (0.45 mg/mL polymer, $\lambda_{\text{ex}} = 674 \text{ nm}$, $\lambda_{\text{em}} = 679 \text{ nm}$) as function of the PS concentration (The insert shows the UV-vis spectrum of the micelle formulation with a $\text{Si}(\text{sol})_2\text{Pc}$ concentration of $0.05 \mu\text{M}$)

The physical state of Si(sol)₂Pc in the micellar core was also studied with fluorescence spectroscopy for various PS concentrations (Figure 4). Clearly, Si(sol)₂Pc concentrations higher than 0.05 μM lead to severe fluorescence quenching likely because the denser packing of PS molecules in the micellar core results in π-π interactions and/or aggregation, as was observed by UV-vis spectroscopy in the highly loaded micelles at 6.2 μM. An absorption spectrum was recorded at the concentration of the maximum fluorescence (0.05 μM, insert of Figure 4), which indeed showed a Q-band similar to that of Si(sol)₂Pc in DMF. This indicates that at this low concentration, the PS is present in the micelles in a non-aggregated, dissolved state. Hence, based on the aggregation state of the encapsulated PS, the Si(sol)₂Pc-loaded micelles (at a polymer concentration of 0.45 mg/mL) are defined as low-loaded micelles (≤ 0.05 μM) and high-loaded micelles (> 0.05 μM), respectively.

Photocytotoxicity of Si(sol)₂Pc-loaded micelles

For the *in vitro* experiments, micellar integrity was assured by taking a final polymer concentration of at least 0.45 mg/mL, which is 15 times above the critical micelle concentration (CMC) of mPEG-*b*-p(HPMAm-Lac₂) micelles (CMC is 0.03 mg/mL)⁴². To assess whether the polymeric micelles are resistant against the illumination procedure used in the PDT experiments, empty micelles (in the absence or presence of 10 % THF) and micelles loaded with Si(sol)₂Pc (6.2 μM) were illuminated at 670 nm for 10 min with a fluence rate of 3.5 mW/cm². The micellar size and PD were measured by DLS before and after the illumination (Table 3). The results indicated that the light alone as well as the ROS formation upon illumination of the Si(sol)₂Pc-loaded micelles did not affect the micellar characteristics.

	before illumination		after illumination	
	Z _{Ave} (nm)	PD	Z _{Ave} (nm)	PD
micelles	62.2	0.06	63.4	0.03
micelles (in presence of 10 % THF)	75.0	0.09	71.2	0.07
micelles containing Si(sol) ₂ Pc (6.2 μM)	76.4	0.24	77.8	0.22

Table 3 Effect of illumination on the integrity of (Si(sol)₂Pc-loaded) mPEG-*b*-p(HPMAm-Lac₂) micelles (polymer concentration 0.45 mg/mL, 10 min illumination at 670 nm with a fluence rate of 3.5 mW/cm²)

The photocytotoxicity of Si(sol)₂Pc-loaded micelles towards B16F10 and 14C cells was determined after 6 hours of incubation in medium with 10 % serum and compared with free Si(sol)₂Pc. The polymer concentration was fixed (0.45 mg/mL) whereas the Si(sol)₂Pc concentration was varied (Figure 5). Both the free PS and the micellar formulation displayed no dark toxicity while the photocytotoxicity of Si(sol)₂Pc-loaded micelles was comparable to that of free PS in both cell lines: the IC₅₀ values of the free PS and the micellar formulation were approximately 5 and 3 nM for B16F10 and 14C cells, respectively.

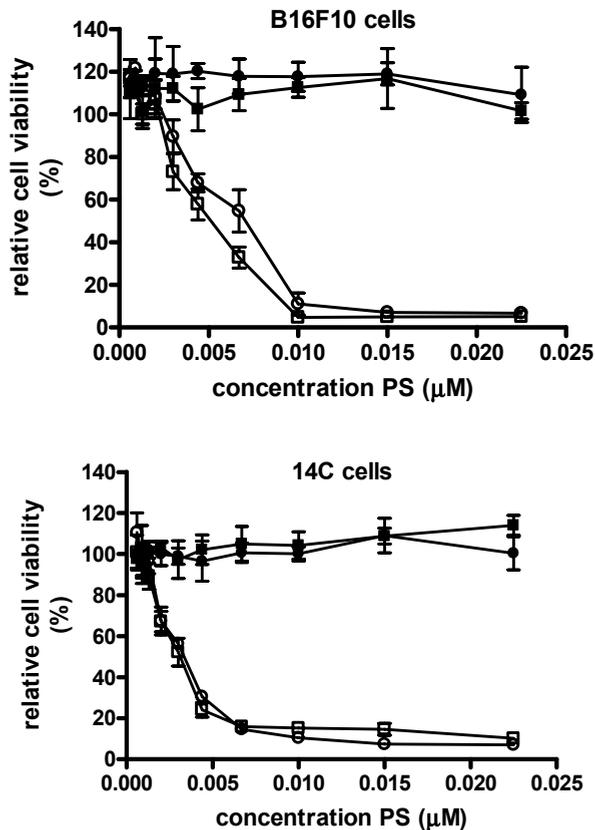


Figure 5 Relative cell viability of B16F10 and 14C cells after 6 hours of incubation in presence of free Si(sol)₂Pc (squares) and Si(sol)₂Pc-loaded micelles (circles, 0.45 mg/mL polymer) in 10 % FBS, followed by illumination for 10 min with 3.5 mW/cm² (open symbols) and without illumination (closed symbols) (n = 3 ± SD)

	0 % FBS	10 % FBS	25 % FBS	50 % FBS
free Si(sol) ₂ Pc	0.8 ± 0.1	3.0 ± 0.2	6.0 ± 0.5	14.0 ± 2.0
Si(sol) ₂ Pc in micelles	0.8 ± 0.1	3.2 ± 0.1	3.5 ± 0.1	8.0 ± 0.1

Table 4 Photocytotoxicity expressed as IC₅₀ values (nM) of free Si(sol)₂Pc and Si(sol)₂Pc loaded in micelles (0.45 mg/mL polymer) in 14C cells after 6 hours of incubation in medium with different concentrations of FBS, followed by illumination for 10 min with 3.5 mW/cm² (n = 3 ± SD)

The photocytotoxicity of Si(sol)₂Pc, either formulated in micelles or in its free form, was also investigated at different serum concentrations in 14C cells (Table 4). Unexpectedly, the photocytotoxicity of both free and micellar encapsulated Si(sol)₂Pc decreased upon increasing FBS concentration (as reflected by higher IC₅₀ values, which for example increased 10 - 15 times in presence of 50 % FBS). Similarly, the photodynamic efficacy of Si(sol)₂Pc-loaded micelles was inversely correlated with the FBS concentration (Table 4).

Cellular uptake of Si(sol)₂Pc-loaded micelles

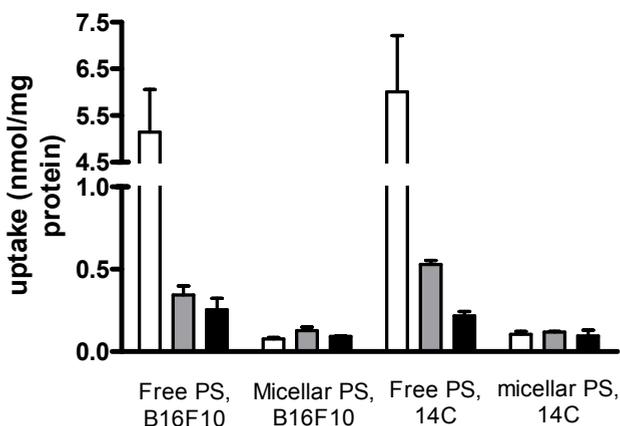


Figure 6 Cellular uptake of Si(sol)₂Pc (nmol/mg protein) by B16F10 and 14C cells after 6 hours of incubation, either administered as free drug or encapsulated in mPEG-*b*-p(HPMAM-Lac₂) micelles (10 μM Si(sol)₂Pc, 0.45 mg/mL polymer) (White bars represent the uptake from medium without FBS, grey bars represent the uptake from medium supplemented with 10 % FBS for B16F10 cells and 5 % FBS for 14C cells and the black bars show the uptake from medium supplemented with 50 % or 25 % FBS for B16F10 and 14C cells, respectively)

Given that the serum concentration adversely affects the photocytotoxicity of the PS, the corresponding cellular uptake of free $\text{Si}(\text{sol})_2\text{Pc}$ or encapsulated in micelles by B16F10 and 14C cells was also investigated at different serum concentrations. Unfortunately, no uptake studies could be carried out with low-loaded micelles, due to the detection limit of the fluorescence measurements, and no photocytotoxicity studies could be carried out with high-loaded micelles because that would require the dilution of the micelles far below the CMC. After 6 hours of incubation with $\text{Si}(\text{sol})_2\text{Pc}$ (in its free form or formulated in high-loaded micelles, 10 μM), the cells were lysed and the intracellular amount of PS was determined by fluorescence spectroscopy (Figure 6). Although free $\text{Si}(\text{sol})_2\text{Pc}$ is aggregated in the incubation medium in absence of FBS (data not shown), the cellular uptake is relatively high, i.e. 5 nmol per mg protein. The uptake of free $\text{Si}(\text{sol})_2\text{Pc}$ decreased substantially in the presence of serum (Figure 6). This finding is in good agreement with the inverse correlation between the photocytotoxicity and the serum concentration (Table 4). Internalisation of the free $\text{Si}(\text{sol})_2\text{Pc}$ is assumed to take place via a diffusion controlled process¹⁷ but apparently, this is inhibited by interactions of the PS with serum components. The uptake of $\text{Si}(\text{sol})_2\text{Pc}$ encapsulated in micelles (in its aggregated state) by both B16F10 and 14C cells was lower than that of free PS, i.e. approximately 60 times in the absence of FBS (Figure 5, white bars) and by a factor of 2 to 5 in the presence of FBS (Figure 5, gray and black bars). Remarkably, the amount FBS present did not affected the uptake of $\text{Si}(\text{sol})_2\text{Pc}$ -loaded micelles. This conflicting influence of the FBS concentration on the uptake versus the photocytotoxicity of $\text{Si}(\text{sol})_2\text{Pc}$ -micellar formulation may be explained by the physical state of the PS in the micellar core. In the photocytotoxicity assay, low-loaded micelles were used in which the PS was molecularly dissolved in the core (see Figure 4). On the other hand, the uptake experiments were performed with high-loaded micelles, i.e. the $\text{Si}(\text{sol})_2\text{Pc}$ is aggregated in the core. Once the PS is molecularly dissolved in the micellar core, it obviously can rapidly diffuse out of the micelles into the aqueous environment or exchange, via close contacts, to other (hydrophobic) sites like the cellular membrane or serum components, leading to photodynamic efficacies comparable with free PS. Conversely, aggregation of $\text{Si}(\text{sol})_2\text{Pc}$ in the micellar core increases the stability of the micellar formulations and minimises the release and cellular uptake of the encapsulated PS.

Intracellular distribution of Si(sol)₂Pc

The intracellular distribution of Si(sol)₂Pc-loaded micelles after incubation with B16F10 cells (high-loaded, 1 μM Si(sol)₂Pc, polymer concentration 0.45 mg/mL, 10 % serum) was investigated with confocal microscopy (Figure 7).

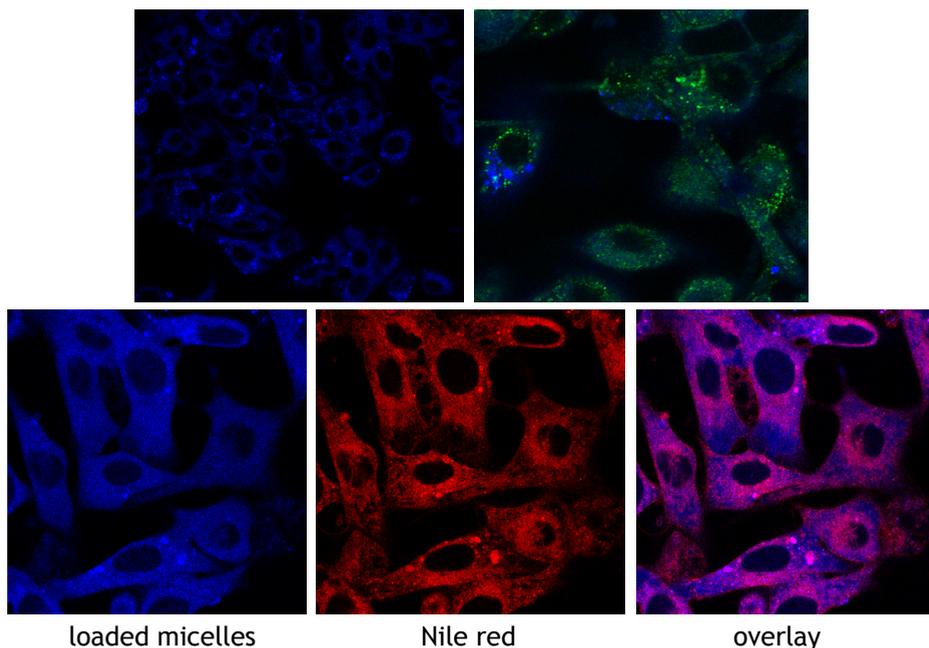


Figure 7 Confocal microscopy of B16F10 cells incubated with Si(sol)₂Pc-loaded micelles and lysotracker (top) or Nile red (bottom) in presence of 10 % serum (images at the right show corresponding fluorescence overlays)

Upon cellular incubation with either free Si(sol)₂Pc or PS encapsulated in micelles (high-loading), both in presence of FBS, a spotted fluorescence pattern was observed. To determine the identity of these spots, cells pre-incubated for 6 hours with Si(sol)₂Pc were washed with PBS and incubated with either lysotracker or Nile red to stain the lysosomes and intracellular lipid droplets, respectively¹⁷. The spots observed after incubation with Si(sol)₂Pc-loaded micelles did not co-localise with lysotracker (Figure 7, top). This observation is not in line with the expected pattern after intact micelles internalisation.

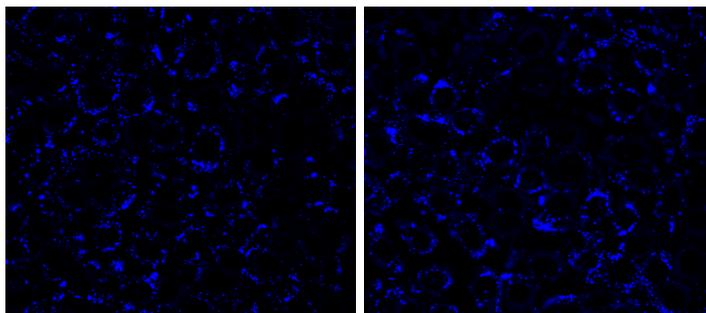


Figure 8 Intracellular localisation of $\text{Si(sol)}_2\text{Pc}$ ($0.05 \mu\text{M}$) either administered in its free form (left) or loaded in mPEG-*b*-p(HPMAm-Lac₂) micelles (right, 0.45 mg/mL polymer) in 14C cells with 5 % FBS in the incubation medium (fixed microscope settings)

Cellular uptake of nanoparticles is assumed to take place via endocytosis, by which they accumulate in the early endosomes and finally end up in lysosomes²⁷. The divergent localisation of lysotracker and PS-loaded micelles is in accordance with the uptake experiments, where $\text{Si(sol)}_2\text{Pc}$ encapsulated in micelles was to a lesser extent taken up than free PS (Figure 5). On the other hand, co-localisation of $\text{Si(sol)}_2\text{Pc}$ with Nile red was observed after incubation of cells with PS-loaded micelles (Figure 7, bottom) which corresponds with the presence of $\text{Si(sol)}_2\text{Pc}$ in lipid droplets, similar to cells incubated with free PS¹⁷. This suggests that the limited uptake (vide supra) and the intracellular distribution observed for $\text{Si(sol)}_2\text{Pc}$ -loaded micelles is attributed to a trace amount of free photosensitiser that has been released from the micelles into the medium.

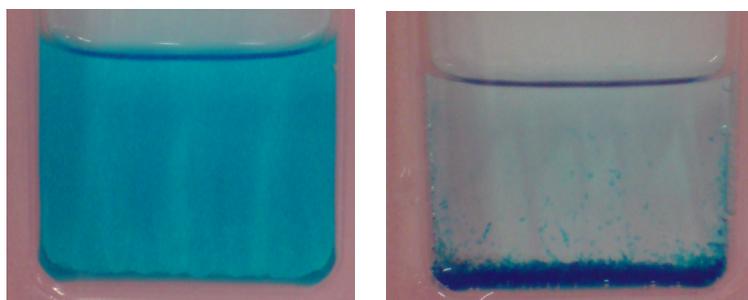


Figure 9 $\text{Si(sol)}_2\text{Pc}$ -loaded micelles ($250 \mu\text{M}$ PS, 9 mg/mL polymer) after dialysis for 10 days at pH 5 (left) and after 8 hours at pH 8.5 (right) at $37 \text{ }^\circ\text{C}$

The intracellular distribution was also examined upon incubation with low-loaded micelles (0.05 μM $\text{Si}(\text{sol})_2\text{Pc}$, 0.45 mg/mL polymer). At this concentration, $\text{Si}(\text{sol})_2\text{Pc}$ showed the highest fluorescence (see Figure 4) since the PS is molecularly dissolved in the micellar core. Cells were also incubated with medium containing the same concentration of free $\text{Si}(\text{sol})_2\text{Pc}$ (Figure 8). The observed intracellular fluorescence pattern and intensity were the same for both formulations, which corroborates the previous hypothesis that in the low-loading regime the dissolved PS molecules easily and fully diffused out of the micelles and were internalised in their free form, causing the same photocytotoxicity as the photosensitiser added directly in its free form (Figure 5).

Release properties of $\text{Si}(\text{sol})_2\text{Pc}$ -loaded micelles

The above experiments suggest that $\text{Si}(\text{sol})_2\text{Pc}$ was fully released from the low-loaded micelles and probably partly released from the high-loaded micelles during *in vitro* incubation. The release of $\text{Si}(\text{sol})_2\text{Pc}$ from high-loaded micelles (250 μM PS, 9.0 mg/mL polymer) was studied via dialysis (MWCO 3.5 kDa) against buffer (pH 5 or 8.5) at 37°C. At pH 5, where ester hydrolysis is minimised and the micelles remain intact for more than 10 days⁴², the micellar dispersion containing aggregated PSs remained homogeneously coloured inside the bag up to at least 10 days (Figure 9, left). The concentration of $\text{Si}(\text{sol})_2\text{Pc}$ in the micellar dispersion did not change in time and spectrophotometrically no PS was detected in the dialysate, indicating that a stable formulation was formed which displayed no detectable release of the $\text{Si}(\text{sol})_2\text{Pc}$ in an aqueous environment. In contrast, blue precipitates were visibly observed at pH 8.5 after approximately 6 hours (Figure 9, right), which might indicate that the micelles had dissociated after this time period and subsequently released their contents. Indeed, it was previously reported that mPEG-*b*-p(HPMAm-Lac₂) micelles started to disassemble after 4 to 5 hours at pH 9 as a result of extensive ester hydrolysis of the lactate side chains, at the time point where the increased CMT passed the incubation temperature⁴¹. Although large precipitates of the $\text{Si}(\text{sol})_2\text{Pc}$ were clearly detected inside the dialysis bag, no $\text{Si}(\text{sol})_2\text{Pc}$ was measured in the dialysate which is ascribed to the very low aqueous solubility of $\text{Si}(\text{sol})_2\text{Pc}$. Even when 1 % BSA (a known solubiliser for hydrophobic compounds^{26, 31}) was added to the dialysate, no $\text{Si}(\text{sol})_2\text{Pc}$ was detected in this medium.

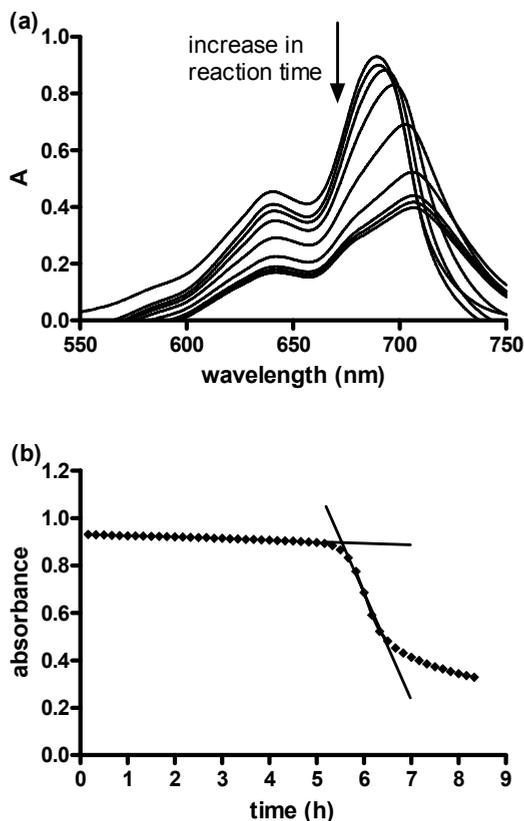


Figure 10 Incubation of $\text{Si}(\text{sol})_2\text{Pc}$ -loaded micelles in 25 % serum at pH 8.7 ($12.5 \mu\text{M}$ PS, 0.45 mg/mL polymer): (a) absorption spectra from 0 to 7 hours, (b) absorption of $\text{Si}(\text{sol})_2\text{Pc}$ at 689 nm in time

The release of aggregated $\text{Si}(\text{sol})_2\text{Pc}$ from high-loaded micelles ($12.5 \mu\text{M}$ $\text{Si}(\text{sol})_2\text{Pc}$, 0.45 mg/mL polymer) at pH 8.7 was also spectrophotometrically monitored in time in the presence of 25 % serum (Figure 10). In agreement with Figure 3, the UV-vis spectrum of $\text{Si}(\text{sol})_2\text{Pc}$ -loaded micelles showed that the photosensitiser was in an aggregated form present in the micellar core. During the first 5 hours of incubation, the UV-absorption spectra hardly changed in time which demonstrated that $\text{Si}(\text{sol})_2\text{Pc}$ was stably encapsulated in the micelles. After approximately 5.5 hours, the absorbance suddenly decreased (Figure 10, right) and the Q-band shifted to longer wavelengths (Figure 10, left). Both aspects correspond with an abrupt change of the $\text{Si}(\text{sol})_2\text{Pc}$ environment and indicate further aggregation and/or precipitation of the PS.

These results are in excellent agreement with the dialysis experiment where precipitation was observed after approximately 6 hours as a result of micellar dissociation. The effect of FBS on the release of $\text{Si}(\text{sol})_2\text{Pc}$ from the micelles was also monitored fluorometrically. The $\text{Si}(\text{sol})_2\text{Pc}$ -loaded micelles were 1:1 diluted with various FBS solutions at $t = 0.05$ hour (final pH 8.7, 12.5 μM PS, 0.45 mg/mL polymer, Figure 11).

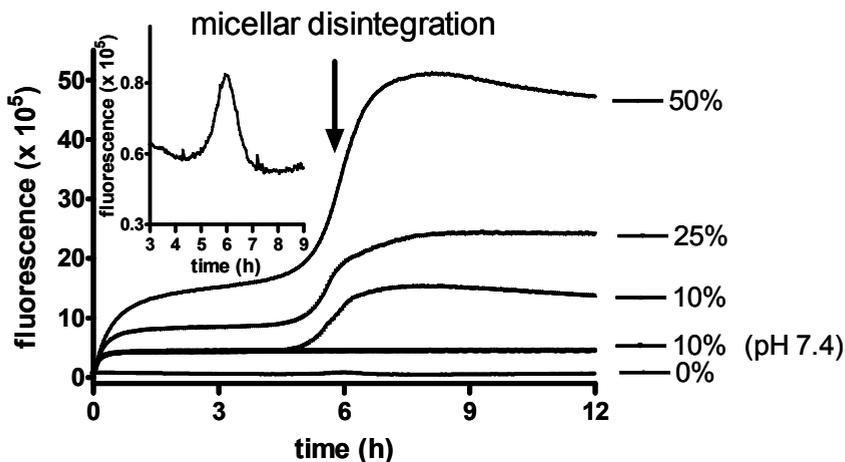


Figure 11 The fluorescence ($\lambda_{\text{ex}} = 674$, $\lambda_{\text{em}} = 679$ nm) of $\text{Si}(\text{sol})_2\text{Pc}$ -loaded micelles in time at 37 °C and pH 8.7, in the presence of various concentrations FBS, and with 10 % FBS at pH 7.4 (The insert shows an enlargement of the curve obtained without FBS; FBS was added at $t = 0.05\text{h}$, final concentrations: 12.5 μM $\text{Si}(\text{sol})_2\text{Pc}$, 0.45 mg/mL polymer)

Initially, the fluorescence of these high-loaded micelles was extensively quenched as seen before (see Figure 4). At pH 8.7, micellar dissociation starts after approximately 5.5 hours (see Figure 9 and 10), thereby releasing the encapsulated $\text{Si}(\text{sol})_2\text{Pc}$. As control, one sample containing 10 % FBS was also incubated at conditions where the micelles were stable during the incubation, i.e. at pH 7.4 (Figure 11). In the absence of FBS at pH 8.7, a small increase in the fluorescence signal was observed after 5.5 hours (insert Figure 11), which coincides with the start of micellar dissociation (Figure 9). The addition of various amounts of serum to the micelles at $t = 0.05$ hour gave a concentration-dependent increase of the fluorescence intensity by a factor 3, 5 or 10 for 10 %, 25 % and 50 % FBS, respectively. Apparently, some $\text{Si}(\text{sol})_2\text{Pc}$ is released from

the (non-degraded) micellar core, solubilised by serum and these PS-protein complexes resulted in an increased fluorescence signal. Previously, the affinity of serum proteins for hydrophobic (drug) molecules was already shown to accelerate the release of drugs from other polymeric assemblies^{23, 26, 31}. After 6 hours, a further increase in the fluorescence was observed for the samples at pH 8.7, which coincides with micellar dissociation (Figure 10). Again, the observed fluorescence intensity was almost linearly dependent on the amount of FBS present, indicating that saturation binding to the serum components occurred, and the excess of released photosensitiser probably aggregated and/or precipitated. Contrary, after the initial increase in fluorescence due to addition of 10 % FBS, the fluorescence of Si(sol)₂Pc-loaded micelles in 10 % FBS at pH 7.4 remained constant since side chain hydrolysis is much slower at this pH and intact micelles were still present after 12 hours.

In a separate experiment, a linear relationship between the fluorescence intensity and the Si(sol)₂Pc concentration in serum-containing medium was found for low concentrations of Si(sol)₂Pc, i.e. up to approximately 0.03 μM in 10 % and 25 % FBS and 0.05 μM in 50 % FBS (results not shown). In this concentration range, the fluorescence intensity of serum-bound Si(sol)₂Pc was comparable to similar concentrations of Si(sol)₂Pc dissolved in DMF, which suggests the presence of Si(sol)₂Pc as unimers in FBS containing medium. When the fluorescence intensity from the release experiments (Figure 11) was compared to these control solutions, the observed initial release of Si(sol)₂Pc after FBS addition was estimated to be approximately 0.05 %, 0.08 % and 0.16 % of the total Si(sol)₂Pc content in the presence of 10 %, 25 % and 50 % FBS, respectively. Apparently, in presence of serum components, Si(sol)₂Pc was partially released from the micelles although this amount was very low. The major fraction (>99 %) of Si(sol)₂Pc remained stably incorporated in the micellar core until the micelles disintegrated.

The properties of the Si(sol)₂Pc-loaded mPEG-*b*-p(HPMAm-Lac₂) micelles make them very interesting carriers for the targeted delivery of the hydrophobic Si(sol)₂Pc *in vivo*. At concentrations higher than 0.05 μM, Si(sol)₂Pc is in an aggregated form in the micellar core. Importantly, in aggregated form the PS is hardly released from the micelles, even in the presence of serum. Ideally, the PS-loaded micelles remain intact after i.v. administration, thereby displaying several attractive features. The most important aspect is that the small size will lead to an enhanced tumour accumulation via the EPR effect. After extravasation at the targeted site, the lactate side chains of the

hydrophobic core are hydrolysed, which ultimately leads to micellar dissociation, thereby releasing the encapsulated $\text{Si}(\text{sol})_2\text{Pc}$. It is expected that the PS aggregates will (partly) dissociate upon release as a result of interaction with proteins or (intracellular) membranes. Subsequent illumination will result in the production of ROS, thereby confining the photodynamic activity of the PS to the site where it is released. The small size, aggregated state of encapsulated $\text{Si}(\text{sol})_2\text{Pc}$ and transient stability of the thermosensitive biodegradable micelles are favourable characteristics in achieving a controlled release of the encapsulated PS in the tumour tissue.

Conclusion

In this study, it is shown that thermosensitive biodegradable mPEG-*b*-p(HPMAM-Lac₂) micelles efficiently encapsulated Si(sol)₂Pc up to a final concentration of 2 mg/mL without affecting the micellar size of approximately 75 nm. At concentrations lower than 0.05 μM, the Si(sol)₂Pc was molecularly dissolved while at higher concentrations, it was aggregated in the micellar core. The Si(sol)₂Pc micelles with a low loading showed a similar high photocytotoxicity as the free PS although this cytotoxicity was inversely correlated to the serum concentration present. The uptake studies indicated that intact high-loaded micelles were minimally taken up by the cells. In presence of 50 % FBS, initially only 0.2 % Si(sol)₂Pc was released from the highly loaded micelles while the remaining, aggregated PS was only released upon micellar disintegration. Overall, the stability of high-loaded micelles in presence of serum, the controlled release and the high photocytotoxicity of the Si(sol)₂Pc are advantageous properties and currently these micellar formulations are evaluated *in vivo*.

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chapter 6

In vitro cellular internalisation of rhodamine-labelled polymeric micelles

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Abstract

In general, the possible uptake and intracellular localisation of nanoparticles is examined by confocal laser scanning microscopy (CLSM), which requires the attachment or encapsulation of a fluorescent dye in the nanoparticle. In this study, thermosensitive block copolymers were prepared containing a covalently linked fluorescent label through copolymerisation of *N*-(2-hydroxypropyl)methacrylamide-dilactate (HPMAm-Lac₂) with rhodamine methacrylate (RhodMA). After extensive purification, a marginal amount (0.22 %) of free label was still present while on average - 90 % of the polymer chains contained a fluorescent rhodamine moiety. Cellular uptake experiments in B16F10 and 14C cancer cells demonstrated that the uptake of covalently bound rhodamine was two orders of magnitude lower than free label, but was not fully inhibited. Strikingly, the intracellular fluorescence as observed with CLSM was attributed to the trace amount of free label that was still present in the micelles and not to the labelled micelles themselves. The intracellular presence of micelles could not be visualised by CLSM, presumably because their fluorescence was quenched.

In conclusion, the PEGylated thermosensitive micelles are to a very low extent taken up by B16F10 and 14C cancer cells. Besides, while monitoring the cellular internalisation of nanoparticles with confocal fluorescence microscopy, it is necessary to have fluorescent nanoparticle formulations that are devoid of (trace amounts) unbound label as this can lead to erroneous conclusions.

Introduction

Polymeric micelles are applied to encapsulate a broad variety of hydrophobic agents. For example, they are used to solubilise poorly water soluble drugs and carry them *in vivo* to their target sites, e.g. tumours or inflamed tissues. Once reached the target site, the drug-loaded micelles can be taken up by cells either by phagocytosis, macropinocytosis and/or (non) clathrin-mediated endocytosis¹⁸ and subsequently release their content. Alternatively, the micelles can release the loaded drug in close proximity of the target cells and the drug can subsequently be taken up by passive diffusion (Figure 1). A special class of polymeric micelles (pluronic[®]) additionally inhibit the P-glycoprotein (P-gp), thereby preventing the cellular excretion of drugs².

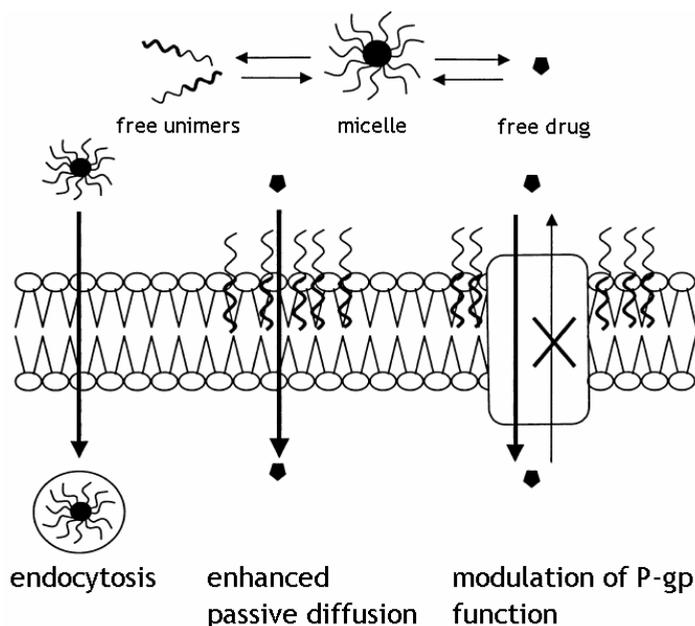


Figure 1 Proposed mechanisms of cellular uptake of drug originally encapsulated in polymeric micelles²⁹

To elucidate the mechanisms of cellular uptake and intracellular localisation, fluorescently labelled drug carriers are frequently used in *in vitro* cell experiments and visualised by confocal laser scanning microscopy (CLSM)¹¹. A more advanced technique is fluorescence resonance energy transfer (FRET) which enables to monitor molecular interactions in detail⁶. Various labelled

nanosized structures (e.g. dendrimers, micelles, nanoparticles and liposomes) display cellular uptake via the endocytotic pathway^{1, 4, 8, 17, 22}. However, the internalisation route and intracellular trafficking is strongly dependent on the type of particle, the particle size^{3, 18}, the presence of a targeting ligand²⁷ and the cell type³⁰. Furthermore, the fluorescent label itself might modify the final particle properties, thereby also affecting the (route of) internalisation and intracellular distribution^{13, 22}.

Various labelling agents are used, which can be physically entrapped in the micelles (sometimes the drug itself is fluorescent, e.g. doxorubicin)^{28, 29} or covalently bound^{1, 5, 15}. Since in the case of free dyes no direct evidence is provided on the internalisation of the nanoparticles themselves, chemical conjugation to the nanoparticle is preferred. For example, tetramethylrhodamine-5-carbonyl azide was attached to the hydroxyl end of a polycaprolactone (PCL) chain^{9, 22} while another rhodamine-labelling was carried out via isothiocyanate coupling to an amine-functionalised polymer²⁴. Furthermore, fluorescein isothiocyanate was coupled to amines^{2, 7} or to alcohol groups⁸ whereas fluorescein carboxylate was used to label pluronics® via the terminal hydroxyl groups¹⁷. After chemical conjugation, the polymers are usually purified via dialysis or (preparative) gel permeation chromatography to remove unreacted (free) label. Despite these purification procedures, the residual percentage of free label in the various formulations investigated is never quantified.

The aim of this study was to evaluate the cellular internalisation of mPEG-*b*-poly(methacrylamide-oligolactates) micelles. Above their so-called critical micelle temperatures (CMT), micelles of 60 - 70 nm diameter are formed due to dehydration of the thermosensitive blocks^{21, 25}. The polymers were covalently labelled with rhodamine by copolymerisation of *N*-(2-hydroxypropyl)methacrylamide-dilactate with rhodamine-methacrylate and subsequently extensively purified. The cellular uptake of the resulting micelles was explored *in vitro* and compared with free label.

Materials & methods

Materials

Acetonitril (ACN), diethyl ether and dimethylformamide (DMF) were purchased from Biosolve Ltd. (Valkenswaard, The Netherlands). Rhodamine-methacrylate (RhodMA) was purchased from PolySciences Europe (Eppelheim, Germany). All buffers were filtered through 0.22 μm filters (Schleicher & Schuell MicroScience GmbH, Dassel, Germany) prior to use. ACN was dried over molecular sieves before use; the other chemicals were used as received. The synthesis of *N*-(2-hydroxypropyl)methacrylamide-dilactate (HPMAM-Lac₂) and macroinitiator (mPEG₅₀₀₀)₂-ABCPA was done as described before^{19, 25}.

Synthesis of mPEG₅₀₀₀-*b*-p((HPMAM-Lac₂)-*co*-RhodMA)

The synthesis of RhodMA labelled mPEG-*b*-p(HPMAM-Lac₂) polymer was carried out according to previously reported polymerisation procedures^{21, 25}. In brief, RhodMA, HPMAM-Lac₂ (molar ratio 1:99) and (mPEG)₂-ABCPA macroinitiator (molar ratio monomer to initiator was 150:1, scale ~ 700 mg monomer) were dissolved in ACN. After flushing with nitrogen for at least 15 minutes at room temperature, the solution was stirred overnight at 70 °C. Subsequently, the obtained polymer was precipitated in diethyl ether, dissolved in water and purified twice via gel filtration at 4 °C (20 mg/mL in water, 2.5 mL applied per PD10 column). Other purification steps involved dialysis against either ACN:H₂O (1:1) or demineralised H₂O while frequently refreshing the dialysis medium. After each purification step, the polymer was recovered by freeze drying. The CMT of the polymer obtained after the different purification steps was determined as described previously^{21, 25}.

Gel Permeation Chromatography (GPC)

Two serial Plgel 3 μm MIXED-D columns (Polymer Laboratories) were used with a Waters System (Waters Associates Inc., Milford, MA, USA) and with DMF containing 10 mM LiCl as eluent. The molecular weights were determined using a calibration curve based on poly(ethylene glycol) standards of defined molecular weights which were detected by a differential refractometer Model 410. UV-detection was done at 548 nm (absorption maximum of rhodamine), the flow rate was 0.7 mL/min and the column temperature was 40 °C. After

overnight dissolution at a concentration of approximately 5 mg/mL in the eluent, the polymer samples were filtered through a 0.45 μm filter and 50 μL was injected for analysis. Peak areas were determined with Empower Software Version 1154 (Waters Associates Inc.). A linear calibration curve of RhodMA (R_t - 26 min) was obtained from 0.03 - 146 nmol.

Micelle formation

Micelles were formed via the rapid heating procedure as described before^{21, 24}. In brief, the rhodamine-labelled block copolymers were dissolved (10 mg/mL) overnight at 4 °C in ammonium acetate buffer (pH 5, 120 mM). The solutions were incubated at 0 °C for at least 15 min in glass vials. Subsequently, the vials were rapidly heated from 0 to 50 °C by putting them into a water bath while vigorously stirring to form micelles. After 1 min of incubation at 50 °C, the dispersions were slowly cooled to room temperature. To remove any possible aggregates, the micelles were finally filtrated through a 0.45 μm filter. Dynamic light scattering (DLS) was used to determine the particle size (Z_{Ave}) and size distribution (PD) of the polymeric micelles as reported previously^{21, 25}.

Cells

The murine B16F10 cell line was kindly provided by Prof. Dr. Ernst Wagner (Pharmaceutical Biology-Biotechnology, Department of Pharmacy, Ludwig-Maximilians-Universität, Munich, Germany) and was originally obtained from I.J. Fidler (Texas Medical Center, Houston, USA). B16F10 cells were cultured in DMEM 1 (Dulbecco's modification of Eagle's Medium, with 3.7 g/L sodium bicarbonate, 1 g/L glucose, Gibco BRL, Breda, The Netherlands), completed with antibiotics/antimycotics (100 IU penicillin G sodium/mL, 100 μg streptomycin sulphate/mL and 0.25 μg amphotericin B/mL, Gibco BRL, Breda, The Netherlands), L-glutamine (2 mM, Gibco BRL, Breda, The Netherlands), and 10 % foetal bovine serum (FBS, Integro, Zaandam, The Netherlands). The human head and neck squamous cell carcinoma cell line UM-SCC-14C (developed by Dr. TE Carey, Ann Arbor, MI, and further abbreviated as 14C) was kindly provided by Prof. Dr. GAMS van Dongen (Department of Otolaryngology/Head and Neck Surgery, VU University Medical Center, Amsterdam, The Netherlands). This cell line was cultured in DMEM 2 (Dulbecco's modification of Eagle's Medium, with 3.7 g/L sodium bicarbonate, 4.5 g/L glucose, Gibco BRL, Breda, The Netherlands), completed with antibiotics/antimycotics and 5 % FBS.

Cellular uptake

B16F10 cells and 14C cells were seeded in a 24 well plate at 15×10^4 cells per well (1000 μL cell suspension) and the plates were incubated overnight at 37 °C under a 5 % CO_2 atmosphere. The medium was removed and 500 μL fresh medium was added, containing either a 20-fold dilution of 10 mg/mL mPEG-*b*-p((HPMAm-Lac₂)-co-RhodMA) micelles (final concentration 20.6 μM RhodMA) or free RhodMA (final concentration of 20.6 μM or 45 nM). The cells were incubated for the indicated time (hours) at 37 °C under a 5 % CO_2 atmosphere. Medium was removed and the cells were washed twice with 500 μL PBS. To lyse the cells, 500 μL of lysis buffer (50 mM Tris/HCl (pH 8), 150 mM NaCl and 1 % Triton X-100) was added followed by incubation on ice for 20 min. The concentration of RhodMA in the cell lysate was determined by dilution of 100 μL of the cell lysate with 900 μL ethanol and measuring the fluorescence of the samples ($\lambda_{\text{ex}} = 548 \text{ nm}$, $\lambda_{\text{em}} = 570 \text{ nm}$, Horiba Fluorolog fluorometer). A calibration curve was prepared by dilution of RhodMA stock solutions in ethanol with cell lysate to a final lysate concentration of 10 % (v/v). An aliquot (20 μL) of each lysate was used for determination of the cellular protein content, with the Micro BCA™ protein assay²³ (Pierce, Rockford, USA), according to the instructions of the supplier. The uptake of RhodMA was calculated as nmol per mg of cellular protein and also given as percentage taken up by the cells relative to the amount added.

Intracellular localisation

To promote cell adhesion on the plates, 35 mm glass bottom microwell dishes (MatTek Corporation, Ashland, USA) were pre-coated with gelatin (Sigma) solution (1:10 dilution in PBS) by incubation for 4h at 37 °C. Cell suspensions of B16F10 or 14C cells (2×10^5 cells in 1.5 mL) were added to the plates and they were incubated overnight at 37 °C under a 5 % CO_2 atmosphere. Freshly prepared mPEG-*b*-p((HPMAm-Lac₂)-co-RhodMA) micelles (10 mg/mL) were 20-fold diluted in medium to obtain a final RhodMA concentration of 20.6 μM . The final polymer concentration was 0.5 mg/mL (23.1 μM based on a M_n of 21600), which is well above the critical micelle concentration of 0.03 mg/mL²⁵. To determine the effect of free label on the intracellular fluorescence pattern, a control experiment was done with free RhodMA that was dissolved in ethanol and diluted in the incubation medium to achieve a final concentration of 45 nM, i.e. the amount free label present in the mPEG-*b*-p((HPMAm-Lac₂)-co-RhodMA)

micelles. The incubation medium was removed from the cell cultures before the sample solutions (1.5 mL) were added. The cells were incubated for 6 hours at 37 °C under a 5 % CO₂ atmosphere. To visualise the lysosomes, cells pre-incubated for 6 hours with or without RhodMA (micelles) were incubated for 5 minutes at 37 °C with lysotracker green (Invitrogen, 130 nM, from 0.1 mM stock in DMSO). Next, live cell imaging was performed with a laser scanning confocal fluorescence microscope (Nikon Eclipse TE2000-U microscope), equipped with a C1 scanning unit, two excitation lasers i.e. Argon-Ion laser (488 nm) and He-Ne laser (543 nm) and two bandpass emission filters, i.e. 515 ± 15 nm and 585 ± 15 nm.

Results & discussion

Polymer synthesis

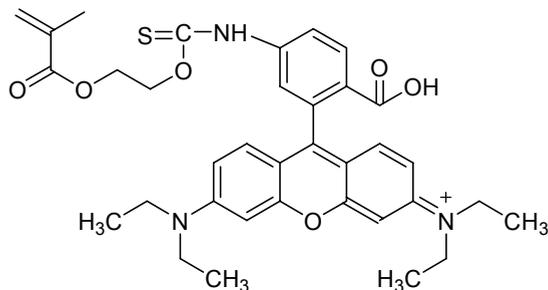


Figure 2 Chemical structure of rhodamine-methacrylate (RhodMA)

The fluorescently labelled block copolymer $m\text{PEG-}b\text{-p}((\text{HPMAm-Lac}_2)\text{-co-RhodMA})$ was synthesised through free radical copolymerisation of HPMAm-Lac_2 and RhodMA (Figure 2) (feed ratio 99:1) with a $(m\text{PEG}_{5000})_2$ -macroinitiator. GPC analysis (refractive index detection) of the crude reaction mixture revealed, besides the block copolymer, also some unreacted macroinitiator (at a retention time (R_t) of ~ 20 min) and $m\text{PEG}_{5000}\text{-OH}$ ($R_t \sim 21$ min) (Figure 3a). Furthermore, the GPC was equipped with a UV-detector set at 548 nm to assess the presence of the rhodamine fluorophore. The chromatogram of the crude reaction product showed that RhodMA was indeed built in the polymer backbone as both RI and UV displayed a comparable peak at a retention time of 15 - 20 min. In the GPC system, the RI-detector was serially attached to the UV-detector causing a slight shift in the signal peaks. In addition, the observed shift may be attributed to the increased probability for longer polymer chains to contain a RhodMA moiety. A substantial amount of free RhodMA was observed as a double peak at $R_t \sim 26$ min (corresponding to 14.7 % of the total rhodamine present, Table 1). Injection of RhodMA also resulted in a double peak (Figure 3A, insert) presumably due to adsorption onto the column material or the formation of multimers. Various purification steps were performed to diminish the amount of free RhodMA (Table 1). Gel filtration chromatography at 4°C ^{2, 4, 7} and dialysis against $\text{ACN:H}_2\text{O}$ (1:1) were used to remove most of the unreacted RhodMA monomer. After that, dialysis against water at 4°C was done till no trace of rhodamine in either the external solvent or in the dialysis

membrane (i.e. after removal of the polymer solution) could be visually detected^{8, 9, 17, 22}. However, even then, a trace amount of free label (0.22 % of the total RhodMA content) was detected in the mPEG-*b*-p((HPMAm-Lac₂)-*co*-RhodMA) (Figure 3b, insert). For poly(sodium 4-styrenesulphate), π - π stacking between rhodamine and the polymer was held responsible for observed low diafiltration rates¹⁴. In our case, hydrophobic interactions between the lactate side chains and the rhodamine fluorophore presumably prevented quantitative removal of RhodMA by dialysis.

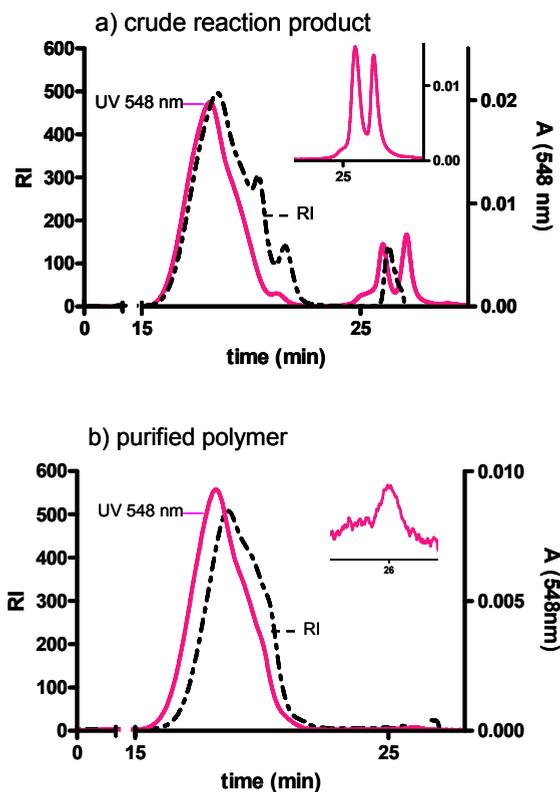


Figure 3 GPC chromatogram of mPEG-*b*-p((HPMAm-Lac₂)-*co*-RhodMA) polymer: black dashed line is RI-detection (left y-axis) and pink line is UV-detection at 548 nm (right y-axis); a) crude reaction product, the insert showing the chromatogram of RhodMA (8.8 nmol); b) polymer after extensive purification (polymer 4, see Table 1) (the insert shows the absorbance at $R_t \sim 26$ min (548 nm) corresponding with free label present)

	purification method	% free RhodMA versus total amount RhodMA present
1	crude polymer	14.7 %
2	column chromatography	9.3 %
3	dialysis ACN:H ₂ O	1.6 %
4	dialysis H ₂ O	0.22 %

Table 1 The effect of the subsequent purification methods on the reduction of the amount of free RhodMA present in mPEG-*b*-p((HPMAm-Lac₂)-co-RhodMA) (The amount of rhodamine was determined by GPC with UV detection at 548nm)

GPC analysis (RI-detection) of the purified polymer revealed an M_n of 21600 g/mol ($M_w/M_n = 1.5$) which is common for these type of polymers^{21, 25}. The unreacted mPEG macroinitiator and mPEG₅₀₀₀-OH were removed during the various purification steps. UV-analysis showed that the fluorescently labelled polymer contained 10.7 nmol RhodMA per 12 nmol polymer which means that on average 89 % of the polymer chains contained one RhodMA moiety. Similar to non-labelled mPEG-*b*-p(HPMAm-Lac₂)²⁵, mPEG-*b*-p((HPMAm-Lac₂)-co-RhodMA) displayed thermosensitive behaviour in aqueous solution with a CMT of 6.5 °C; i.e. the small percentage RhodMA (less than 1 %) did not influenced the CMT. The purified rhodamine-labelled mPEG-*b*-p((HPMAm-Lac₂)-co-RhodMA) polymer (4, Table 1) was used to prepare micelles in a way similar to that for non-labelled polymers, i.e. via rapidly heating an ice-cold polymer solution to above its CMT while stirring^{21, 25}. The micelles formed had a Z_{Ave} of 63 nm (PD of 0.15).

Cellular uptake

Micelles were diluted in culture medium and incubated with B16F10 cells. As explained above, 89 % of the chains of polymer 4 carried a RhodMA moiety and the polymer concentration in the medium was 23.1 μM, thus the final concentration of RhodMA in the mPEG-*b*-p((HPMAm-Lac₂)-co-RhodMA) micellar dispersion was 20.6 μM. According to the GPC analysis, 0.22 % free RhodMA was present, corresponding with a concentration of 45 nM. Therefore, control experiments were carried out with either 20.6 μM, similar to the concentration of labelled micelles, or 45 nM free RhodMA to study the effect of traces of the free label. After incubation for the indicated time intervals, the

cells were lysed and the amount of RhodMA in the lysates was measured by fluorescence spectroscopy at 570 nm ($\lambda_{\text{ex}} = 548$ nm) after tenfold dilution with ethanol (Table 2).

incubation time (h)	RhodMA micelles		20.6 μM free RhodMA	
	nmol RhodMA/ mg protein	% uptake	nmol RhodMA/ mg protein	% uptake
1	0.035 \pm 0.003	0.022 %	7.1 \pm 1.1	4.4 %
4	0.11 \pm 0.01	0.076 %	14.0 \pm 0.6	8.6 %
6	0.13 \pm 0.02	0.095 %	14.0 \pm 0.9	8.2 %
incubation time (h)	45 nM free RhodMA			
	nmol RhodMA/ mg protein	% uptake		
1	0.014 \pm 0.001	7.1 %		
4	0.014 \pm 0.001	8.5 %		
6	0.015 \pm 0.001	8.0 %		

Table 2 Cellular uptake of RhodMA expressed as nmol RhodMA/mg protein in B16F10 cells after incubation of mPEG-*b*-p((HPMAm-Lac₂)-*co*-RhodMA) micelles (polymer 4, 20.6 μM) or free RhodMA (20.6 μM or 45 nM) for 1 to 6 hours at 37 °C (n = 3, % uptake is defined as the percentage of the amount in the cells relative to the amount added to the cells)

After one hour, mPEG-*b*-p((HPMAm-Lac₂)-*co*-RhodMA) micelles displayed a low but detectable uptake (0.022 %) which increased over the next 5 hours to a concentration of 0.13 nmol RhodMA per mg protein (0.095 %). In contrast, free RhodMA incubated at the same concentration (20.6 μM) showed a high uptake by the cells in time up to 14 nmol/mg protein (i.e. 8.2 % of the added amount). In other words, the uptake of RhodMA-labelled micelles was inhibited by two orders of magnitude as compared to equivalent concentrations of free RhodMA. Upon incubation with a concentration of free RhodMA that corresponds with the amount of free label present in the RhodMA micelles (i.e. 45 nM), again 8.0 % of the added amount was taken up but the absolute amounts were 8 times less than those for the labelled micelles. Hence, besides uptake of the trace amount of free RhodMA present in polymer 4, internalisation of labelled polymeric micelles must contribute to the intracellular rhodamine concentration. Besides B16F10 cells, similar results were obtained after incubation of RhodMA micelles

with 14C cells (results not shown) and in both cases, the total micellar uptake was low (≤ 0.1 %). The localisation of RhodMA micelles and free label after cellular incubation was investigated by confocal microscopy as described in the next section.

Confocal microscopy

The cellular internalisation of mPEG-*b*-p((HPMAm-Lac₂)-*co*-RhodMA) micelles (polymer 4, containing in total 20.6 μ M RhodMA, see Figure 4d) and free RhodMA (45 nM, Figure 4a) as control was investigated in B16F10 cells. The fluorescence pattern of both samples was rather evenly distributed although cells incubated with RhodMA-labelled micelles displayed a few spots with an enhanced fluorescence signal (see for example the enlarged image, Figure 4g). Besides, the intracellular fluorescence intensity in the cells incubated with both samples was comparable at fixed instrument settings, despite the ~ 450 -fold higher RhodMA concentration of the micelles during incubation. To obtain more insight into the origin of the few intense fluorescent spots observed in cells incubated with RhodMA micelles, a co-localisation study with lysotracker was performed to stain the lysosomes (Figure 4). The cellular incubation studies with RhodMA-labelled micelles revealed that most of these spots do not overlap with the lysotracker fluorescence (see the overlay pictures in Figure 4: c, f and i). Hence, no direct evidence is given for micellar uptake via endocytosis, which is the common internalisation pathway of nanoparticles¹². Although the results of Table 2 show that the RhodMA micelles are to some extent taken up by cells, this is not clear from CLSM studies. It is presumed that the intracellular presence of labelled micelles is not observed with confocal microscopy due to fluorescence quenching.

The clear similarity of the CLSM pictures with the labelled micelles and with the free label, in terms of fluorescence pattern and intensity, suggests that mainly the free label present in RhodMA-labelled micelles accounts for the intracellular fluorescence pattern after uptake. In many studies, no attention is given to a quantitative analysis of fluorescently labelled polymers^{4, 8, 17}. Usually, purification is performed by dialysis until no traces of the fluorophore are (visually) observed in the medium. However, in our case, GPC analysis in DMF revealed the presence of 1.6 % free label after initial dialysis. Therefore, we continued the purification procedure and after additional (extensive) dialysis of the unimers (i.e. below the CMT) in H₂O resulted in a low but detectable amount of 0.22 % free RhodMA still present and even this trace amount of free label gave rise to significant intracellular fluorescence. This finding emphasises that if one

ignores the presence of free label, false conclusions may be drawn from fluorescence microscopy studies.

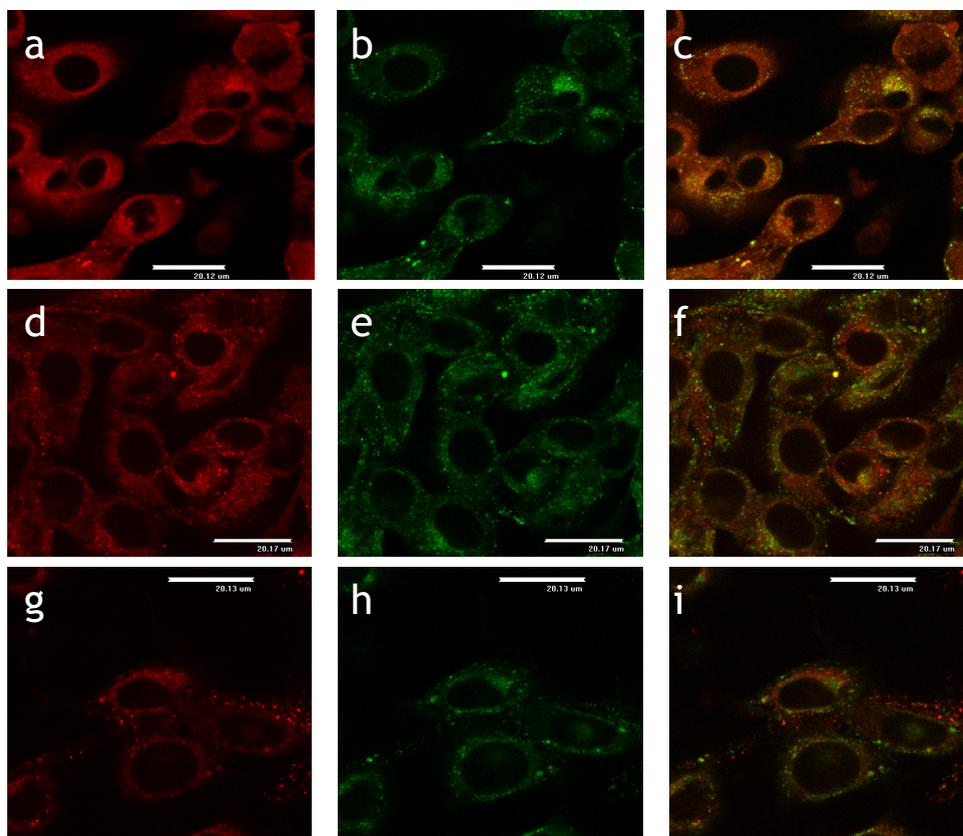


Figure 4 B16F10 cells incubated for 6 hours at 37 °C with free RhodMA corresponding with the concentration of free label present in the micelles (45 nM, a - c), with mPEG-*b*-p((HPMAm-Lac₂)-*co*-RhodMA) micelles (polymer 4, 20.6 μM, d - f) and enlarged images after incubation of RhodMA micelles (g - i). The middle column corresponds with lysotracker incubation while the images at the right (c, f, i) show the corresponding fluorescence overlays. (fixed microscope settings)

The low uptake of our RhodMA-labelled micelles (more than two orders of magnitude less than similar amounts of free label) was obvious. Strikingly, the micelles as described in this study comprised similar physicochemical parameters, i.e. the size (≤ 200 nm)^{18, 30} and the PEG length¹⁰, as for various

other micellar systems that were reported to be internalised as indicated by, amongst others, confocal studies^{17, 24}. The distance between neighbouring mPEG chains on the shell surface of mPEG-*b*-p(HPMAm-Lac₂) micelles was determined to be ~ 3 nm, which indicates a rather high mPEG density¹⁶. Possibly, the dense and extensively hydrated mPEG₅₀₀₀-coating surrounding the mPEG-*b*-p((HPMAm-Lac₂)-*co*-RhodMA) micelles may inhibit the adhesion to the cell surface, which is the first step of cellular internalisation. Nevertheless, the micelles showed some, although limited, uptake which may be ascribed to the internalisation of either a small fraction of intact micellar structures or only labelled unimers. To discriminate between these two possibilities, cellular uptake experiments are planned with core crosslinked (CCL) rhodamine-labelled micelles. These stabilised CCL micelles can not dissociate into unimers²⁰ and can only be internalised in their intact form. Furthermore, it would be very attractive to couple targeting ligands (e.g. antibodies, folate, RGD peptides) to the micellar shell to promote specific cellular recognition and subsequent active uptake²⁶.

In conclusion, the cellular uptake of mPEG-*b*-p((HPMAm-Lac₂)-*co*-RhodMA) micelles is very low whereas trace amounts of free label lead to significant intracellular fluorescence which is distracting and may lead to a misinterpretation of confocal fluorescence microscopy results.

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chapter 7

Hydrolysable core crosslinked thermosensitive polymeric micelles

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Abstract

Upon intravenous administration of drug-loaded polymeric micelles, dilution below the critical micelle concentration (CMC) or interactions with blood components lead to premature destabilisation of the micelles with concomitant release of encapsulated compounds. In order to prevent early dissociation of biodegradable thermosensitive micelles based on mPEG₅₀₀₀ and *N*-(2-hydroxyethyl)methacrylamide-oligolactates (mPEG-*b*-p(HEMAm-Lac_n)), a strategy towards core crosslinking (CCL) was developed. Rapidly heating ice-cold solutions of partially methacrylated (3 - 12 %) block copolymers to above their critical micelle temperature (CMT), followed by illumination in presence of Irgacure 2959 generated CCL micelles of 68 ± 7 nm with a PD of 0.07 ± 0.05 . Either below the CMT or after addition of sodium dodecyl sulphate (SDS), the non crosslinked (NCL) micelles rapidly disintegrated whereas the CCL micelles kept their integrity. NCL micelles fell apart after 5 hours in pH 7.4 at 37 °C as a result of the hydrolysis of lactate side chains. In contrast, the destabilisation kinetics of CCL micelles were strongly dependent on the percentage of methacrylation. The fluorescence of pyrene loaded micelles revealed that CCL disintegration took place only after cleavage of the crosslinked ester bonds. The increased physical stability of these degradable core crosslinked micelles renders them to be highly potential as controlled drug delivery carriers.

Introduction

In recent years, nanosized polymeric micelles (PM) attract a lot of attention because of their very favourable properties as carriers for low molecular weight drugs, genes and imaging agents^{9, 28, 32, 44}. The micelles are mainly kept together either by hydrophobic forces between the hydrophobic blocks (e.g. polylactide^{7, 17}, polycaprolactone^{5, 26}, poly(meth)acrylamides^{36, 42, 43}) of amphiphilic block copolymers or by electrostatic interactions e.g. between negatively charged DNA and cationic block copolymers (polyion complexes)⁹. Among the most advantageous properties for micelles are their core which can accommodate various compounds (e.g. drugs), the brushlike hydrophilic surface (mainly polyethylene glycol (PEG) to prevent opsonisation and subsequent recognition by the reticulo-endothelial system) and their small size (~ 10 - 100 nm) that enables tumour accumulation due to the enhanced permeation and retention effect (EPR)¹². In water, amphiphilic block copolymers self-assemble into core-shell structures above the critical micelle concentration (CMC) due to the above mentioned intermolecular hydrophobic interactions. The value of the CMC denotes the equilibrium state between free unimers and micelles in solution under static conditions, i.e. without environmental influences. The CMC as well as various other micellar properties are mainly a function of the type of amphiphiles, e.g. the chemical composition, relative polarity, the overall chain length, and the ratio of the hydrophilic and hydrophobic blocks³⁸.

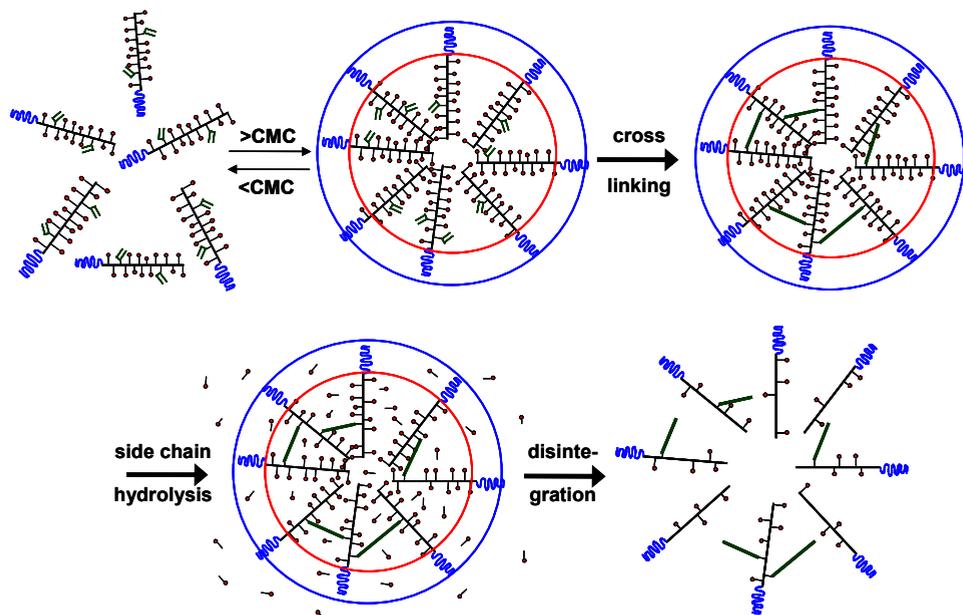
Ideally, the hydrophobic micellar core serves as a (protected) reservoir and delivery vehicle for hydrophobic drugs, whereas its hydrophilic shell is responsible for a favourable pharmacokinetic behaviour of the PM. Despite the equilibrium state of micelles above the CMC, at physiological conditions, adsorption of the polymers is possible to plasma proteins (such as albumin or lipoproteins) that possess affinity for the hydrophobic blocks thereby disrupting the equilibrium. *In vitro* studies showed indeed that the addition of either human albumin or serum to drug-loaded micelles substantially decreased their stability, thereby inducing rapid release of the encapsulated drug^{16, 22, 24, 31}. Furthermore, intravenous (i.v.) administration brings about not only the interactions with these blood components but might also dilute the micelles below their CMC. Both aspects result in unwanted early disintegration of the micelles with a concomitant premature (burst) release of the loaded compounds and unfavourable biodistribution^{14, 39, 40, 48}. Apparently, the micellar-driving non covalent hydrophobic forces are not strong enough to

maintain the micellar integrity. Consequently, for *in vivo* applications, PMs require stronger interactions to be able to resist the physiological destabilisation forces. Increasing the micellar stability can be established by either physical or chemical crosslinking of the shell, intermediate layer or core of the micelles^{9, 29, 38}.

In physically stabilised micelles, the physical state of the core forming block is of importance; a crystalline structure or stereocomplex formation yielded substantially more stable micelles than their amorphous counterparts.^{14, 48} Modifying the core forming block with aromatic moieties also stabilised (mainly via π - π interactions) micellar morphologies as was indicated by an extensively decreased CMC^{5, 26}. Covalently shell crosslinked PM were prepared by chemical or photo-induced reactions^{3, 13, 19, 20, 37} while also the intermediate layer (moiety between hydrophobic and hydrophilic block) was exploited to crosslink polymeric nanoparticles^{8, 23, 49}.

The functionalisation of hydroxyl moieties present in the hydrophobic block with polymerisable groups (e.g. (meth)acrylate) precedes core stabilisation^{2, 11, 21}. After micelle formation, the hydrophobic blocks are linked together via thermal^{11, 21} or photoinduced polymerisation^{15, 34}, or Michael addition with multifunctional thiol compounds¹⁸ which resulted in core crosslinked (CCL) micelles. CMC determination or addition of a destabilising agent (e.g. sodium dodecyl sulphate (SDS)) proved the increased stability of above mentioned crosslinked particles versus their non crosslinked equivalents^{14, 15}. Crosslinking appeared to fixate the micellar morphology, while drug release could be controlled by the extent of crosslinking¹⁰ and furthermore, the stimuli-responsiveness (e.g. pH, temperature, salt concentration) was retained^{1, 2, 25}. However, so far, core crosslinked thermosensitive micelles that are still biodegradable have not yet been reported. Previously, our department has reported on biodegradable polymeric micelles where thermosensitive blocks form the hydrophobic micellar core above the critical micelle temperature (CMT) and mPEG₅₀₀₀ creates the hydrophilic micellar shell. The thermosensitive polymeric backbones are composed of either *N*-(2-hydroxypropyl)methacrylamide (HPMAm) or *N*-(2-hydroxyethyl)methacrylamide (HEMAm) which are derivatised with various monodisperse oligolactates (HP/EMA_m-La_{c_n})^{36, 42}. The CMT depends on the starting comonomer ratio in the polymer and can be tailored to be between 0 °C and body temperature. Drug loaded micelles are easily obtained by quickly heating an aqueous polymer-drug mixture till above the CMT. Upon incubation at physiological conditions, the hydrophobic lactate side chains are cleaved of by ester hydrolysis which results

in an increasing CMT. Disintegration of the micelles will only occur once the CMT has passed 37 °C, which is then, ideally, associated with a concomitant release of the loaded drug. However, paclitaxel-loaded micelles did not result in very high paclitaxel tumour accumulation in tumour bearing mice after 24 hours⁴⁰. After i.v. injection, this is likely due to too rapid release, possibly caused by premature micelle disruption since also the micelles themselves did not accumulate in the tumour to a high extent.



Scheme 1 Concept of core crosslinked biodegradable thermosensitive polymeric micelles

In order to counterbalance the aforementioned physiological conditions, crosslinking the micellar core would prevent preliminary disintegration and promote a prolonged circulation of PMs in the blood stream. Derivatisation of the hydroxyl groups of the lactate side chains of mPEG-*b*-p(HEMAm-Lac_n) polymers with methacryloylchloride introduces polymerisable groups onto the thermosensitive block that allow core crosslinking by thermal or UV-induced polymerisation (Scheme 1). Overall, the aim is to synthesise a thermosensitive micellar drug delivery system with an increased physical stability but with a retained biodegradability.

Material & methods

Materials

Methacryloyl chloride, 4-methoxyphenol (99 %), lithium chloride (LiCl), monomethylether of poly(ethylene glycol), M_w is 5000 g/mol (mPEG₅₀₀₀), 4,4'-azobis(4-cyanopentanoic acid) (ABCPA) and pyrene were obtained from Fluka Chemie AG (Buchs, Switzerland). Deuterated dimethyl sulphoxide (99.9 %, DMSO- d_6) and deuterated water (99.9 %, D₂O) were obtained from Acros (Zwijndrecht, The Netherlands). Triethylamine was obtained from Merck (Darmstadt, Germany). Acetonitrile (ACN), diethyl ether, dimethylformamide (DMF), and tetrahydrofuran (THF) were purchased from Biosolve Ltd. (Valkenswaard, The Netherlands), Irgacure 2959 from Ciba Specialty Chemicals Inc and sodium dodecyl sulphate (SDS) from Biorad Laboratories (Hercules, USA). Methacryloyl chloride was distilled under nitrogen and THF was purified by reflux distillation over sodium prior to use. All buffers were filtered through 0.2 μ m filters (Schleicher & Schuell MicroScience GmbH, Dassel, Germany) prior to use. The other chemicals were used as received. The monomers HEMAm-Lac_n and the (mPEG₅₀₀₀)₂-ABCPA macroinitiator were synthesised as described before^{27, 35}.

¹H-NMR Spectroscopy

¹H-NMR spectra were recorded with a Gemini 300 MHz spectrometer (Varian Associates Inc., NMR Instruments, Palo Alto, CA). Spectra were obtained in DMSO- d_6 where the central line of DMSO at 2.49 ppm was used as reference line; in D₂O the H₂O line lay at 4.75 ppm.

Block copolymer synthesis

Block copolymers with HEMAm-Lac_n (various ratios of mono- and dilactate) as thermosensitive block and mPEG₅₀₀₀ as hydrophilic block were prepared by free radical polymerisation using (mPEG₅₀₀₀)₂-ABCPA as macroinitiator (ratio of monomer/initiator was 150:1 mol/mol) essentially as described previously³⁶. In short, the concentration of the starting materials (monomers plus macroinitiator) was 300 mg/mL in ACN in airtight glass vials. The solution was flushed with nitrogen for at least 10 min, heated to 70 °C, and stirred for 24 hours. Next, by drop wise addition of the solution to an excess of diethyl ether, the polymers were precipitated. After centrifugation, the residue

was dried in a vacuum oven. Afterwards, the polymers were dissolved in water and dialysed (membrane with a cut-off of 12-14 kDa) against water for at least 24 hours with recovery by freeze drying.

¹H-NMR (DMSO, *d*₆): 7.5 (b, CO-NH-CH₂), 5.5 (b, CH-OH (HEMAm-Lac₂), 5.3 (b, CH-OH (HEMAm-Lac₁), 5.0 (b, CO-CH(CH₃)-O), 4.1 (b, CO-CH-(CH₃)-OH), 4.0 (b, CH₂-CH₂-O of HEMAm), 3.6 (b, PEG methylene protons, O-CH₂-CH₂), 3.4 (b, NH-CH₂-CH₂), 1.4, (b, CO-CH-CH₃), 1.3 (b, HO-CH-CH₃), 1.0-0.6 (pHEMAm-Lac_n main chain protons). The percentage of HEMAmLac₁ in the block copolymer was determined based on the ¹H-NMR spectra: $L_1 / (L_1 + L_2) * 100 \%$; 'L₁' and 'L₂' are assigned as the protons peaks of the hydroxyl group of HEMAm-Lac₁ and HEMAm-Lac₂, respectively.

Determination of the Critical Micelle Temperature (CMT)

The CMT of the synthesised block copolymers in aqueous solutions was determined with static light scattering using a Horiba Fluorolog fluorometer (650 nm at an angle of 90 °). The polymers were dissolved overnight at 4 °C in ammonium acetate buffer (pH 5, 120 mM) at a concentration of 2 mg/mL. The scattering intensity was measured every 0.2 °C during heating (heating rate approximately 1 °C/min) thereby stirring the solution in the cuvette. The onset on the X-axis, obtained by extrapolation of the intensity-temperatures curves to intensity zero, was considered as the CMT.

Derivatisation with methacryloylchloride

Methacrylate groups were introduced to the thermosensitive block by reaction of methacryloylchloride with the terminal hydroxyl groups of the lactate side chains. The aimed molar percentage of methacrylate moieties per monomer units was 3 till 12 % (~ 10 - 40 µL methacryloylchloride). The reactions were performed under dry conditions in freshly distilled THF (scale ~ 200 mg polymer, polymer concentration ~ 15 mg/mL) and in presence of an equimolar amount (with respect to methacryloylchloride) of triethylamine. After overnight reaction, the reaction mixture was dialysed (membrane with a cut-off of 12-14 kDa) against water and freeze dried. The percentage OH groups in the polymer derivatised with methacrylate groups were determined with NMR in DMSO-*d*₆ as follows: $((m+n)/2) / ((m+n)/2) + L_1 + L_2 * 100 \%$. The letters 'm' and 'n' correspond with the 2 protons of the double bond of methacrylate groups attached either to HEMAm-Lac₁ or HEMAm-Lac₂ chains. 'L₁' is the proton of the free alcohol functionality of HEMAm-Lac₁, 'L₂' is the proton

of the free alcohol functionality of HEMAm-Lac₂, and '(m+n) / 2' - the number of derivatised HEMAm-Lac₁ and HEMAm-Lac₂ chains.

Gel Permeation Chromatography (GPC)

The molecular weights of the different polymers and their distributions were determined by GPC. Two serial Plgel 3 μm MIXED-D columns (Polymer Laboratories) were used with a Waters System (Waters Associates Inc., Milford, MA) with a differential refractometer model 410. Poly(ethylene glycol)s of defined molecular weights were used as standards. The eluent was DMF containing 10 mM LiCl. The samples were dissolved overnight at a concentration of 5 mg/mL in the eluent and filtered through a 0.45 μm filter prior to analysis. The flow rate was 0.7 mL/min and the column temperature was 40 °C. Peak areas were determined with Empower Software Version 2 build 1154 (Waters Associates Inc).

Micelle formation & crosslinking

Micelles were formed via the 'heat shock' procedure as described previously^{36, 41}. In brief, the block copolymers were dissolved (2 mg/mL) overnight at 4 °C in ammonium acetate pH 5 buffer (120 mM). For the ¹H-NMR study, polymer solutions of 15 mg/mL in ammonium acetate pH 5 (50 mM) in D₂O were prepared. The solutions were incubated at 0 °C for at least 15 min in glass vials. To the polymer solutions that were to be crosslinked after micellar formation, 2 - 10 μL of an Irgacure[®] 2595 solution in water (5 mg/mL) was added to obtain a final molar ratio of methacrylate versus initiator of 25:1. Subsequently, the vials were rapidly heated from 0 to 50 °C by putting them into a water bath while vigorously stirring to form micelles. After 1 min of incubation at 50 °C, the mixtures were slowly cooled to room temperature. Crosslinked micelles were obtained by irradiating the micellar solution (~ 2 - 5 mL) with a Bluepoint lamp 4 (350-450 nm, Honle UV technology, Germany, light intensity was ~ 210 mW/cm² as measured with an Orion PD 300-SH at 410 nm) for 10 minutes while stirring the solution. The (non) irradiated micelles were finally filtrated through a 0.22 μm filter.

Dynamic Light Scattering (DLS)

Dynamic light scattering (DLS) was used to determine the particle size and size distribution of the polymeric micelles. The equipment consisted of a Malvern CGS-3 multi-angle goniometer (Malvern Ltd, Malvern, UK) with He-Ne

JDS Uniphase laser ($\lambda = 632.8$ nm, 22 mW output power), an optical fibre based detector, a digital LV/LSE-5003 correlator, and a temperature controller (Julabo Waterbath). Time correlation functions were analysed using the ALV-60.0 software V.3.X provided by Malvern. Scattering of the micellar solutions was measured at an angle of 90° and at 25°C in an optical quality 8 mL borosilicate cell giving rise to the hydrodynamic diameter of the micelles (Z_{Ave}) and their polydispersity (PD).

Micellar stability

Various experiments were performed to assess the thermal stability (DLS and NMR), the physical stability (SDS addition) and the chemical stability (DLS incubation at various pHs). For the thermal stability study, micelles were incubated first at 25°C , followed by stepwise cooling down to -3°C and stepwise heating back to 25°C . During both the DLS and NMR measurements at different temperatures, the samples were incubated for at least 10 minutes at the indicated temperature to obtain equilibrium. The chemical stability of the polymeric micelles was studied at 2 mg/mL with DLS; either in ammonium acetate buffer pH 5 (120 mM), phosphate buffer pH 7.4 (100 mM) or by threefold dilution of a 6 mg/mL polymer solution in ammonium acetate pH 5 (30 mM) with phosphate buffer pH 7.4 (100 mM), borate buffer pH 9 (100 mM), or carbonate buffer pH 10 (100 mM) to reach final pH values of 7.3, 8.5 or 9.5 respectively. A cell with approximately 1 mL micellar solution was incubated in the DLS machine at 37°C and the scattering was measured at regular time intervals. The physical stability was examined by the stepwise addition of 20 μL SDS (20 % solution) to 1 mL of a (non) crosslinked micellar dispersion; after each addition the DLS cell was gently mixed followed by immediate DLS measurement at 25°C .

Change of micellar hydrophobicity upon chemical destabilisation studies

Pyrene was used as a fluorescent probe to detect hydrophobic to hydrophilic changes in the micellar core upon chemical destabilisation⁴⁷. (Non) crosslinked micelles of different block copolymers were formed as described above in 30 mM ammonium acetate buffer (pH 5.0) at a concentration of 6 mg/mL. Subsequently, pyrene dissolved in acetone (30 μL , 12 $\mu\text{g}/\text{mL}$) was added to 1 mL micellar solution to achieve a final pyrene concentration of $6 \cdot 10^{-7}$ M. Samples were incubated overnight at room temperature in the dark to

allow equilibration of the samples and evaporation of the acetone. Before fluorescence measurements, the pyrene loaded micelles were 1:3 diluted with either pH 5 (120 mM), phosphate buffer pH 7.4 (100 mM), or carbonate buffer pH 10 (100 mM) yielding a final polymer concentration of 2 mg/mL and reaching final pH values of 5, 7.3 or 9.5, respectively. Fluorescence excitation spectra of pyrene were obtained using a Horiba Fluorolog fluorometer (90 ° angle). The excitation spectra were recorded every 10 minutes at 37 °C from 300 - 360 nm with the emission wavelength at 390 nm. The excitation and emission band slits were 4 and 2 nm, respectively. The intensity ratio of I_{338}/I_{333} was plotted against the time to detect changes of the micelle's core hydrophobicity in time.

Results & discussion

Synthesis of mPEG-*b*-p((HEMAM-Lac₁)-*co*-(HEMAM-Lac₂)) block copolymers

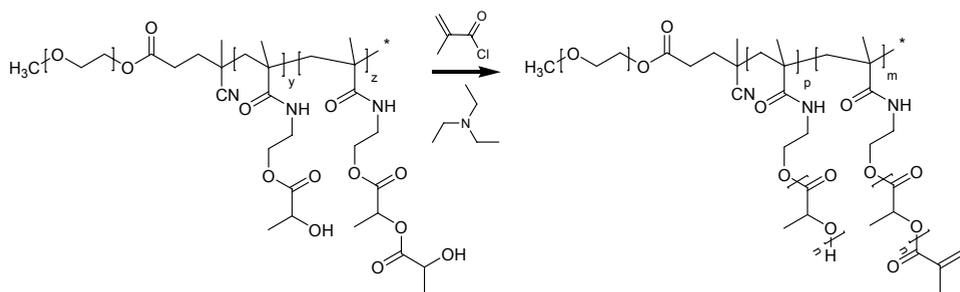
Various block copolymers composed of a random block of HEMAM-Lac₁ and HEMAM-Lac₂ and a hydrophilic mPEG block were obtained by radical polymerisation with a mPEG-macroinitiator and obtained in good yields (~ 80 %). ¹H-NMR revealed that the amounts of HEMAM-Lac₁ comonomer incorporated in mPEG-*b*-p((HEMAM-Lac₁)-*co*-(HEMAM-Lac₂)) corresponded well with the feed ratios (Figure 1, Table 1) as was also previously observed³⁶. GPC analysis showed that the block copolymers had an M_w of ca. 44000 g/mol with a polydispersity of ca. 1.9 (Table 1) which is normal for this type of free radical polymerisation. Similar to an earlier report³⁶, a linear correlation between the percentage of the more hydrophilic monomer HEMAM-Lac₁ in the polymer and the corresponding CMT was found (Table 1). In other words, the CMT of these block copolymers based on HEMAM-Lac_n can be tailored by the comonomer composition.

Methacrylation of mPEG-*b*-p((HEMAM-Lac₁)-*co*-(HEMAM-Lac₂)) block copolymers

Part of the lactate side chains (3 - 12 mol %) of the mPEG-*b*-p((HEMAM-Lac₁)-*co*-(HEMAM-Lac₂)) block copolymers was derivatised with methacrylate groups (Scheme 2) using methacryloylchloride as reactant and triethylamine as HCl scavenger. The percentage derivatisation of the hydroxyl groups of HEMAM-Lac₁ or HEMAM-Lac₂ with methacrylate moieties was determined by ¹H-NMR (Figure 1). After methacrylation, three additional peaks at 6.15, 5.8 and 2.1 ppm appeared which are assigned to the protons of the coupled methacrylate groups. The coupling efficiency of methacryloylchloride was low (~ 20 % of the added amount), but highly reproducible. Higher coupling efficiencies were obtained at longer reaction times and elevated temperatures, however, this was frequently associated with unwanted preliminary polymerisation of the methacrylate groups. GPC analysis of the methacrylated block copolymers displayed equal M_w and PD as before (Table 1) indicating that no preliminary interpolymer reaction of methacrylate had occurred.

P	feed % Lac ₁	% Lac ₁ (NMR)	M _w	M _w /M _n	CMT	% M	after methacrylation				after UV-illumination			
							M _w	M _w /M _n	CMT	ΔCMT	Z _{ave}	PD	Z _{ave}	PD
1	25	25	43500	1.9	41	3.6	43300	1.9	33.5	7.5	n.d.	n.d.	n.d.	n.d.
2	20	19	38300	1.7	38	5	36600	1.8	28.8	9.2	206 ± 9.4	0.06 ± 0.01	225 ± 4.2	0.02 ± 0.02
3	20	18.5	44300	1.8	35	6.5	49500	1.9	10.9	24.1	87.3 ± 5.4	0.05 ± 0.03	89.5 ± 11.3	0.09 ± 0.05
4	25	25	46000	1.9	35.6	8.1	46500	1.9	13.2	22.4	90.7 ± 2.4	0.06 ± 0.03	92.1 ± 1.3	0.08 ± 0.01
5	20	18.5	44300	1.8	35	8.6	45200	1.9	7.3	27.7	79.0 ± 5.2	0.07 ± 0.02	79.3 ± 9.1	0.08 ± 0.06
6	25	25	46000	1.9	35.6	9.5	46100	1.9	8.9	26.7	91.3 ± 4.2	0.06 ± 0.01	98.0 ± 1.4	0.04 ± 0.02
7	25	25	43500	1.9	41	10	45000	1.9	20.3	20.7	66.7 ± 7.6	0.1 ± 0.05	67.8 ± 7.2	0.07 ± 0.05
8	25	25	43500	1.9	41	11.6	45500	1.9	17.3	23.7	62 ± 2.0	0.08 ± 0.03	54.3 ± 4.0	0.06 ± 0.01
9	10	13	23700	1.4	33.2	13.6	29700	1.5	1.4	31.8	73.0 ± 4.2	0.11 ± 0.01	79.5 ± 14.8	0.08 ± 0.01
10	25	23	25100	1.4	42.3	14	34700	1.5	5.3	37	68.5 ± 5.0	0.09 ± 0.03	73.5 ± 9.2	0.07 ± 0.01
11	25	25	46000	1.9	35.6	15.1	45400	1.9	<0	n.d.	n.a.	n.a.	n.a.	n.a.
12	20	22	44300	1.8	35	17.8	43400	1.8	<0	n.d.	n.a.	n.a.	n.a.	n.a.

Table 1 Characteristics of mPEG-*b*-p((HEMAm-Lac₁)-*co*-(HEMAm-Lac₂)) block copolymers (n.d. = not determined, n.a. = not applicable, the micellar size and PD are determined by DLS and are depicted as the average ± standard deviation of three independently prepared micellar formulations, M_w and M_w/M_n are determined by GPC, % M is percentage methacrylation, ΔCMT is the difference in CMT before and after methacrylation)



Scheme 2 Derivatization of OH groups of mPEG-*b*-p((HEMAm-Lac₁)-*co*-(HEMAm-Lac₂)) with methacryloylchloride ($n = 1, 2$; y is % HEMA_m-Lac₁, z is % HEMA_m-Lac₂; m varies between 3 and 12 %)

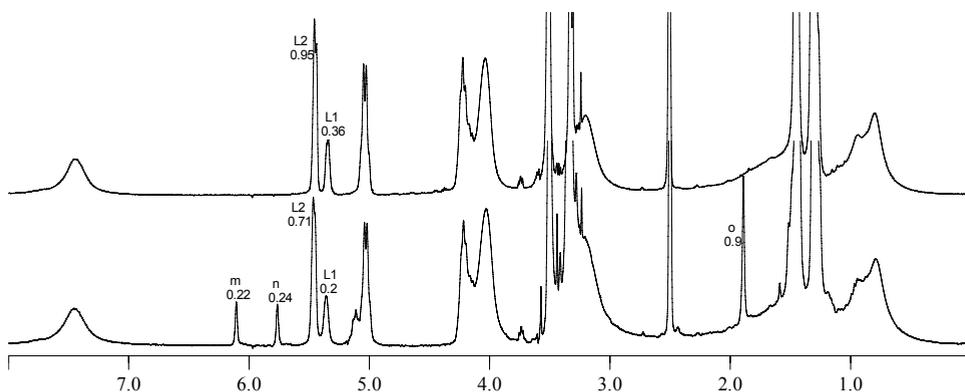


Figure 1 ¹H-NMR spectrum of mPEG-*b*-p((HEMA_m-Lac₁)-*co*-(HEMA_m-Lac₂)) in DMSO-*d*₆ (L₁ is the peak deriving from the hydroxyl group of HEMA_m-Lac₁ and L₂ corresponds to the hydroxyl group of HEMA_m-Lac₂). Upper spectrum displays the non modified polymer; lower spectrum corresponds with the methacrylated block copolymer (peaks *m*, *n* and *o* are attributed to the methacrylate groups)

Similar to a previous report¹⁸, coupling of hydrophobic methacrylate groups ($\log P = 0.58$) to a thermosensitive polymer decreased the CMT (Table 1). This lowering in CMT was linearly with the percentage of methacrylation. Polymers with a degree of methacrylation > 15 % (while keeping the ratio HEMA_m-Lac₁ and HEMA_m-Lac₂ constant at 25:75) were insoluble in water (i.e. CMT < 0 °C, P11 and P12). For practical reasons, it was aimed to obtain a methacrylated polymer which is present as dissolved unimers in water above 0 °C but as micelles at room temperature. Consequently, depending on the desired amount of methacrylate groups, a starting polymer mPEG-*b*-p((HEMA_m-

Lac₁)-co-(HEMAm-Lac₂)) was synthesized with a HEMA_m-Lac₁ to HEMA_m-Lac₂ ratio that yielded a polymer with a CMT between 0 °C and 25 °C after methacrylation. Polymers 3-10 (Table 1) fulfilled this criteria and their potential to generate biodegradable crosslinked micelles was examined.

Crosslinking of the micelles: methacrylate conversion & temperature effect

Micelles were formed by rapidly heating an ice-cold polymer solution to above the CMT of the hydrophobic block. Subsequently, micelles were photopolymerised using Irgacure[®] 2959 as initiator. During micelles formation, it is likely that the photoinitiator, because of its hydrophobic character (log P = 0.89), will accumulate in the micellar core. Furthermore, Irgacure has a good cytocompatibility profile which is an extra argument to use this photoinitiator⁴. Micelles based on 10 % methacrylated mPEG-*b*-p((25%HEMA_m-Lac₁)-co-(75%HEMA_m-Lac₂)) were prepared in D₂O and NMR spectra were recorded at 37 °C before and after UV-irradiation for 10 minutes (Figure 2).

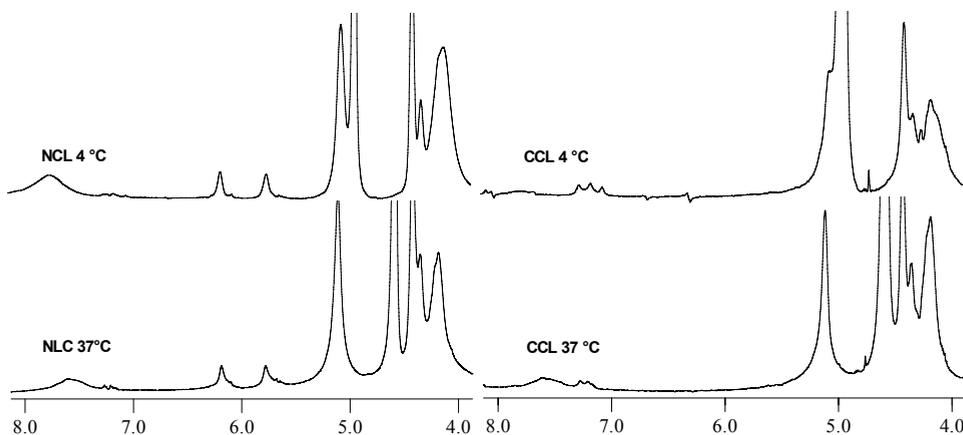


Figure 2 ¹H-NMR spectra of micelles based on 10 % methacrylated mPEG-*b*-p((25%HEMA_m-Lac₁)-co-(75%HEMA_m-Lac₂)) (P7) in D₂O before (left) and after (right) UV-illumination at either 4 or 37 °C (Note: the residual solvent peak in D₂O shifted from 5.0 ppm to 4.7 ppm upon the temperature increase)

Before illumination, the methacrylate moieties are clearly observed in the ¹H-NMR spectrum of the micelles in D₂O at 37 °C and were similar to but somewhat broader than the corresponding peaks in DMSO-*d*₆ (Figure 1). In

contrast, these peaks could not be detected anymore in the UV-irradiated micelles (Figure 2). Apparently, 10 minutes of illumination was already sufficient to achieve complete conversion of the methacrylate groups which is very attractive in controlling the reaction steps of (eventually drug loaded) biodegradable systems. In literature, crosslinked systems are reported that required 12¹ till 24 hours^{8, 11, 33} to complete. Photochemical polymerisation shortened the reaction time to 2 (unknown intensity)³⁵ or 4 hours (13 - 14 mW/m²)¹⁵ but this was still significant longer than the crosslinking time for the methacrylated thermosensitive micelles presented here; the light intensity presumably influences this efficiency.

The thermal stability of non and crosslinked micelles in D₂O was assessed by ¹H-NMR at temperatures above and below the CMT. At 37 °C (above the CMT), the non crosslinked (NCL) micelles displayed a significant lower signal of the amide-protons (~ 7.5 ppm) than observed in the corresponding spectrum in DMSO-*d*₆. This indicates that dehydration of amide moieties above the phase transition temperature occurs as previously also found for *N*-isopropylacrylamide polymers³⁰. Cooling to 4 °C revealed the peaks that point to rehydration of the polymers (Figure 2). The CCL micelles had, besides the absence of the methacrylate moieties, a similar NMR spectrum at 37 °C (Figure 2) with regard to the NCL micelles at the same temperature. However, below the CMT the revival of the amide protons was not observed (Figure 2). Likely, the crosslinking step restricted the polymer chains in their mobility (e.g. semi-solid phase) so that proton resonance could not be observed. The particle size and polydispersity of these polymeric micelles were measured with DLS at 25 °C before and after UV-illumination (Table 1). No change in particle size was found, e.g. P7 NCL micelles had a diameter of 67 ± 8 nm (PD 0.10 ± 0.05) while after crosslinking the size was 68 ± 7 nm with a PD of 0.07 ± 0.05 (n = 3).

Thermal stability

The thermal stability of micelles (6.5 % methacrylated, P3) was investigated before and after photopolymerisation (Figure 3). The untreated and UV-illuminated micelles had equal sizes (~ 83 nm) and PDs (~ 0.1) at 25 °C (above the CMT). Cooling the temperature to the CMT (10.9 °C for P3) provoked a strong increase in micellar size for the NCL micelles. This swelling is a result of the rehydration of the thermosensitive block copolymers at the CMT. Below 11 °C, this swelling continued while the scattering signal decreased and the PD increased dramatically to 1. The polymers phase transition from a hydrophobic to hydrophilic character fades away the micellar driving

hydrophobic aggregation forces until the NCL thermosensitive micelles are fully destabilised below their CMT. Bringing this polymer solution back to 25 °C, small micelles (94 nm) with a low polydispersity (0.08) were detected again (Figure 3a).

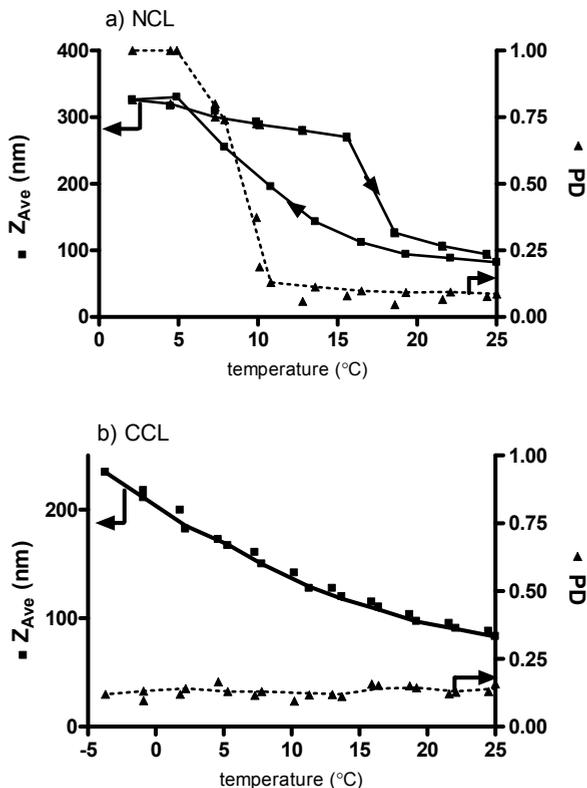


Figure 3 Thermal stability of micelles based on P3: 6.5 % methacrylated mPEG-*b*-p((19%HEMAm-Lac₁)-*co*-(81%HEMAm-Lac₂)), CMT 11 °C; —■— is diameter (Z_{Ave}) -▲- is polydispersity (PD)), a) NCL, b) UV-illuminated micelles

In contrast, the UV-illuminated micelles displayed a complete different behaviour (Figure 3b). At 25 °C, the micelles had a particle size of 83 nm. Cooling these micelles from 25 °C down to -3 °C (15 °C below CMT) caused a gradual increase in the particle size to 230 nm with a constant low polydispersity (< 0.15) and a constant scattering intensity. The photopolymerisation of methacrylate moieties apparently prevented micellar disintegration and demonstrates that the core was indeed crosslinked. Heating the micellar solution back to 25 °C resulted in a gradual shrinkage to finally

reach almost the original particle size, being 88 nm. This reversible swelling is in good agreement with shell crosslinked thermosensitive micelles as described in literature^{1, 20, 33}. Photopolymerised micelles composed of a block copolymer with 10 % methacrylate moieties (P7) showed less swelling below their CMT; their size was 59 and 68 nm at 4 and 37 °C, respectively. This is in good agreement with the expectations that the micellar cores (10 % methacrylation) are more strongly crosslinked than micelles with only 6.5 % methacrylate moieties. In literature, destabilisation of crosslinked systems was assessed by the addition of an organic solvent and also led to micellar swelling which was controlled by the crosslinking density^{11, 34, 37}.

Physical stability

The physical stability of the core crosslinked micelles was also investigated in the presence of a surfactant, sodium dodecyl sulphate (SDS)^{11, 14, 15}.

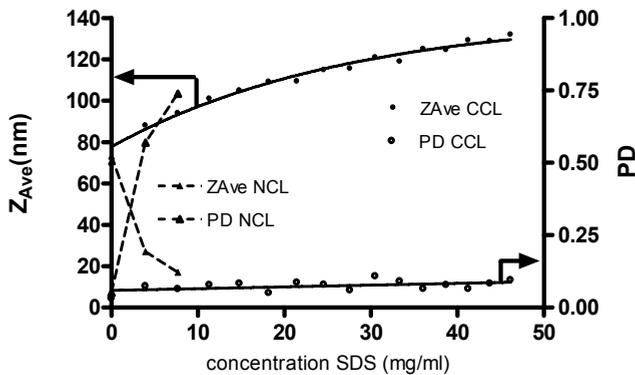


Figure 4 Effect of addition SDS on NCL (dashed lines) and CCL (solid lines) micelles based on P5 (9 % methacrylated mPEG-*b*-p((19%HEMAm-Lac₁)-co-(81%HEMAm-Lac₂)))

NCL micelles immediately fell apart at a SDS concentration of 4 mg/mL (above the CMC of SDS; which is 1,7 - 2,3 mg/mL or 8 mM) as seen by the rapid drop in size and huge increase in PD (Figure 4). It is known that the addition of SDS to block copolymer micelles speeds up the intermicellar exchange rate at low concentrations (< CMC) of SDS while at higher concentrations, the SDS micelles themselves are interacting with the block copolymers⁴⁶. So, in the case of NCL micelles, SDS destabilised the micelles by the extraction of

aggregated polymers which then results in particle disintegration. In contrast, CCL micelles retained their unimolecular size ($PD < 0.1$) upon SDS addition although the size increased from 69 to 132 nm (Figure 4). In a control experiment, SDS alone in pH 5 buffer above its CMC showed no particles with DLS (the size of SDS-micelles are below the detection limit). This again demonstrates that the crosslinked micelles have an excellent physical stability. The particle size increase might be ascribed to absorption of SDS by the micelles^{11, 14, 15}.

Chemical stability

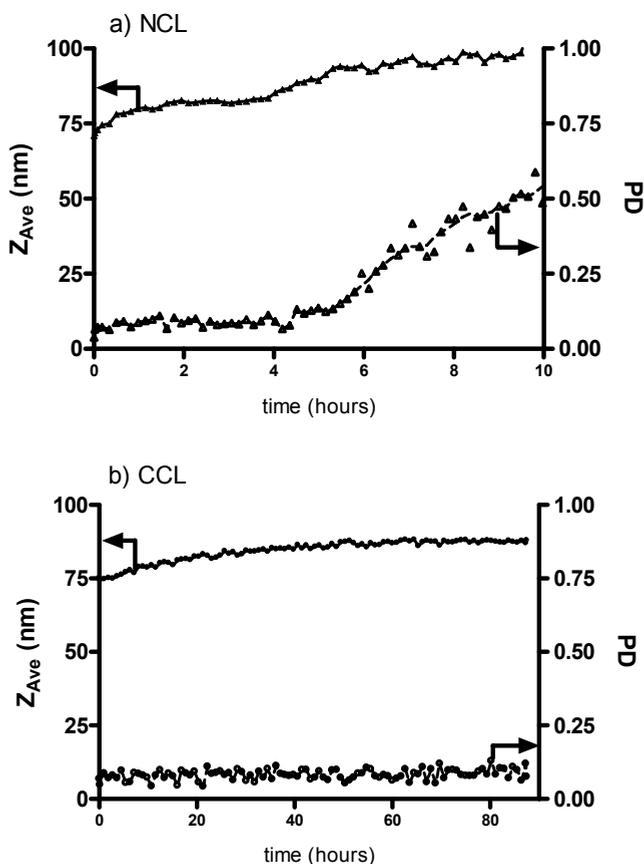


Figure 5 The size (Z_{Ave}) and PD of a) NCL and b) CCL micelles based on P7; 10 % methacrylated mPEG-*b*-p((25%HEMAm-Lac₁)-co-(75%HEMAm-Lac₂)) in pH 7.4 at 37 °C monitored in time

The destabilisation kinetics of (non) crosslinked micelles as a result of ester hydrolysis was examined. Incubation of NCL micelles in pH 7.4 buffer at 37 °C displayed a pattern that was already observed previously (Figure 5a)³⁶. During the first 4 hours, the particle size and PD remained stable. However, after 5 hours, the PD started to increase rapidly whereas the scattering intensity dropped (data not shown), indicating that the NCL micelles started to disintegrate after 5 hours incubation at physiological conditions. The destabilisation kinetics of CCL micelles were drastically slowed down as shown in Figure 5b. Even after almost 90 hours, the particle size only slightly increased, the scattering intensity was minimally lowered while the PD remained constant thus indicating the presence of intact structures after this time. The hydrolysis half lives of the monomers HEMAm-Lac₁ and HEMAm-Lac₂ at pH 7.4 and 37 °C are 58 and 5.6 hours respectively³⁶. The rapid degradation of HEMAm-Lac₂ is attributed to the so-called ‘back-biting’ attack of the terminal alcohol onto the carbonyl moiety two lactic acid units further in the side chain. To obtain the CCL micelles, some of the hydroxyl moieties were end capped with methacrylate groups and therefore random chain scission is required to break the crosslinks (Scheme 3). For a similar type of monomer, i.e. HPMAm-oligolactate, a 100-fold reduction of the hydrolysis rate was observed when the hydroxyl groups were end capped with an acetyl unit⁴⁵. Therefore, a similar reduction in degradation rate is anticipated for the crosslinks in these CCL micelles.

Within the time span of the CCL micelles destabilisation study at pH 7.4, enough non derivatised side chains will be hydrolysed to increase the polymers CMT till above 37 °C. However, the covalent crosslinks present in the rehydrated hydrophilic core hampers micellar disintegration. Consequently, the micellar core only swells, as was also observed by DLS measurements of CCL micelles at low temperatures (vide supra). Only when the methacrylate ester crosslinks for both HEMAm-Lac₁ and the HEMAm-Lac₂ derivatives are broken, the micelle can dissociate (Scheme 3). The destabilisation kinetics of CCL micelles were examined at 37 °C and at pH 8.5 (P4) and 9.5 (P8) to accelerate the hydrolysis of the ester bonds. The micelles with relatively low crosslink density (8.1 % methacrylated) showed strong swelling and disintegrated after 3.5 hours at pH 8.5 (Figure 6). The highly crosslinked micelles with 11.6 % methacrylate moieties destabilised only after ~ 12 hours at pH 9.5 as judged from the lowered scattering intensity (data not shown). Once again, the crosslink density controls the micellar stability, i.e. the extent of swelling and the final disintegration time. The disintegration values obtained at these elevated pH

can be extrapolated to physiological conditions because the hydrolysis rate of esters is a first order reaction in OH^- at pH values above 5⁶. The results of the accelerated degradation studies were used to calculate the disintegration times at pH 7.4 at 37 °C and appeared to be ~ 40 and 1300 hours respectively for P4 and P8. Indeed, incubation of CCL micelles prepared from P4 in pH 7.4 buffer at 37 °C resulted in destabilisation after 35 hours.

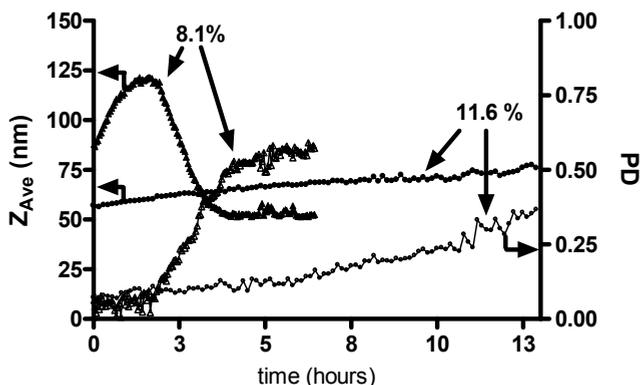


Figure 6 Incubation of CCL micelles: size (Z_{Ave}) and polydispersity (PD) of 8.1 % methacrylated (P4) in pH 8.5 or 11.6 % methacrylated (P8) in pH 9.5 buffer at 37 °C as measured by DLS

Pyrene fluorescence during chemical destabilisation

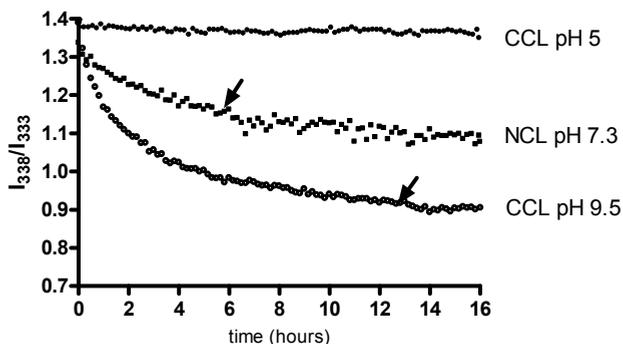
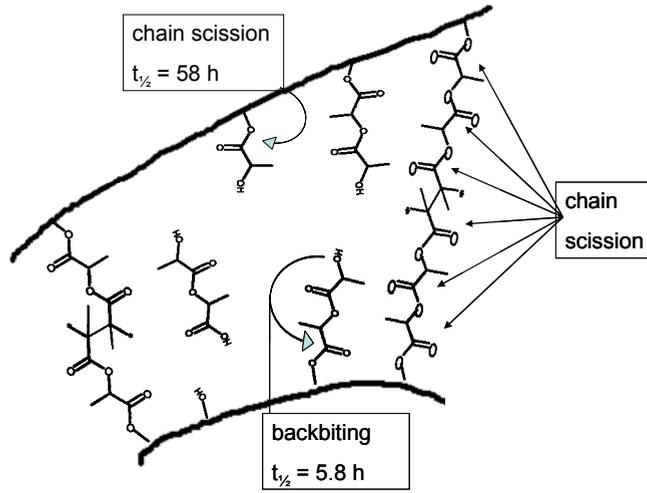


Figure 7 Pyrene fluorescence (I_{338}/I_{333}) followed in time for NCL and CCL micelles based on P8 under degradation conditions at 37 °C (the arrows indicate the start of micelle disintegration as measured with DLS)

The results of the thermal and chemical stability experiments (Figure 3 - 6) suggested that below the CMT or after lactate side chain hydrolysis, a hydrophilic micellar core is formed which is only kept together by the methacrylate crosslinks (Scheme 3). The polarity of the core was examined in time by using pyrene as a fluorescent probe. Upon environmental changes, i.e. from hydrophobic to hydrophilic, pyrene displays a shift in its fluorescence signal⁴⁷. Pyrene loaded (non) crosslinked micelles were prepared at pH 5, diluted with different buffers and the fluorescence signal was followed in time during incubation at 37 °C (Figure 7). At pH 5, the fluorescence signal of pyrene loaded micelles is stable since hydrolysis of ester moieties is minimised and consequently the hydrophobicity of the micellar core does not change. The NCL micelles showed a gradual decrease in the I_{338}/I_{333} ratio at pH 7.3; which is caused by an increase in the core's hydrophilicity in time since the hydrophobic lactate side chains are gradually hydrolysed. Due to this hydrolysis, the corresponding CMT of the polymer also increases and once this passes 37 °C the micelles will disintegrate, as was indeed observed after 5 hours with DLS (Figure 5). In contrast, incubation of CCL micelles at pH 9.5 caused immediately a large decrease in the I_{338}/I_{333} ratio, indicating a drastic change of the hydrophobicity of the core. However, during DLS measurements, the micelles only started to fall apart after approximately 12 hours (Figure 6). Taken together, this proves that degradation of core crosslinked micelles occurs in two steps. First, the non modified lactate side chains are rapidly hydrolysed as a result of the 'back-biting' reaction and generate a hydrophilic core that swells in size depending on the crosslinking density. Only after the slower hydrolysis of the ester bonds in the crosslinks, the micelles will disintegrate (Scheme 3).



Scheme 3 Hydrolysis mechanisms during disintegration of core crosslinked micelles

Conclusion

UV-illumination of micelles based on methacrylated thermosensitive mPEG-*b*-p((HEMAM-Lac₁)-*co*-(HEMAM-Lac₂)) block copolymers resulted in core crosslinking. The increased stability of these CCL versus the corresponding NCL micelles was clearly indicated by the intact micellar structure after addition of a large excess of SDS. Furthermore, the nanostructure kept its integrity at temperatures below the CMT since the crosslinks hampered the rehydrated hydrophilic core to disintegrate. NCL micelles start to fall apart after 5 hours at physiological conditions while the corresponding CCL micelles with 11 % crosslinked were stable for at least 90 hours and only in pH 9.5 disintegrated after 12 hours. Pyrene fluorescence measurements in time showed that this degradation was accompanied with a transition from a hydrophobic to a hydrophilic core far before the CCL micelles fell apart.

These hydrolysable core crosslinked polymeric micelles are considered to be very attractive for *in vivo* controlled drug delivery purposes. After *in vivo* injection the drug loaded CCL micelles are expected to be stable during circulation through the bloodstream and upon degradation of the lactate side chains (which is controllable by the type of polymer backbone and type of side chains), encapsulated drug will be released by (slight) swelling of the micelles. Ultimately, the micellar structure itself falls apart and the individual polymer chains will be excreted via the kidneys.

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chapter 8

Biodistribution of (paclitaxel-loaded) degradable core crosslinked polymeric micelles after intravenous administration

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Abstract

In chapter 7 of this thesis, it is shown that the photopolymerisation of thermosensitive micelles based on methacrylated mPEG-*b*-p((20%HEMAM-Lac₁)-*co*-(80%HEMAM-Lac₂)) polymers resulted in core crosslinked (CCL) micelles with a superior physical stability as compared to non crosslinked micelles (NCL). In this study, the circulation kinetics and biodistribution of these CCL micelles (with and without encapsulated paclitaxel (PTX)) after intravenous (i.v.) administration are explored. PTX-loaded micelles were crosslinked by potassium persulphate induced radical polymerisation. ³H-labelled CCL and NCL micelles were i.v. injected in 14C-tumour bearing mice. The biodistribution of ¹⁴C-PTX-loaded micelles was compared with ¹⁴C-PTX formulated in cremophor/ethanol (i.e. ¹⁴C-Taxol). The circulation kinetics and biodistribution of empty CCL micelles were considerably different as compared to NCL micelles with the same size (57 nm), i.e. 58 % of the injected dose (ID) of CCL versus 6 % of NCL micelles was recovered in the circulation 4 hours post injection. Furthermore, the liver uptake of the CCL micelles (10 % ID) was much lower than that of the NCL micelles (24 % ID) 4 hours after administration, while tumour accumulation was almost 6 times higher for the CCL micelles, reaching 5.8 % ID per gram tumour 48 hours post injection. Likely, NCL micelles dissociated after i.v. administration and/or were opsonised after adsorption of plasma proteins while the rigid PEG shell of fixed CCL micelles made them less prone towards opsonisation. Clearly, CCL polymeric micelles had a circulation profile resembling that of clinically applied PEGylated long-circulating liposomes while less uptake by the mononuclear phagocyte system was observed. The particle size of PTX-loaded CCL micelles was 76 nm (PD 0.08) and the particles had a PTX-loading capacity of 13 % (w/w, final polymer concentration was 15 mg/mL; PTX concentration was 2 mg/mL). Upon i.v. administration of ¹⁴C-PTX-loaded ³H-labelled micelles, the tissue distribution of the two radiolabels was markedly divergent. The distribution of ¹⁴C-Taxol was comparable to ¹⁴C-PTX loaded in (non) crosslinked micelles, indicating rapid release and/or extraction of encapsulated PTX from the micellar core. In conclusion, thermosensitive biodegradable CCL micelles demonstrated a very favourable circulation and biodistribution profile but 95 % of the encapsulated PTX was released and/or extracted from these micelles within 30 minutes. The entrapment of drugs that have a higher compatibility with the CCL core will be beneficial in achieving enhanced tumour accumulation of drug-loaded micelles.

Introduction

Polymeric micelles (PM) are presently under investigation for the (targeted) delivery of both low molecular weight drugs and biotherapeutics (e.g. pDNA, siRNA)^{12, 54}. Their small size (10 - 100 nm) and hydrophilic outer shell (usually composed of polyethylene glycol (PEG)) prevent opsonisation in the blood stream and subsequent uptake by the mononuclear phagocyte system (MPS), which is favourable for prolonged blood circulation kinetics. Both a small size and long circulatory half-life are considered to be essential to achieve tumour accumulation via the enhanced permeation and penetration (EPR) effect^{31, 38}. Until now, *in vivo* studies performed with drug-loaded PM are mainly focused on therapeutic effects rather than the biodistribution of the drug-carrier complex itself. Of the micellar systems investigated so far, only a few have favourable circulation kinetics. For example, 25 % of the administered dose of PEG-*b*-PDLLA micelles⁵⁷ and 20 % of mPEG-*b*-polycaprolactone assemblies²⁹ was still present in the circulation 24 hours post injection. In general, PM accumulate to a large extent in the MPS-organs (i.e. liver and spleen^{2, 17}), are non-specifically distributed over other tissues and do not (substantially) prolong the circulation time of encapsulated drug or enhance its tumour accumulation. Apparently, PM are often prone to premature dissociation and/or preliminary drug release occurs upon intravenous (i.v.) administration, either due to dilution below their critical micelle concentration (CMC) or by destabilisation by blood components^{29, 44}. The relatively rapid elimination of many of the PM formulations asks for stabilisation strategies. One of the proposed routes to circumvent dissociation upon i.v. administration is covalent crosslinking^{37, 42} of either the micellar shell (SCL)^{3, 51} or the core (CCL)^{16 23}. Since crosslinking will affect the degradability of polymeric assemblies, sophisticated micelles with labile or reversible crosslinks are currently under investigation⁵⁵, and comprise for example reducible disulfide bonds^{21, 27, 33, 36} or photo-induced dimerisation of cinnamoyl containing block copolymers^{19, 20}.

Drug-loaded crosslinked micelles are prepared in two ways, either via soaking empty crosslinked micelles in a solution of a hydrophobic drug or by crosslinking drug-loaded micelles. The latter is most frequently done for SCL micelles^{15, 27, 58} and retarded the release of paclitaxel¹⁵ and dipyrimadole²⁷ when compared to non crosslinked (NCL) counterparts. Some core crosslinking strategies are successfully applied to stabilise preloaded micelles^{18, 28}, but most reactions are done by (temperature- or light-induced) radical

polymerisation^{16, 23}, which are potentially harmful for encapsulated drugs^{5, 23, 46}. Core crosslinking can enhance the drug-loading capacity and prolong the *in vitro* drug release⁴³. For example, an eightfold higher amount of triclosan could be encapsulated in the core of PEG-*b*-lipid micelles (crosslinked by vinylic termini) relative to the NCL assemblies⁵³. The release of doxorubicin from PCL-(chitosan-oligosaccharide)-PEG micelles was extended from 4 to 8 days upon crosslinking²⁸ and the camptothecin release rate from crosslinked PLA-*b*-PEG-*b*-PLA nanoparticles was inversely dependent on the crosslinking density²⁶.

Paclitaxel (PTX) is a potent cytotoxic agent that arrests the cell division through stabilisation of the microtubuli¹³. This very hydrophobic compound has a low water solubility of ~ 6 µg/mL (25 °C)¹⁵. Therefore, the commercial formulation (Taxol) contains the non-ionic surfactant cremophor (polyoxyethylated castor oil) and ethanol as solubilising agents although the castor oil is correlated with various hypersensitivity and toxicity issues^{11, 56}. Several nanoparticulate systems are currently investigated as potential delivery vehicles for PTX^{10, 13}. Among these, micellar encapsulated PTX displayed equal distribution profiles as the Taxol formulation presumably due to rapid release/extraction of the drug from the carrier and/or micellar dissociation^{7, 25, 47}. Nevertheless, these micelles were advantageous over Taxol because of an increased maximum tolerated dose (MTD)^{6, 25}, which was similar to that of micelles with PTX conjugated to the polymer backbone via labile bonds⁴. The use of CCL micelles provides a possible way to retain the drug more stably and to increase its target accumulation. The only crosslinked micellar system tested *in vivo* so far were DNA containing reducible CCL micelles which showed effective liver transfection³³. Crosslinked micelles loaded with low molecular weight drugs have not yet been explored *in vivo*.

In chapter 7 of this thesis, degradable CCL micelles are described which were obtained by introducing methacrylate moieties onto the degradable oligolactate grafts of the core forming thermosensitive polymer (up to 12 % of the grafts were derivatised). After micelle formation by heating an aqueous polymer solution to above the critical micelle temperature (CMT), UV-illumination in the presence of a photoinitiator resulted in polymerisation of the methacrylate groups by which the core of the micelle were crosslinked. These CCL micelles had a superior physical stability as compared to their NCL counterparts, as for example evidenced by the fact that they stayed intact upon incubation with SDS. The CCL micelles retained their degradability. Upon incubation at physiological conditions, the NCL micelles disintegrated after ~ 5 hours as a result of side chain hydrolysis⁴⁰. In the same time frame, unmodified

lactate grafts present in the CCL micelles were also cleaved, but the crosslinks kept the micellar structure intact. Disassembly of the CCL occurred only upon ester hydrolysis of these crosslinks and consequently the dissociation time was dependent on the crosslink density and varied between 30 hours to 60 days⁴⁰. Since the core of these thermosensitive biodegradable micelles is composed of hydrophobic lactate side chains, various hydrophobic drugs can potentially be encapsulated. The aim of this study is to evaluate the circulation kinetics and biodistribution profile including tumour accumulation of PTX-loaded hydrolysable CCL micelles based on methacrylated mPEG-*b*-p((20%HEMAm-Lac₁)-*co*-(80%HEMAm-Lac₂)) in 14C-tumour bearing mice.

Materials & methods

Materials

Acetonitril (ACN) and tetrahydrofuran (THF) were purchased from Biosolve Ltd. (Valkenswaard, The Netherlands). Tritium labelled (^3H -)acetic anhydride was a product of Amersham (Roosendaal, The Netherlands) while ^{14}C -paclitaxel (^{14}C -PTX) was obtained from Campro Scientific BV (Veenendaal, The Netherlands). Ultima Gold liquid scintillation cocktail and Solvable tissue solubiliser were purchased from Perkin Elmer Bioscience BV (Groningen, The Netherlands). Hydrogen peroxide and potassium persulphate (KPS) were both obtained from Merck (KGaA, Darmstadt, Germany). Irgacure 2959 (Ciba Specialty Chemicals Inc), 4-(*N,N*-dimethylamino)pyridine (DMAP, Fluka) and *N,N,N',N'*-tetramethylethylenediamine (TEMED, Fluka Chemie AG, Buchs, Switzerland), paclitaxel (PTX, MP Biomedicals Inc, Illkirch, France), Taxol (Mayne Pharma, Brussels, Belgium), deuterated water (D_2O) and trifluoroacetic acid (TFA, Sigma Aldrich, Zwijndrecht, The Netherlands) were all used as received. THF was freshly distilled and dried overnight over molecular sieves before use. A 20 % solution of sodium dodecyl sulphate (SDS) from Biorad Laboratories (Hercules, USA) was diluted 1:1 with buffer pH 5 (ammonium acetate, 120 mM). All buffers were filtered through 0.22 μm filters (Schleicher & Schuell MicroScience GmbH, Dassel, Germany) prior to use. ^1H -NMR spectra were recorded with a Gemini 300 MHz spectrometer (Varian Associates Inc., NMR Instruments, Palo Alto, CA, USA).

Animals

Balb/c nude female mice were housed in groups of 4 - 8 animals and had free access to water and food. All animal studies were performed in compliance with guidelines set by national regulations and were approved by the local animal experiments ethical committee. The head and neck squamous-cell carcinoma line 14C was introduced in the flank of the mice by inoculation with 1×10^6 14C-tumour cells suspended in 100 μL ^{14}C . Palpable subcutaneous tumours had developed over a period of approximately one month.

Polymer synthesis & micelle formation

A methacrylated (12 % of the terminal hydroxyl moieties) block copolymer mPEG-*b*-p((20%HEMAm-Lac₁)-*co*-(80%HEMAm-Lac₂)) was synthesised

and characterised essentially as described before⁴⁰. In brief, after polymerisation of the monomers HEMAm-Lac_n with a mPEG-macroinitiator, part of the hydroxyl groups were derivatised with a methacrylate moiety via reaction with methacrylic anhydride in dry THF in presence of a catalytic amount of DMAP. Radioactive labelled methacrylated polymers were obtained by derivation of less than 1 % of the terminal hydroxyl groups with ³H-acetate moieties. For this modification, the methacrylated block copolymer (~ 50 mg) was dissolved in THF (~ 5 mL) together with a catalytic amount (~ 1 mg) of DMAP in a septum capped glass vial and ³H-acetic anhydride (11 µL, ~ 20MBq) was added. After flushing N₂ through the solution via the septum for 15 minutes, the solution was stirred overnight at room temperature. The polymers were purified by extensive dialysis (Pierce dialysis bag, MWCO 10 kDa) against pH 5 buffer (ammonium acetate, 30 mM) at 4 °C until no radioactivity could be traced in the dialysate. Counting the radioactivity of the dialysed 12 % methacrylated mPEG-*b*-p((20%HEMAm-Lac₁)-*co*-(80%HEMAm-Lac₂)) solution demonstrated that approximately five percent of the ³H-acetic anhydride had reacted with the hydroxyl termini.

The ³H-labelled polymer (2.5 mg/mL in ammonium acetate buffer pH 5, 30 mM) was supplemented with non-labelled methacrylated polymer to achieve a final polymer concentration of 10 mg/mL and radioactivity of approximately 70 kBq/mL. Non- and core crosslinked empty micelles were formed as described before⁴⁰. In brief, micelles were formed by rapidly heating an ice cold polymer solution to above the CMT while stirring. The crosslinking occurred upon illumination (Bluepoint lamp 4 (350-450 nm, Honle UV technology, Germany) of the micelles during 10 minutes in presence of the photoinitiator Irgacure. Afterwards, the solutions were brought to pH 7.4 and iso-osmotic conditions with NaOH and NaCl respectively. The mean particle size (Z_{Ave}) and polydispersity index (PD) of the micelles was determined with dynamic light scattering (DLS) using a Malvern ALV/CGS-3 Goniometer⁴¹. Radioactivity of the micellar dispersions were determined in an Ultima Gold liquid scintillation cocktail and counted in a Packard Tricarb 2200 CA liquid scintillation counter.

Biodistribution of empty ³H-labelled polymeric micelles

For the biodistribution study of empty micelles, 200 µL of 10 mg/mL ³H-labelled micelles was administered via the tail vein of the mice. Blood samples were obtained via orbital puncture after 0.5, 2 or 6 hours and the animals were sacrificed after 1, 4 or 24 hours, respectively (4 mice per group). In case of CCL micelles, an extra group was used of which the animals were blood sampled

after 24 hours and sacrificed after 48 hours. The organs and tissues of interest were removed and their wet weight was measured. To 50 μL of the blood samples, 100 μL Solvable tissue solubiliser and 100 μL 35 % hydrogen peroxide were added and these mixtures were incubated at room temperature overnight. A similar procedure was applied for the dissected liver, spleen and tumour although the time required for full dissolution was longer (3 days). Ten mL Ultima Gold scintillation cocktail was added to these samples and the resulting mixture was counted for radioactivity. The percent injected dose (% ID) and the percent injected dose per gram tissue (% ID/g) values were calculated using the following equations:

$$\% \text{ ID} = (\text{Bq in tissue sample} / \text{Bq in original micelle formulation}) * 100 \%$$

$$\% \text{ ID}_{\text{tissue}}/\text{g} = (\% \text{ ID}_{\text{tissue}} / \text{weight of tissue in g})$$

Preparation of paclitaxel-loaded micelles

PTX was severely degraded upon UV-exposure (demonstrated by UPLC analysis), and thus, photopolymerisation with Irgacure could not be performed to prepare PTX-loaded CCL micelles. Therefore, KPS-TEMED initiation, as previously used for hydrogel formation^{49, 50}, was explored as alternative to polymerise the methacrylate moieties present in the micellar core. In detail, solutions of 5 mg/mL KPS (freshly prepared), 20 mg/mL TEMED and 20 mg/mL polymer (dissolved overnight at 4 °C) (all in pH 5 ammonium acetate buffer, 150 mM) as well as 20 mg/mL PTX in ethanol were placed in thin wall glass vials on ice. To 608 μL of the polymer solution, the following solutions were added: 40 μL TEMED, 72 μL KPS and 80 μL PTX. Next, the resulting solution was rapidly heated by immersion of the glass vial into a water bath of 60 °C for 1 minute while stirring. Subsequently, the micelles were quickly transferred into a septum capped glass vial and N_2 was flushed through the mixture via the septum for 10 minutes. Thereafter, the micellar dispersion was left at room temperature for 60 minutes, followed by filtration of the micelles through a 0.2 μm filter to remove possible drug-aggregates. During the formation of NCL micelles, the KPS and TEMED solutions were replaced by equivalent volumes pH 5 buffer (ammonium acetate, 150 mM). NMR analysis was done as described before⁴⁰ and for that purpose, all excipients (except the PTX) were dissolved in deuterated pH 5 buffer (ammonium acetate 100 mM).

UPLC analysis of paclitaxel

For the determination of the PTX concentration, micellar dispersions were 100 times diluted in ACN/H₂O (50:50 v/v). Next, 5 µL of this solution was analysed by UPLC (Acquity, Waters, Etten-Leur, the Netherlands). A C8 column was used with a gradient from 70 % eluens A (95 % H₂O/5 % ACN/0.1 % TFA) to 60 % B (95 % H₂O/5 % ACN/0.1 % TFA) in 2 minutes with a flow of 1 mL/min and UV-detection at 227 nm. Paclitaxel standards dissolved in ethanol were used to prepare a calibration curve (linear between 0.5 and 400 µg/mL).

Biodistribution of ¹⁴C-PTX-loaded ³H-labelled micelles

A trace amount of ¹⁴C-PTX was mixed with non-labelled PTX dissolved in ethanol to achieve a final PTX concentration of 20 mg/mL and radioactivity of approximately 100 kBq/mL. This solution was used to prepare PTX-loaded ³H-labelled NCL and CCL micelles as described above. The biodistribution of these ¹⁴C-PTX-loaded ³H-labelled micelles as well as ¹⁴C-PTX supplemented Taxol was studied in ¹⁴C-tumour bearing mice. Hundred µL of a 2 mg/mL PTX formulation (15 mg/mL polymer) was administered via the tail vein corresponding with a final dosage of 10 mg PTX per kg mice. Blood samples were taken via orbital puncture at 0.5, 2 or 6 hours after injection and animals were sacrificed after 1, 4 or 24 hours respectively (4 mice per group) followed by dissection of the liver, spleen, tumour and a piece of the abdominal skin. In case of CCL micelles, an extra group was evaluated of which the animals were blood sampled after 24 hours and sacrificed after 48 hours. Analysis of the samples was done as described above.

Results & discussion

Biodistribution of empty ^3H -labelled polymeric micelles

The evaluation of CCL and NCL micelles as carrier system for PTX was preceded by a study of the circulation kinetics and biodistribution of the empty carriers, which are composed of 12 % methacrylated mPEG-*b*-p((20%HEMAm-Lac₁)-co-(80%HEMAm-Lac₂)) block copolymers. The CMT of this polymer ($\sim 11^\circ\text{C}$) was in a practical range, i.e. below room temperature and above 0°C allowing particle formation by simply heating a polymer solution to above its CMT ⁴⁰. A tritium-labelled polymer was obtained by reacting some of the hydroxyl groups of the lactate side chains ($< 1\%$) with ^3H -acetic anhydride (Figure 1).

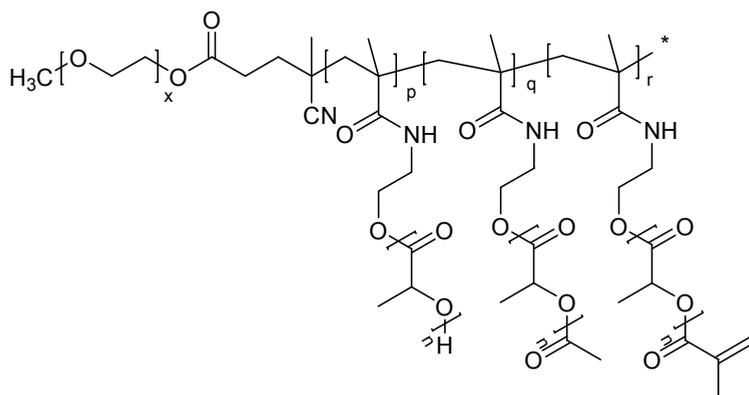


Figure 1 Chemical structure of ^3H -labelled partially methacrylated mPEG₅₀₀₀-*b*-p((20%HEMAm-Lac₁)-co-(80%HEMAm-Lac₂)) ($n = 1$ or 2 ; p , q and r corresponded with 87, ~ 1 and 12 % respectively, while the PEG₅₀₀₀-block comprised on average 114 units (x))

The resulting ^3H -labelled polymer retained its temperature-sensitivity with a CMT of 14°C . The CMT of the polymer before the labelling procedure was 11°C , which indicates that some (ester) hydrolysis of the side chains had occurred during the dialysis step to remove unbound label (pH 5 at 4°C). Rapidly heating a 10 mg/mL polymer solution to above the CMT in presence of a photoinitiator (Irgacure) and subsequent UV-illumination yielded ^3H -labelled

CCL micelles with a Z_{Ave} of 57 nm (PD of 0.07). Applying this procedure for the same polymer solution but without the Irgacure and illumination yielded ^3H -labelled NCL micelles with the same size and polydispersity. The biodistribution of both CCL and NCL micelles was investigated in ^{14}C -tumour bearing mice via blood and tissue sampling at 1, 4 and 24 hours (Figure 2; only in case of CCL micelles, mice were sampled 48 hours post administration).

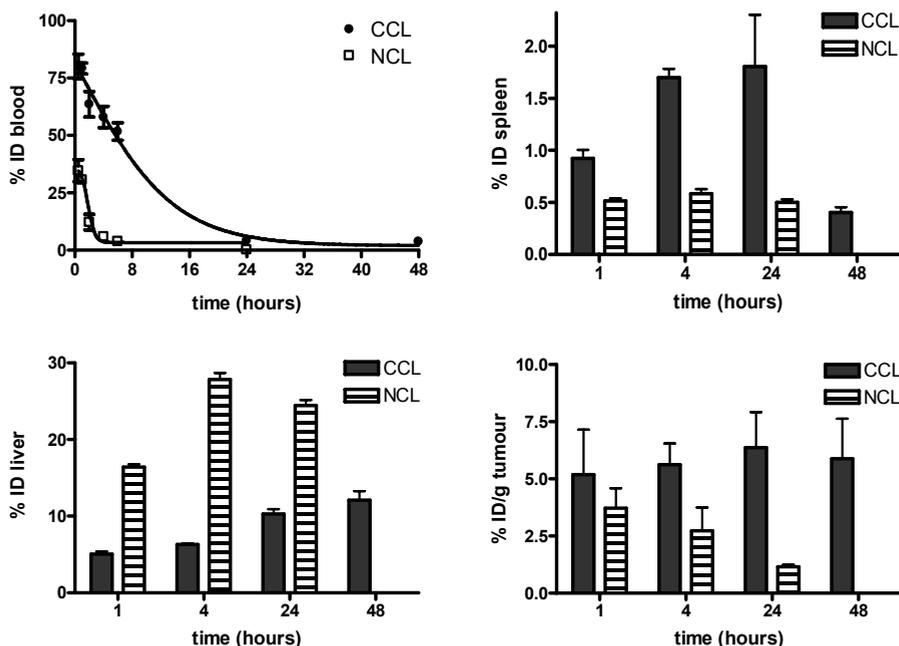


Figure 2 The biodistribution profile of non crosslinked (NCL, dashed bars) and core crosslinked (CCL, filled bars) micelles was evaluated in ^{14}C -tumour bearing mice using ^3H -labelled polymers (10 mg/mL): percentage of the injected dose (% ID) in blood (upper left), spleen (upper right), liver (lower left) and tumour (% ID/g, lower right) (Note: only in case of CCL micelles, mice were also sampled 48 hours post administration)

Both micellar formulations displayed good tolerability as no infusion related side effects, direct toxicity or other micellar associated negative symptoms were observed. The NCL micelles were rapidly eliminated from the circulation and only 6 % of the ID was present after 4 hours. Low amounts (< 1 % of the ID) resided in the spleen while the liver uptake was high (28 % ID after 4 hours). Furthermore, NCL micelles showed some initial tumour localisation (2.5 % ID 4 hours post injection), however at these early time points,

radioactivity in the blood can still contribute substantially to overall tumour radioactivity levels. Indeed, only 1.1 % ID/g was recovered in the tumours after 24 hours, indicating the absence of significant tumour extravasation and retention of these NCL micelles. The administered injected dose of NCL micelles resulted in a blood polymer concentration of 1 mg/mL directly after injection which is far above the CMC (0.08 mg/mL). Additionally, it was previously calculated for a similar micellar system (mPEG-*b*-p((80%HEMAm-Lac₂)-*co*-(20%HEMAm-Lac₄))), that the distance between neighbouring PEG chains on the micellar surface was 3.6 nm⁴¹. Such a high PEG density on the surface should be an effective steric barrier for adsorption of serum proteins, thereby minimising opsonisation and MPS-phagocytosis². Despite these two favourable characteristics, a rapid blood clearance of the NCL micelles was observed. After i.v. injection, interactions with plasma proteins are likely to induce either premature dissociation of the micellar structure or the surface of the micelles is modified due to protein adsorption which leads to (enhanced) recognition by macrophages. Both events might explain the relatively high uptake of the NCL micelles by the liver: either the unimers associate to hydrophobic blood components (e.g. lipoproteins) and transported to the hepatocytes²² or intact micelles are phagocytosed by Kupffer cells³⁴.

Contrary to NCL assemblies, CCL micelles displayed significantly prolonged circulation times since more than 50 % ID was still present in the systemic circulation after 6 hours. Consequently, the AUC_{blood (0-24h)} of CCL was significantly higher than the AUC_{blood} of NCL micelles (990 versus 136 % ID × h/mL blood). After 24 hours, the CCL micelles showed a higher uptake in the spleen than the NCL micelles (1.8 % versus 0.5 % ID), whereas their liver accumulation was more than 2-fold lower than that of NCL structures (10 % versus 24 % ID). The % ID of CCL micelles in the tumour appeared already fairly high after 1 hour (5 % ID/g) but this can probably be accounted for by high blood contributions. More importantly, a considerable tumour accumulation was still observed after 48 hours (5.8 % ID/g) although the blood levels of ³H-labelled CCL micelles were negligible by then. This clearly indicates that the small CCL micelles extravasated from the blood vessel into the tumour tissue, likely as a result of their high AUC which contributed to the EPR effect³¹. The prolonged blood circulation of CCL micelles leads to enhanced exposure and favours a high degree of tumour localisation similarly as observed for long-circulating liposomes⁴⁵, which display blood half-lives of 5 - 20 hours^{1, 24}. In case of liposomes, the predominant factor for prolonged blood retention and effective tumour accumulation is their small size^{32, 45}. Furthermore, even

though the PEG-coating of liposomes minimises opsonisation, complement activation reactions are observed⁵², and especially the spleen and liver play important roles in the blood clearance of PEGylated liposomes¹⁷. In general approximately 2 - 3 % of the administered liposome dose still resides in the blood 48 hours post injection, ~ 20 % has accumulated in the liver while 10 % is recovered in the spleen¹. In contrast, the CCL micelles studied here were taken up by the liver to a lower extent (12 %) and a much lower splenic accumulation was observed (0.5 %) at 2 days post injection. Upon crosslinking the micellar core, presumably a rather rigid and dense PEG₅₀₀₀-coating is formed which more effectively prevents opsonisation as compared to the liposomes which are covered with a relatively low density (generally 5 mol %) of PEG₂₀₀₀-chains^{1, 32, 39}. In addition, as splenic uptake is strongly correlated with particle size³⁵, the smaller size of CCL micelles as compared to PEGylated liposomes may contribute to this observation.

Paclitaxel loading of CCL micelles

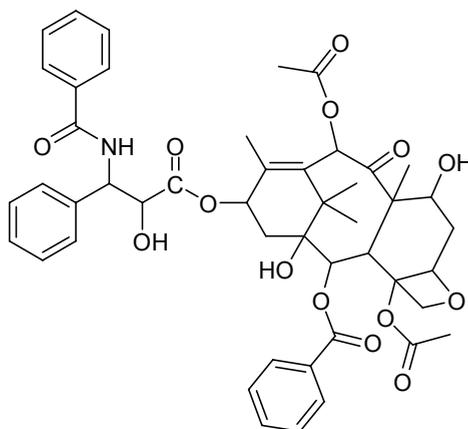


Figure 3 Chemical structure of paclitaxel

UPLC analysis demonstrated that paclitaxel (Figure 3) was severely degraded upon UV-illumination, but it was unaffected upon exposure to an alternative polymerisation initiator system (i.e. TEMED-catalysed KPS radical formation⁵⁰). This initiator system was therefore applied to crosslink the PTX-loaded micelles. It turned out to be essential to ensure a rapid heating of the polymer solution during micelle formation to achieve almost quantitative encapsulation (> 95 %) of the PTX. As the standard therapeutic dose is approximately 10 mg/kg mouse, a micellar dispersion was prepared with a final

PTX concentration of ~ 2 mg/mL and polymer concentration of 15 mg/mL (i.e. PTX loading of 13 % w/w). The KPS-TEMED crosslinked micelles were studied with DLS. Micelle formation in presence of relatively high concentrations of initiator (final concentrations were 9 μM KPS and 32 μM TEMED as used for hydrogel formation⁵⁰) resulted in particles with a Z_{Ave} of 193 nm (PD of 0.2) which were much bigger than NCL particles (~ 60 nm). Likely, because the initiator is added to the ice-cold polymer solution (i.e. before micelles are formed), some radicals are already generated immediately after KPS-TEMED addition, thereby initiating polymerisation of the methacrylated groups and linking polymer chains together before micelle formation could occur. These clustered chains obviously prevented the formation of small monodisperse micelles during the subsequent heating of the polymer solution. A 4-fold reduction of the amount of initiator and catalyst (final concentrations were 2 μM KPS and 9 μM TEMED) yielded particle sizes closer to those of UV-illuminated (empty) micelles, i.e. ~ 60 nm. The PTX-loaded CCL and NCL micelles had a Z_{Ave} of 76 nm (PD 0.08) and 66 nm (PD 0.07), respectively. When compared to empty micelles, the size of loaded NCL micelles was slightly larger due to the presence of 10 % ethanol in the solution as previously observed for mPEG-*b*-p(HPMAm-Lac₂) micelles⁴⁸. The crosslinking in presence of 13 % PTX (w/w) apparently increased the micellar diameter (by ~10 nm) but the particles formed were still nicely monodisperse. Upon formation of CCL micelles in D₂O, no unreacted methacrylate peaks were detected with NMR analysis indicating full conversion which is in agreement with previous results⁴⁰.

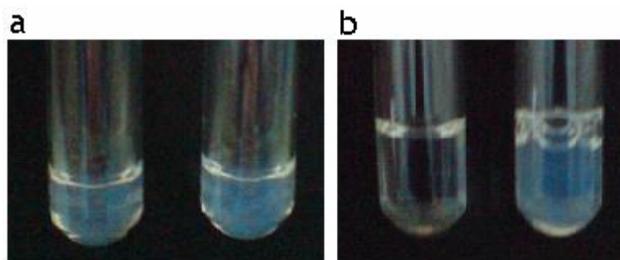


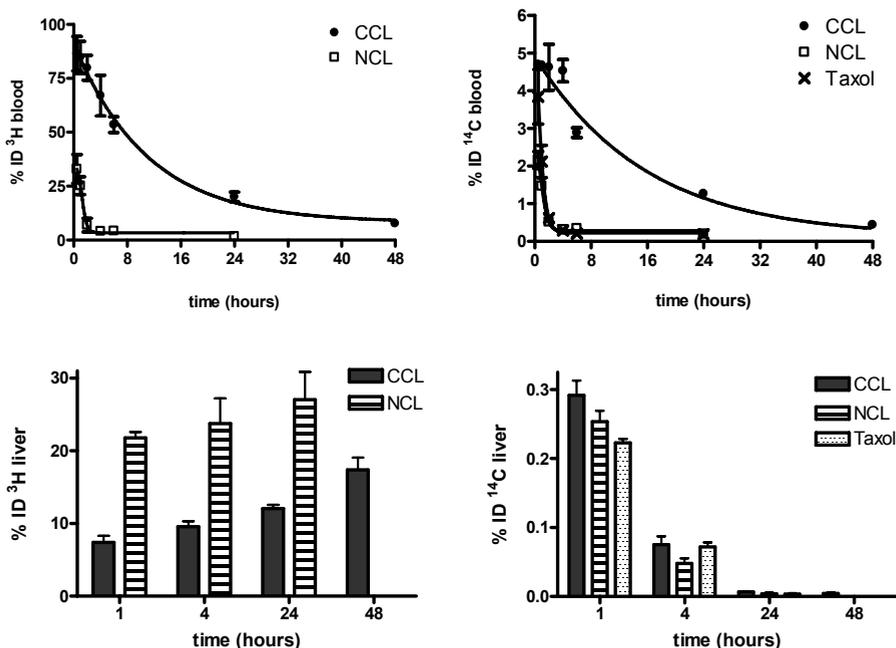
Figure 4 a) PTX-loaded NCL (left) and CCL (right) micelles (15 mg/mL polymer, 2 mg/mL PTX), b) PTX-loaded NCL (left) and CCL (right) micelles after addition of 200 μL 10 % SDS

Both crosslinked and non crosslinked micellar dispersions were stable in pH 5 buffer at room temperature for several days. The physical stability of the PTX-loaded micelles was evaluated upon addition of SDS (Figure 4). As clearly seen by the transition from an opaque to a clear solution, addition of

SDS resulted in the immediate dissociation of NCL micelles (Figure 4b, left). In contrast, the CCL micellar dispersion retained its opalescence indicating the presence of intact micelles (Figure 4b, right) as previously demonstrated with DLS experiments⁴⁰. In case of the disrupted NCL micelles, no PTX precipitates were observed since the SDS micelles solubilised the PTX.

Biodistribution study of ¹⁴C-PTX-loaded ³H-labelled polymeric micelles

The circulation kinetics and biodistribution of ¹⁴C-labelled paclitaxel-loaded ³H-CCL micelles was monitored in time in ¹⁴C-tumour bearing mice, with ¹⁴C-PTX-loaded ³H-NCL micelles and Taxol formulation as controls (Figure 5).



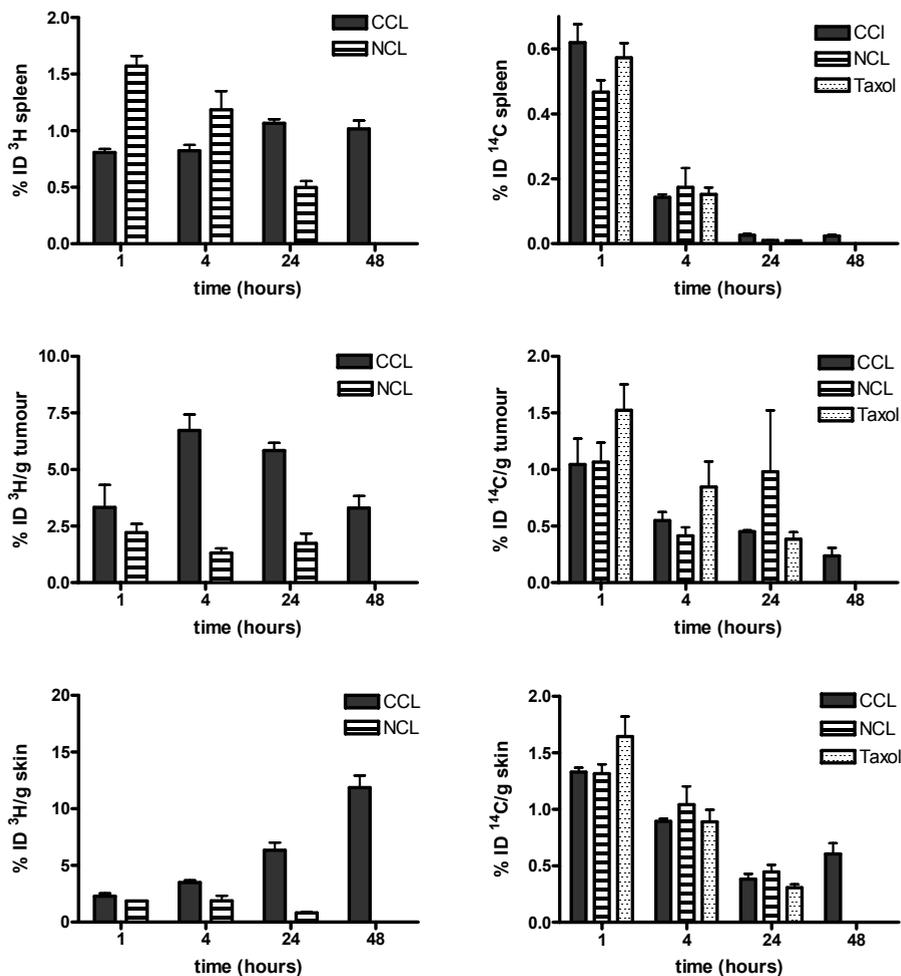


Figure 5 The blood, organ (spleen, liver and skin) and tumour distribution profiles of ^3H -labelled NCL (dashed bars) and ^3H -labelled CCL micelles (filled bars) loaded with ^{14}C -PTX and of ^{14}C -PTX-supplemented Taxol (dotted bars) in ^{14}C -tumour bearing mice (Note: only in case of CCL micelles, mice were sampled 48 hours post administration)

The biodistribution of ^3H -labelled NCL and CCL micelles (both loaded with ^{14}C -PTX) were in good agreement with the results obtained for empty assemblies (Figure 2). Similarly to small long-circulating liposomes (≤ 100 nm)^{8, 9}, ^3H -CCL micelles showed relatively high accumulation in the abdominal skin in time, i.e. ~ 12 % ID/g after 48 hours. Assuming a homogenous distribution of

micelles over the entire skin (comprising on average 2.7 g per nude balb/c mouse), this tissue ultimately contained 32 % of the injected dose. Presumably, the long circulation and small size allow the CCL micelles to extravasate in pressure areas and/or micro-traumatised areas in the skin as previously reported for liposomes ⁹.

Thirty minutes after administration, the blood levels of ¹⁴C-paclitaxel were decreased to 2.0 % ID (NCL), 3.9 % ID (Taxol) and 4.7 % ID (CCL). Although encapsulation of ¹⁴C-PTX in CCL micelles resulted in a twofold higher blood concentration than PTX loaded in NCL micelles, 95 % of the injected dose of PTX was removed from the circulation, indicating that PTX was rapidly released and/or extracted from the crosslinked cores followed by rapid tissue uptake and/or elimination. The latter is supported by the low amounts of PTX recovered from the other sampled tissues, i.e. in all investigated organs less than 2 % of the administered dose of PTX was found. The PTX-tumour accumulation was comparable for all three formulations, i.e. approximately 1 - 1.5 % ID after 1 hour and 0.5 - 1 % ID after 24 hours.

Ideally, loaded micelles should release the encapsulated drug only after arrival and accumulation at its target site. This study shows however that the biodistribution of ¹⁴C-PTX does not coincide with the ³H-labelled micelles (both NCL and CCL), showing that PTX is not stably encapsulated in both micelle types. Similar results were previously reported for (non crosslinked) PTX-loaded mPEG-*b*-poly(D,L-lactide) ⁷ and mPEG-*b*-p(HPMAm-Lac₂) micelles ⁴⁷ and was ascribed to either rapid dissociation of the micelles after injection or fast extraction of the PTX by serum components. In the case of CCL micelles, the micellar integrity is guaranteed by covalent crosslinks and only upon hydrolysis of the lactate chains (i.e. after approximately 5 hours at physiological conditions) the core is hydrophilised ⁴⁰. The premature drug release 30 min post injection must therefore be the result of rapid diffusion of PTX out of the micelles and/or extraction by blood constituents. In the CCL micelles, the crosslink density is obviously not sufficient to prevent the release of PTX upon i.v. administration. Therefore, potential routes to decrease the release rate is by increasing the crosslink density and/or to use triblock copolymers where the middle block is densely crosslinkable ^{27, 30} or by increasing the compatibility between the drug and the crosslinked core .

Conclusion

This study shows that non crosslinked micelles were rapidly cleared from the systemic circulation and displayed a high liver uptake. In contrast, CCL micelles demonstrated a superior circulation profile as compared to NCL micelles, thereby even resembling the blood half-life of PEGylated liposomes. More than 50 % of the CCL micelles still resided in the blood after 6 hours and an increased tumour accumulation, likely as a result of the EPR effect, was observed. Both micelles were well tolerated with no symptoms of acute toxicity. The micelles were loaded with PTX followed by core crosslinking via KPS - TEMED induced polymerisation. The biodistribution profile of ^{14}C -PTX-loaded ^3H -labelled CCL micelles revealed that the encapsulated PTX was rapidly released from the micellar core, which requires further optimisation in order to take full advantage of the favourable circulation kinetics of the CCL micelles.

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chapter 8

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chapter 9

Synthesis & characterisation of shell functionalised core crosslinked polymeric micelles with thermoresponsive & biodegradable properties

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Abstract

In chapter 8, it is shown that core crosslinked (CCL) polymeric micelles are very interesting as drug delivery systems because they have shown prolonged systemic circulation kinetics *in vivo*, probably as a result of low binding of opsonising proteins and low non-specific cellular uptake. This study aims at the preparation of CCL micelles suitable for active tumour targeting via ligand-receptor interactions, by introducing functional moieties on the micellar shell to which ligands can be attached. Previously, a ω -methoxy-poly(ethylene glycol₅₀₀₀) (mPEG) macroinitiator was used to induce the free radical polymerisation of *N*-(2-hydroxypropyl)methacrylamide-dilactate (HPMAM-Lac₂). To introduce reactive functional groups at the PEG terminus for coupling targeting ligands, a novel macroinitiator synthesis route was developed starting from an α -hydroxy ω -amine PEG. After protection of the amine group of NH₂-PEG₅₀₀₀-OH with a *tert*-butyloxycarbonyl (Boc) group, the product was esterified with 4,4-azobis(4-cyanopentanoic acid) (ABCPA) to yield the azomacroinitiator with 82 % coupling efficiency. After debocylation and coupling of the amine to the bifunctional linker *N*-succinimidyl 3-(2-pyridyldithio)-propionate (SPDP), more than 90 % of the (2-pyridyldithio)-propionate (PDP)-functionalised macroinitiator (PDP-NH-PEG)₂-ABCPA was recovered. Next, polymerisation with HPMAM-Lac_n monomers generated thermosensitive block copolymers with the PDP thiol-reactive moiety at the PEG terminus. Finally, 11 % of the lactate units of the PDP-block polymer were methacrylated by reaction with methacrylic anhydride. Micelles were formed by heating an aqueous solution of the thermosensitive polymer and an equal amount of rhodamine-labelled 11 % methacrylated mPEG-*b*-p(HPMAM-Lac_n) to above their critical micelle temperature (CMT). Subsequent polymerisation of the methacrylate units upon KPS-TEMED initiation yielded fluorescently labelled shell functionalised CCL micelles. These particles had a diameter of 56 nm (polydispersity 0.04) while the PDP moieties attached to the PEG termini were all reactive (as indicated by their cleavage upon addition of dithiothreitol (DTT)). This means that approximately 800 thiol-reactive groups were present on the micellar surface, i.e. the micelles can potentially be surface modified with thiol-containing ligands. The core crosslinked micelles presented in this study are suitable for further modification with targeting ligands aiming at active drug targeting.

Introduction

The concept of drug targeting is primarily applied in cancer therapy since either fast blood clearance, inefficient tumour accumulation and tumour cell entry or detrimental side effects limit the use of highly potent drug molecules (e.g. cytostatics, photosensitisers) after intravenous (i.v.) administration. The active compounds can be directed to the aimed areas by passive or active targeting of a drug carrier system (e.g. polymer-drug conjugates, drug-loaded liposomes and polymeric micelles). Passive targeting is the result of the enhanced permeation and retention (EPR) effect, i.e. the leaky vasculature of diseased and inflamed tissues is permeable for colloidal particles that are smaller than 200 nm while, due to an impaired function of the lymph system, the extravasated particles remain localised in the pathological area¹⁵. For a full exploitation of the EPR effect, drug-loaded particles should be long-circulating and are therefore generally coated with hydrophilic poly(ethylene glycol) (PEG) to prevent opsonisation by proteins and subsequent recognition by cells of the mononuclear phagocyte system (MPS). However, this hydrophilic PEG layer also prevents cellular uptake by the target cells²⁴. Hence, an additional active uptake mechanism is required for the internalisation of drug-loaded particles after passive accumulation at their target tissue.

For active targeting, a drug-loaded carrier system is functionalised with a targeting ligand¹⁶. Ideally, interaction of the ligand with its receptor at the target site leads to internalisation of the whole carrier, and once inside the cell the drug is preferably released in a controlled manner²⁰. Frequently used targeting ligands are (fragment(s) of) antibodies³³, folic acid¹³, transferrin³⁴, sugar moieties¹⁰ and other specific peptides (for example RGD peptides^{12, 18})⁹. A variety of conjugation techniques have been described to attach the ligand to the carrier system, using carboxylic acid, amine, thiol or aldehyde functionalities of the carrier³¹, or by introducing linkers that contain nitrophenyl or succinimidyl esters, or maleimide units^{18, 11}. Obviously, it is essential to maintain the biospecificity of the targeting moieties during coupling reactions between ligands and the carriers. So far, liposomal surfaces have been decorated with a variety of ligands^{8, 30} but the field of targeted micelles is less explored. However recently, some routes to synthesise targeted polymeric micelles have been described which comprise mainly the attachment of targeting ligands to functionalised block copolymers either before or after

micelle formation^{13, 18, 34, 35}. Pyridyl disulphide derivatives (for example 3-(2-pyridyldithio)-propionate, PDP) are interesting functional moieties because of their selective and quantitative reactivity with thiol containing ligands (e.g. peptides, antibodies) under mild conditions^{2-4, 7}. Besides, reduction of the disulphide bond in the PDP groups results in thiol-decorated polymeric micelles that may be of interest because of their mucoadhesive properties e.g. for oral administration^{1, 4}.

The coupling of various ligands to a micellar shell may affect the micellar stability. Especially in the case of large/bulky ligands (e.g. antibodies), the hydrophobic interactions in the core might not be sufficiently strong to keep the targeted block copolymer entrapped in the micellar morphology. To assure the structural integrity of shell functionalised assembled micelles, it is favourable to stabilise the micellar cores. Previously, biodegradable thermosensitive polymeric micelles were prepared by heating ice-cold aqueous solutions of ω -methoxy-PEG₅₀₀₀-*b*-poly(methacrylamidoalkyl-oligolactates) (e.g. mPEG-*b*-p(HPMAm-Lac₂)) to above their critical micelle temperature (CMT)^{25, 28}. The partial methacrylation of the lactate side chains of the core-forming block and subsequent polymerisation generated core crosslinked (CCL) micelles (chapter 7). These particles were resistant to dilution and to dissociation (for example upon addition of a surfactant), but they ultimately disintegrate as a result of hydrolysis of the crosslinks²³. Importantly, the CCL micelles displayed a significantly prolonged circulation as compared to non crosslinked micelles after i.v. injection (chapter 8 of this thesis). Moreover, they substantially extravasate and accumulate in tumour tissue as a result of the EPR effect. For example, almost 6 % of the injected dose had accumulated in the tumour tissue in mice 48 hours post administration²². However, *in vitro* cellular incubation studies (as described in chapter 6) revealed that the extent of internalisation of PEGylated micelles is low ($\leq 0.1\%$)²⁴. Hence, the coupling of various ligands would be interesting to pursue active targeting and to promote cellular binding and internalisation. Additionally, a fluorescent label should be attached to be able to follow the fate of the micelles *in vitro* and *in vivo*²⁴. It is the aim of this study to synthesise CCL micelles with thiol-reactive groups at the surface. Therefore, a partially methacrylated thermosensitive block copolymer based on HPMAm-Lac_n with a hydrophilic PEG block containing a thiol-reactive end group was synthesised. Combining this polymer with a rhodamine-containing partially methacrylated non-functional block copolymer and subsequent crosslinking the self-assembled micelles, should render fluorescently labelled core crosslinked micelles to which various thiol-containing targeting ligands can be attached.

Materials & methods

Materials

Acetonitril (ACN), diethyl ether, tetrahydrofuran (THF), dichloromethane (DCM) and dimethylformamide (DMF) were purchased from Biosolve Ltd. (Valkenswaard, The Netherlands). Lithium chloride (LiCl), *p*-toluene sulfonic acid monohydrate (*p*TSA), Hyflo, the monomethyl ether of poly(ethylene glycol) (mPEG₅₀₀₀), dimethyl sulphoxide (DMSO), 4,4'-azobis(4-cyanopentanoic acid) (ABCPA) and *N,N,N',N'*-tetramethylethylenediamine (TEMED) were obtained from Fluka Chemie AG (Buchs, Switzerland). *N,N'*-dicyclohexylcarbodiimide (DCC), 4-(dimethylamino)pyridine (DMAP), trichloroacetyl isocyanate (TAIC), deuterated chloroform (99.9 %, CDCl₃) and deuterated dimethyl sulfoxide (99.9 %, DMSO-*d*₆) were products of Acros (Zwijndrecht, The Netherlands). A 20 % solution of sodium dodecyl sulphate (SDS) from Biorad Laboratories (Hercules, USA) was diluted 1:1 with buffer pH 5 (ammonium acetate, 120 mM). Rhodamine-methacrylate (RhodMA) was purchased from PolySciences Europe (Eppelheim, Germany), NH₂-PEG₅₀₀₀-OH from NOF (Kyoto, Japan), potassium persulphate (KPS) from Merck (KGaA, Darmstadt, Germany) and 2,4,6-trinitrobenzene sulfonic acid (TNBSA) from Pierce (Perbio Science Nederland B.V., Etten-Leur, The Netherlands). Trifluoroacetic acid (TFA), di-*tert*-butyldicarbonate (Boc-*o*-Boc), dithiothreitol (DTT), methacryl anhydride, (*N*-succinimidyl 3-(2-pyridyldithio)-propionate) (SPDP) were products from Sigma Aldrich (Zwijndrecht, The Netherlands). 4-(dimethylamino)pyridinium-4-toluene sulfonate (DPTS) was prepared from DMAP and *p*TSA in the presence of dry toluene as described by Moore et al.¹⁷. THF was purified by reflux distillation over sodium and stored over molecular sieves, and DCM and ACN were dried over molecular sieves before use. All buffers were filtered through 0.2 µm filters (Schleicher & Schuell MicroScience GmbH, Dassel, Germany) prior to use. The other chemicals were used as received. The synthesis of HPMAM-Lac_{*n*} and (mPEG₅₀₀₀)₂-ABCPA was carried out as reported previously^{21, 28}.

¹H-NMR spectroscopy

¹H-NMR spectra were recorded with a Gemini 300 MHz spectrometer (Varian Associates Inc., NMR Instruments, Palo Alto, CA, USA). Spectra were obtained in either CDCl₃ or DMSO-*d*₆. The central lines of CDCl₃ at 7.26 ppm and

DMSO at 2.49 ppm were used as reference line, the values for chemical shifts (δ) given in ppm relative to TMS.

Gel Permeation Chromatography (GPC)

The molecular weights and the molecular weight distributions of the different polymers (macroinitiator and the (methacrylated) thermosensitive block copolymers) were determined by GPC. For the analysis of the macroinitiator, two serial Mesopore columns were applied, while for the thermosensitive block copolymers two serial Plgel 3 μm MIXED-D columns (Polymer Laboratories) were used, with a Waters System (Waters Associates Inc., Milford, MA, USA) with a differential refractometer Model 410. Poly(ethylene glycol)s of defined molecular weights were used as calibration standards. The eluent was DMF containing 10 mM LiCl²⁷. The samples were dissolved overnight at a concentration of 5 mg/mL in the eluent and filtered through a 0.45 μm filter prior to analysis. The flow rate was 0.7 mL/min and the column temperature was 40 °C. Peak areas were determined with Empower Software Version 1154 (Waters Associates Inc.).

Quantification of primary amines (TNBSA-assay)

Samples were dissolved in freshly prepared sodium bicarbonate buffer (pH 8.5, 100 mM; the thermosensitive block copolymers polymers were dissolved overnight at 4 °C). Standard solutions for calibration were prepared from a stock solution of glycine in the same buffer. Next, 250 μL of a 0.01 % (w/v) solution of TNBSA in buffer was added to 0.5 ml of a sample or standard solution. The PEG and macroinitiator were kept at RT for 4 hours while for the analysis of the block copolymers, all samples and standards were put at 4 °C for at least 7 hours (i.e. below the CMT of the block copolymers). Next, 250 μL of 10 % SDS and 125 μL of 1 N HCl were added to each sample and the absorbance at 335 nm was measured. The glycine standards generated a calibration curve which was linear between 27 and 130 μM ¹.

Synthesis

Bocylation of the primary amine of NH₂-PEG-OH (1)

Approximately 1 g NH₂-PEG-OH was dissolved in DCM (10 mL) and 2 equivalents of Boc-o-Boc (82 mg) was added. After stirring at room temperature (RT) under a N₂-atmosphere overnight, ~ 8.5 mL DCM was evaporated under

reduced pressure. The remaining solution (~ 1.5 mL) was added to ~ 100 mL diethyl ether to precipitate the Boc-NH-PEG-OH. After centrifugation, the pellet was dried under vacuum (40 °C, overnight) and the product was recovered quantitatively as a white powder.

¹H-NMR(DMSO-*d*₆): δ (ppm) 3.6 - 3.3 (454H, PEG) and 1.4 (9H, Boc).

Macroinitiator synthesis (2)

The Boc-NH-PEG₅₀₀₀-OH was used to synthesise a macroinitiator based by a method that was described previously for mPEG-OH²⁸. In brief, ABCPA, DCC, and DPTS (28, 62 and 16 mg respectively with corresponding molar ratio of 1:3:0.3) were dissolved in a 1:1 mixture of THF and DCM (10 mL) and stirred at RT for 15 minutes under N₂. Two molar equivalents relative to ABCPA of Boc-NH-PEG₅₀₀₀-OH were added (1 g, final concentration 100 mg/mL) and the mixture was stirred for 24 hours at RT under N₂. After filtration over a paper filter and removal of the solvents under reduced pressure, the remaining solid was dissolved in water and stirred for 2 hours. Next, the mixture was filtered over hyflo to remove traces of DCU. The (Boc-NH-PEG₅₀₀₀)₂-ABCPA macroinitiator was recovered by freeze drying and a white powder was obtained in high yield (> 95 %).

¹H-NMR (CDCl₃): δ (ppm) 4.25 (2H, PEG methylene next to ABCPA), 3.8 - 3.5 (452H, PEG), 2.4 (8H, ABCPA methylene), 1.8 (6H, ABCPA methyl), 1.4 (9H, Boc); GPC: M_n = 9600 g/mol, M_w/M_n = 1.05.

Unreacted PEG was determined by ¹H-NMR by addition of TAIC to the sample¹⁴. Thereby, the NMR signal of the methylene group adjacent to the terminal PEG-hydroxyl group shifted downfield from the main methylene PEG peak to 4.4 ppm. After 30 minutes, the amount of unreacted Boc-NH-PEG-OH was determined based on the NMR-peak areas. The amount of monosubstituted Boc-NH-PEG-ABCPA present in the final product was calculated by taking the relative peak area (%) of the GPC-peak at 4900 g/mol minus the percentage unreacted Boc-NH-PEG-OH as determined by NMR.

Debocylation (3)

The amine groups of the macroinitiator were deprotected by the addition of an excess TFA (~ 5 mL) to a solution of approximately 1 g (Boc-NH-PEG)₂-ABCPA in DCM (10 mL). After stirring for 24 hours (RT), the TFA/DCM mixture was removed by rotary evaporation. The residue was dissolved in NaHCO₃ buffer (pH 8.5, 100 mM), stirred for 2 hours and subsequently dialysed

against water at 4 °C for 24 hours and freeze dried⁵. (NH₂-PEG₅₀₀₀)₂-ABCPA was obtained as a white powder in a yield of > 95 % (1 g).

¹H-NMR (CDCl₃ + TAIC): δ (ppm) 4.4 (PEG methylene next to TAIC), 4.25 (2H, PEG methylene next to ABCPA), 3.8 - 3.5 (452H, PEG), 2.4 (8H, ABCPA methylene), 1.8 (6H, ABCPA methyl); GPC: M_n = 9400 g/mol, with a peak from the free PEG and monosubstituted macroinitiator at 4700 g/mol, both with M_w/M_n < 1.1.

Coupling of SPDP to the macroinitiator (4)

The macroinitiator (NH₂-PEG₅₀₀₀)₂-ABCPA was modified with 3-(2-pyridyldithio)-propionate moieties (PDP). Two molar equivalents of SPDP (36 mg) relative to the amine groups of the macroinitiator in DMSO (0.5 mL) was added to a solution of (NH₂-PEG)₂-ABCPA (300 mg) in phosphate buffer (100mM, pH 7.4, 5 mL). After 6 hours stirring at RT, the unreacted SPDP was removed by gel filtration chromatography (PD10 column, eluent H₂O, 2.5 mL per column). The PDP functionalised macroinitiator (PDP-NH-PEG)₂-ABCPA was recovered after freeze drying as white powder in 95 % yield (284 mg). The molar ratio of PDP versus PEG was determined by dissolving the compound in DMSO (~ 3 mg/mL). Fifteen minutes after the addition of 20 µL of a DTT stock solution in DMSO (5 mg/mL), the UV-absorbance was measured at 343 nm. A calibration curve of SPDP incubated with DTT prepared in DMSO was linear between 25 and 800 nM^{6, 26, 32}.

Block copolymer synthesis (5) & micelle formation

The block copolymers PDP-NH-PEG-*b*-p(HPMAm-Lac_n) were synthesised essentially as reported previously^{21, 28}. In short, polymerisation was performed on a scale of ~ 400 mg and a molar ratio of monomer to initiator ((PDP-NH-PEG)₂-ABCPA) of 150:1. The final concentration of the starting materials (monomers plus macroinitiator) was 300 mg/mL in ACN in airtight glass vials. Two PDP-functionalised polymers were prepared with different compositions of the thermosensitive block: PDP-NH-PEG-*b*-p(HPMAm-Lac₂) and PDP-NH-PEG-*b*-p((55%HPMAm-Lac₁)-*co*-(45%HPMAm-Lac₂)). Of the latter polymer, part of the lactate side chains (11 %) were derivatised with methacrylate groups by reaction of the block copolymers with methacryl anhydride as described before²⁴.

¹H-NMR (DMSO, *d*₆): δ (ppm) 7.5 (b, CO-NH-CH₂), 6.1 and 5.8 (b, CO-CCH₃=CH₂), 5.5 (b, CH-OH (HPMAm-Lac₂)), 5.3 (b, CH-OH (HPMAm-Lac₁)), 5.0 (b, CO-CH(CH₃)-O), 4.1 (b, CO-CH(CH₃)-OH), 4.0 (b, CH₂-CH-O van HPMAm), 3.6 (b,

PEG methylene protons, O-CH₂-CH₂), 3.4 (b, NH-CH₂-CH van HPMAM), 1.4 (b, CO-CH-CH₃), 1.3 (b, HO-CH-CH₃), 1.2 (b, CH₂-C(CH₃)-O) 1.0-0.6 (b, pHPMAM-Lac_n main chain protons). The protons of the PDP moiety (theoretical chemical shifts between 7 - 8 ppm and 3 - 4 ppm) were not observed as they overlapped with the broad peak of the amide proton and the PEG protons, respectively. The molecular weight of the block copolymers was determined by GPC (Table 1) and the average number of PDP per block copolymer molecule was determined as described above for the macroinitiator.

A fluorescently labelled mPEG-*b*-p((54%HPMAM-Lac₁)-co-(45%HPMAM-Lac₂)-co-1%RhodMA) block copolymer was prepared starting from a (mPEG₅₀₀₀)₂-ABCPA macroinitiator. After extensive purification by dialysis against ACN:H₂O and H₂O to remove unreacted RhodMA²⁴, 11 % of the lactic acid chains were also methacrylated.

Micelles were obtained as described before by rapidly heating ice-cold polymer solutions (2 mg/mL in pH 5, ammonium acetate 100 mM) to 50 °C (i.e. above their CMT) while stirring²². Core crosslinked micelles of the methacrylated polymers were obtained similarly in presence of 2 μM KPS and 9 μM TEMED as the initiator and catalyst, respectively²².

Characterisation of the block copolymers & micelles

Physical characterisation

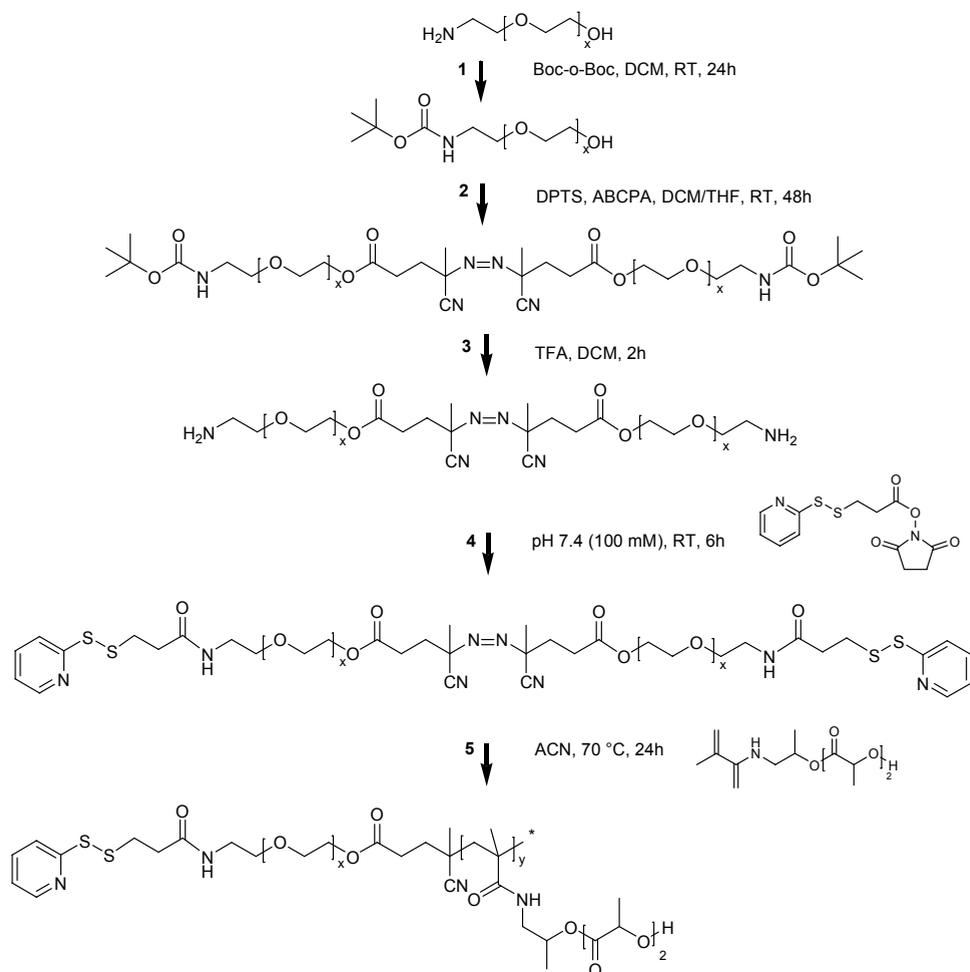
The CMT of different block copolymers was determined by static light scattering as reported previously²⁵ and the mean Z-average hydrodynamic diameter (Z_{Ave}) and polydispersity index (PD) of the micelles were assessed with dynamic light scattering using a Malvern ALV/CGS-3 Goniometer²⁵.

Quantification of the PDP moieties of the micelles

The reactivity of the PDP moieties after micelle formation was analysed with GPC at a temperature above the CMT of the constituting polymer in buffer. The aqueous GPC system comprised a Superdex 200 10/300 GL column (GE Healthcare, Piscataway, USA). The eluent was a pH 6.8 phosphate buffer (100 mM), the flow rate was 0.5 ml/min, the runtime was 70 minutes at RT and dual UV-detection (210 and 343 nm) was performed. Peak areas were determined with Empower Software Version 1154 (Waters Associates Inc.). The micelles and DTT were monitored at 210 nm at retention times (R_t) of 15 and 50.5 minutes, respectively. The polymeric micelles eluted at a retention time corresponding to the void volume of the column, as their size is larger than the

pore sizes of the column. A calibration curve of pyridine 2-thione was obtained by incubation of SPDP in buffer (0.3 - 611 μM) with DTT (0.4 - 744 μM) for 1 hour at RT. Fifty μL of each standard was injected on the GPC system. Pyridine 2-thione eluted at ~ 58 min and was detected 343 nm. A linear correlation of the AUC (~ 58 min, 343 nm) was obtained between 0.01 and 67 nmol pyridine 2-thione. The percentage of thiol reactive PDP groups was determined by GPC (50 μL per injection) after DTT-addition to preformed micelles.

Results & discussion



Scheme 1 Synthesis route for the thiol-reactive biodegradable thermosensitive block copolymer PDP-NH-PEG₅₀₀₀-*b*-p(HPMAm-Lac₂)

For the synthesis of the PDP-functional block copolymer, a route was adapted that was previously used to synthesise similar block copolymers with a non-functional ω -methoxy-PEG block¹⁹. The PEGylated-ABCPA macroinitiator decomposed at 70 °C and initiated the free radical polymerisation of HPMAm-Lac₂²⁸. For the synthesis of the functional block copolymer, the amine group of the bifunctional NH₂-PEG₅₀₀₀-OH was first bocylated by addition of a 2-fold

excess of Boc-o-Boc (Scheme 1, step 1). Boc-NH-PEG₅₀₀₀-OH was recovered quantitatively and no unreacted primary amines could be detected with a TNBSA assay (Table 1)¹.

product	yield (%)	M _n	M _w /M _n	CMT (°C)
(Boc-NH-PEG ₅₀₀₀) ₂ -ABCPA	> 95	9600	1.02	n.a.
		4900	1.01	n.a.
(NH ₂ -PEG ₅₀₀₀) ₂ -ABCPA	> 95	9400	1.02	n.a.
		4700	1.01	n.a.
(PDP-NH-PEG ₅₀₀₀) ₂ -ABCPA	> 95	n.d.	n.d.	n.a.
PDP-NH-PEG- <i>b</i> -p(HPMAm-Lac ₂)	60	27000	1.8	6
11 % methacrylated PDP-NH-PEG- <i>b</i> -p((55%HPMAm-Lac ₁)- <i>co</i> -(45%HPMAm-Lac ₂))	68	25200	1.8	17
11 % methacrylated mPEG- <i>b</i> -p((54%HPMAm-Lac ₁)- <i>co</i> -(45%HPMAm-Lac ₂))- <i>co</i> -1%RhodMA)	71	23600	1.6	13

Table 1 Characteristics of the various intermediate products and final block copolymers (The molecular weight and molecular weight distribution of the polymers were determined with GPC, n.a. = not applicable, n.d. = not determined)

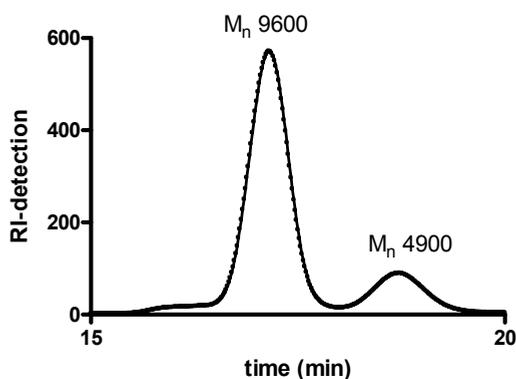


Figure 1 GPC chromatogram (RI-detection) of the product formed by esterification of ABCPA with amine-protected Boc-NH-PEG₅₀₀₀-OH (step 2) after 48 hours reaction time

Moreover, NMR displayed the characteristic peaks of the Boc-methyl protons at 1.4 ppm. Next, the amine-protected PEG was coupled to the azoinitiator ABCPA similar to a previous report ¹⁹, i.e. by a DPTS-catalysed esterification between the carboxylic acid groups of the ABCPA and the hydroxyl moieties of PEG using DCC as coupling agent (step 2). GPC analysis showed that the obtained macroinitiator had a bimodal molecular weight distribution (Figure 1). The peak with a retention time of ~ 17 minutes (corresponding to a molecular weight of 10000 g/mol) is ascribed to the bisPEGylated macroinitiator (Boc-NH-PEG₅₀₀₀)₂-ABCPA whereas the peak with a retention time of ~ 18.7 minutes (corresponding to a molecular weight of 5000 g/mol) can be ascribed to unreacted Boc-NH-PEG-OH and/or monosubstituted Boc-NH-PEG₅₀₀₀-ABCPA. To discriminate between these two products, NMR analysis was performed. TAIC, which reacts with the free hydroxyl group of the unreacted Boc-NH-PEG₅₀₀₀-OH, was added to the product mixture, thereby causing a downfield shift of the peaks of the methylene group adjacent to the hydroxyl group, thus allowing its quantification ¹⁴. After 20 hours of reaction time, NMR-analysis indicated that 32 % unreacted Boc-NH-PEG₅₀₀₀-OH was present. GPC (data not shown) revealed the presence of 56% of the 5000 g/mol compound. This means that the reaction mixture contained 24 % of monosubstituted Boc-NH-PEG₅₀₀₀-ABCPA. Prolonging the esterification reaction time of Boc-NH-PEG₅₀₀₀-OH with ABCPA to 48 hours increased the yield of the bisPEGylated macroinitiator to 82 % according to GPC (Figure 1). In this case, NMR analysis revealed the presence of 11 % unreacted Boc-NH-PEG₅₀₀₀-OH which implies that the reaction mixture contained 7 % of Boc-NH-PEG₅₀₀₀-ABCPA. During polymerisation of HPMAM-Lac_n, the latter will give rise to the formation of a small amount of homopolymer of HPMAM-Lac_n besides the synthesis of the desired block copolymer. This does not necessarily have to be a drawback since it is expected that upon micelle formation, the small fraction of homopolymer will co-assemble in the micellar core. After cleavage of the protecting Boc group by TFA (step 3), the amine moieties of the macroinitiator termini were almost quantitatively recovered as indicated by the TNBSA assay and the absence of Boc-protons in the NMR spectrum. Thermal initiation of HPMAM-Lac₂ polymerisation with this deprotected (NH₂-PEG)₂-ABCPA macroinitiator resulted in a block copolymer with less than 10 % recovery of the primary amines. Presumably, during the polymerisation reaction, aminolysis of some of the ester moieties (of the lactate side chains), which are present in a high molar excess relative to the terminal NH₂ groups, had taken place (Figure 2).

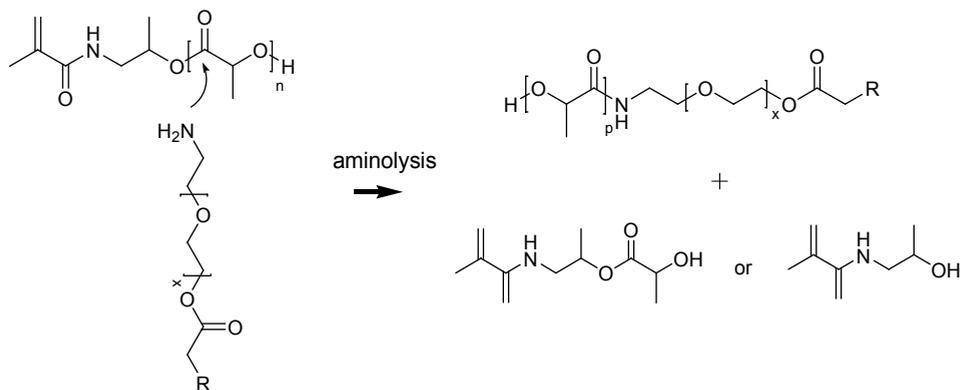


Figure 2 Suggested aminolysis reaction during the polymerisation of HPMAM-Lac₂ with (NH₂-PEG₅₀₀₀)₂-ABCPA (R is the remaining part of the ABCPA unit, see Scheme 1, n is 1 or 2; p is 1 or 2)

To circumvent aminolysis during the polymerisation step, deprotection of the amines after polymerisation of HPMAM-Lac _{n} with (Boc-NH-PEG)₂-ABCPA could be an option. However, we did not investigate this route because the lactate esters are prone to hydrolysis upon TFA-mediated deprotection of the amines and, also, aminolysis between the block copolymers can still take place. Therefore, we chose to attach PDP groups to the NH₂ groups of the deprotected macroinitiator prior to the polymerisation of HPMAM-Lac₂. Therefore, the amines were reacted with the succinimide group of the SPDP linker, followed by gel filtration chromatography to remove unreacted SPDP (step 4). Upon addition of DTT to the PDP-macroinitiator, pyridine 2-thione was split off. This compound has a specific adsorption band at 343 nm, which enables to quantify the extent of derivatisation of the amine groups of the macroinitiator with 3-(2-pyridyldithio)-propionamide (PDP) groups. This analysis revealed that the primary amine termini of the macroinitiator were quantitatively derivatised with PDP moieties. The (PDP-NH-PEG₅₀₀₀)₂-ABCPA macroinitiator was used to initiate the polymerisation of HPMAM-Lac₂ (step 5). The yield of this reaction was 60 % after purification and the M_n of the synthesised PDP-NH-PEG-*b*-p(HPMAM-Lac₂) was 26000 g/mol ($M_w/M_n = 1.8$). Static light scattering experiments revealed a critical micelle temperature (CMT) of 6 °C. These results are all in excellent agreement with previous findings²⁸. The block copolymer was dissolved in DMSO; after addition of DTT, it was shown that more than 90% of the PDP moieties present in the macroinitiator were recovered.

For the preparation of core crosslinked (CCL) micelles, part of the lactate side chains of the block copolymer was substituted with methacrylate units. However, methacrylation of PDP-NH-PEG-*b*-p(HPMam-lac₂) would reduce the CMT below 0 °C²³, which would make the polymer insoluble in water. Therefore, HPMam-Lac₂ was copolymerised with the more hydrophilic HPMam-monolactate (HPMam-Lac₁), which is known to increase the CMT of the resulting block copolymers²⁷. A CMT between 0 °C and room temperature is aimed for, i.e. to obtain water-soluble polymers that form stable micelles at room temperature. Therefore, a ratio of 55 % HPMam-Lac₁ and 45 % HPMam-Lac₂ was chosen. Indeed, PDP-NH-PEG-*b*-p((55%HPMam-Lac₁)-*co*-(45%HPMam-Lac₂)) displayed a CMT of 32 °C, which decreased to 17 °C after methacrylation of 11 % of the lactate side chains (Figure 3).

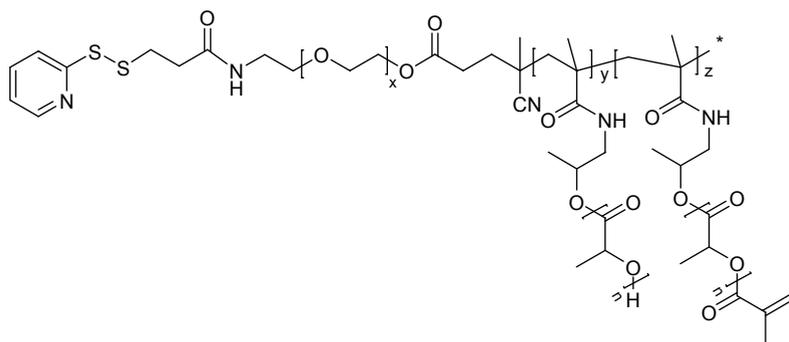


Figure 3 Chemical structure of partially methacrylated PDP-NH-PEG-*b*-p((55%HPMam-Lac₁)-*co*-(45%HPMam-Lac₂)) (n = 1 or 2, y and z correspond to 89 % and 11 %, respectively)

Similarly, a fluorescently labelled polymer was synthesised by copolymerisation of HPMam-Lac₁ and HPMam-Lac₂ with the fluorescent monomer RhodMA using (mPEG₅₀₀₀)₂-ABCPA as initiator. The resulting block copolymer was subsequently partially (11%) methacrylated. The M_n of both final (i.e. fluorescently labelled and PDP functionalised) polymers was ~ 25000 g/mol (Table 1). CCL micelles were prepared by heating aqueous solutions of the methacrylated block copolymers from 0 °C to 50 °C (i.e. above their CMT) in the presence of KPS-TEMED at RT for 1 hour. Non-fluorescent PDP-containing polymer, fluorescent non-functional polymer, and a 1:1 mixture of both polymers were used for the preparation of the CCL micelles (2 mg/mL polymer, Table 2).

micelles	Z_{Ave} (nm)	PD
non-functional	54 ± 1	0.05 ± 0.02
PDP-functional	96 ± 4	0.12 ± 0.03
PDP-functional CCL	76 ± 1	0.05 ± 0.01
non functional fluorescent CCL	49 ± 1	0.06 ± 0.03
PDP-functional fluorescent CCL	56 ± 1	0.04 ± 0.02

Table 2 Hydrodynamic diameter (Z_{Ave}) and polydispersity (PD) of the biodegradable thermosensitive micelles used in this work (2 mg/mL polymer in 100 mM buffer pH 6.8) (The values \pm standard deviation are the average of three independently prepared micelle formulations)

The non crosslinked PDP-NH-PEG-*b*-p(HPMAm-Lac₂) (PDP-functional) micelles had a hydrodynamic diameter of 96 nm, which is slightly larger than the size of corresponding non-functionalised mPEG-*b*-p(HPMAm-Lac₂) assemblies (54 nm). A similar effect was observed for PDP-NH-PEG-*b*-p((55%HPMAm-Lac₁)-co-(45%HPMAm-Lac₂)) (PDP-functional) CCL micelles, although to a lesser extent, i.e. particles with a diameter of 76 nm were formed (PD 0.05). Particles with a size of 56 nm were formed after heating an aqueous solution and crosslinking of the mixed PDP-functional polymer and the non-functional fluorescent polymer (PDP-functional fluorescent CCL micelles).

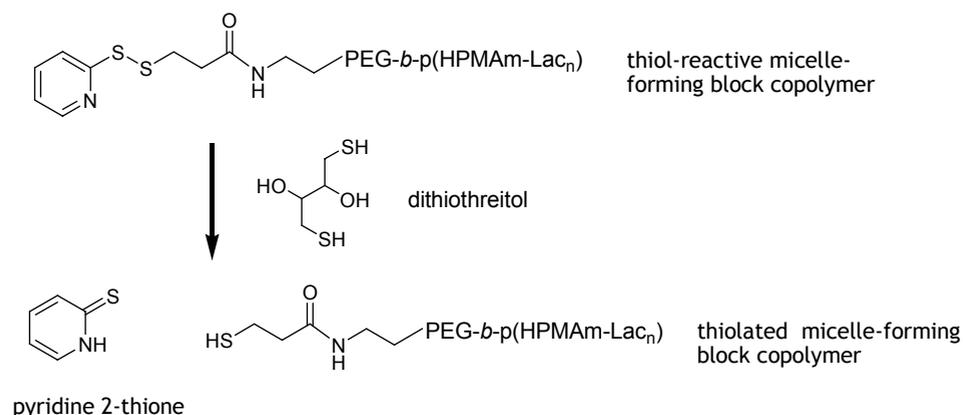


Figure 4 DTT-induced cleavage of the thiol-reactive (PDP-functionalised) micelles in buffer (pH 6.8)

As reported above, the extent of PDP derivatisation of the PEG termini of the intermediate products was assessed by dissolving the polymers in DMSO with subsequent addition of DTT to yield pyridine 2-thione, which can be quantified by UV spectrophotometry (Figure 4). However, the question remained whether the PDP moieties are still thiol-reactive after micelle formation and not shielded by neighbouring PEG chains or entrapped within the micellar core. Hence, the reactivity of the PDP groups of the various micelles (i.e. in buffer pH 6.8) was determined upon DTT addition. The formed pyridine 2-thione could not be measured directly with UV because the micelles scattered the UV-light. Therefore, the PDP-functionalised micelles were treated with DTT and injected on an aqueous Superdex GPC system equipped with an UV-detector to separate and detect the released pyridine 2-thione at 343 nm. The polymeric micelles were detected at 210 nm at the void volume of the column, as their size is larger than the pore sizes of the column. Pyridine 2-thione was only detected after DTT-addition. GPC analysis showed that more than 90% of the present PDP groups of the unlabelled CCL micelles and the fluorescently labelled CCL mixed micelles were split off after addition of DTT, indicating the PDP groups are accessible for DTT. Since it was previously found that the aggregation number of micelles from similar block copolymers was 1700²⁸, it can be estimated that the micellar surface of the fluorescently labelled micelles presented approximately 800 thiol-reactive PEG termini. This amount is anticipated to be more than sufficient for subsequent thiol-containing ligand coupling²⁹.

In conclusion, the thiol-reactive shell functionalised micelles are very attractive for the coupling of various ligands. Their crosslinked core prevents the extraction of block copolymers that are conjugated with a targeting ligand, while the fluorescent label enables monitoring of the cellular internalisation and trafficking. Overall, these sophisticated micelles have a high potential for active targeting purposes.

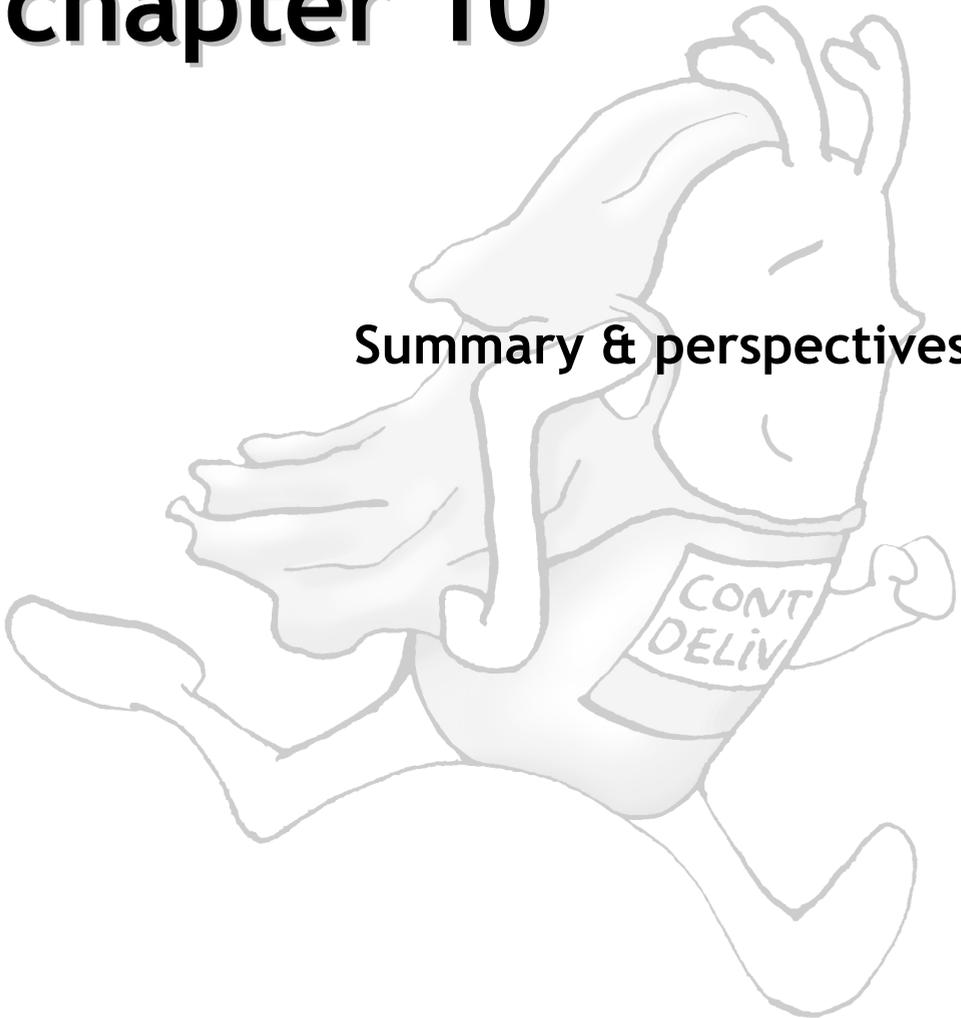
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chapter 10

Summary & perspectives



Summary

In recent years, colloidal systems are increasingly applied as vehicles for controlled drug delivery purposes of which liposomes, nanoparticles and micelles are the most frequently used. The encapsulation of hydrophobic drugs in a micellar core is advantageous for various reasons. Upon intravenous injection, the drug resides inside the micelles. Hence, the active compound can not elicit direct toxicity, is protected against degradation and rapid blood clearance. Ideally, the micellar formulation prolongs the systemic circulation and the drug-loaded micelles selectively accumulate in diseased tissues as a result of the enhanced permeation and retention (EPR) effect. Besides, in the ideal situation, the micelles dissociate only at the target site to allow the drug to exert a local therapeutic effect, while leaving non-target (healthy) tissue unaffected. As explained in **chapter 1**, the aim of this thesis was to synthesise micelles with tuneable destabilisation profiles in which various hydrophobic drugs (e.g. photosensitisers) were stably encapsulated. The release profiles as well as *in vitro* efficacy of these drug-loaded micelles were to be determined. Alternative synthesis routes needed to be developed to generate micelles that were fluorescent or radioactive labelled or to which a targeting ligand could be coupled. Lastly, an increased *in vivo* stability of the micelles was desirable to enhance the blood circulation and to promote tumour accumulation.

After having positioned the polymeric micelles in the broad field of drug delivery carrier systems, **chapter 2** discusses various approaches to enhance the longevity of polymeric micelles and various strategies to increase their stability, as well as options to introduce stimuli-sensitivity in these systems (**chapter 2a**). Two predominant characteristics of micelles to achieve a prolonged circulation are a dense hydrophilic coating (for which mainly polyethylene glycol (PEG) is used) and a small size. Micellar stabilisation is achieved by (reversibly) crosslinking the shell, interfacial layer or core to generate particles that are initially inert to serum proteins. Nevertheless, the encapsulated agents should ultimately be released, preferably via (local) triggers. This aspect is elaborated in **chapter 2b**, in which the potential of local and external stimuli to induce controlled drug release via hydrophobic to hydrophilic conversion of the polymers constituting the micelles is discussed.

Block copolymers comprising a hydrophilic part (e.g. PEG) and a thermosensitive block (e.g. a (meth)acrylamide backbone) self-assemble in water above the cloud point (CP) of the thermosensitive part (critical micelle

temperature, CMT) into spherical micelles. Biodegradability in these systems is ensured by attaching hydrophobic side chains via labile linkages to the methacrylamide backbone. After micelle formation, the side chains are cleaved off under physiological conditions, thereby increasing the hydrophilicity of the polymer. Ultimately, the CMT exceeds 37 °C, and the polymer dissolves in water which leads to micelle dissociation. The monomers used for the preparation of these thermosensitive micelles, being methacrylamidoalkyl-oligolactates (HPMAm-Lac_n or HEMA_m-Lac_n), can be synthesised in a stepwise process on a relatively large scale to obtain very (> 99 %) pure compounds (chapter 3). Static light scattering experiments showed that the homopolymer p(HEMA_m-Lac₂) displayed thermosensitive behaviour with a CP of 22 °C (chapter 4). Copolymerisation with the more hydrophobic HEMA_m-Lac₄ decreased the CP to the desired temperature (i.e. between 0 °C and RT). Amphiphilic block copolymers composed of PEG and a thermosensitive block based on 80 % HEMA_m-Lac₂ and 20 % HEMA_m-Lac₄ self-assembled into micelles of ~ 80 nm above 6 °C. The monomers degraded via first order kinetics and the micellar dissociation time (being ~ 5 hours at pH 7.4 at 37 °C) correlated nicely with the rate of monomer hydrolysis.

A novel solketal-substituted phthalocyanine (Si(sol)₂Pc) demonstrated a very high photocytotoxicity. Nevertheless, a suitable formulation was to be developed before this very hydrophobic photosensitiser (PS) could be evaluated in animals for the photodynamic treatment (PDT) of localised tumours. The mPEG-*b*-p(HPMAm-Lac₂) polymers solubilised Si(sol)₂Pc very efficiently (with 60 - 100 % encapsulation efficiency) above their CMT, thereby self-assembling into micelles with a size of 75 nm, which encapsulated the PS with a final concentration of ~ 2 mg/mL (chapter 5). At low concentrations (≤ 0.05 μM, 0.45 mg/mL polymer), Si(sol)₂Pc was molecularly dissolved in the micellar core whereas at higher loading, the PS was present in an aggregated form inside the micelles as demonstrated with UV-vis spectrophotometry and fluorometry. In B16F10 and 14C cells, the photodynamic efficacy of Si(sol)₂Pc loaded micelles (in which the PS was molecularly dissolved) was similar to free PS. For both formulations, the IC₅₀ was 3.0 ± 0.2 nM in the presence of 10 % serum in 14C cells. The high-loaded Si(sol)₂Pc micelles displayed a very good stability, also in the presence of 50 % serum. The PS was only released upon hydrolysis-induced micellar disassembly which was observed after 5.5 hours at pH 8.7 at 37 °C. The degradation-controlled release of the high-loaded micellar Si(sol)₂Pc formulation and their stability in serum make these micelles interesting for future *in vivo* PDT studies. To track and trace the empty polymeric micelles

intracellularly, a fluorescent label (rhodamine) was introduced by copolymerisation of rhodamine methacrylate (RhodMA) with HPMAM-Lac₂ (chapter 6). Unreacted RhodMA was difficult to remove completely as shown by GPC analysis and 0.22 % free label relative to the total amount of rhodamine was still present after extensive purification. Cellular uptake of RhodMA-labelled micelles by B16F10 or 14C cells demonstrated that micelles were only to a very low extent taken up (0.1 %) after 6 hours of incubation. Moreover, the trace amount free RhodMA accounted for significant intracellular fluorescence as observed by confocal microscopy, whereas the intracellular presence of micelles was not observed, likely due to quenching.

The stability of polymeric micelles *in vivo* is questionable as they appear to be prone to premature dissociation and/or preliminary drug release, either due to dilution below their critical micelle concentration (CMC) or by destabilisation by blood components. Therefore stable micelles were designed in chapter 7 by covalently crosslinking the micellar cores by polymerisation of methacrylate groups that were attached to some of the hydroxyl termini of the lactate side chains (core crosslinked (CCL) micelles). After addition of a surfactant (sodium dodecyl sulphate), non crosslinked micelles dissociated while the crosslinked micelles remained intact. Degradation studies proved that the stabilised micelles are still biodegradable. First, the unmodified side chains were hydrolysed, causing a gradual increase in the hydrophilicity and concomitant swelling of the core during the first 5 hours (pH 7.4, 37 °C). The CCL micelles remained intact until hydrolysis of the crosslinks induced dissociation of the micelles, which was dependent on the extent of crosslinking and varied between 30 hours and 60 days. Tritium labelled CCL micelles demonstrated a significantly prolonged circulation after intravenous administration when compared to non-stabilised micelles in 14C-tumour bearing mice as indicated by the almost ten-fold increase in AUC_{blood} (chapter 8). Moreover, the liver and spleen uptake was low while a significant tumour accumulation was observed, indicating that the small stabilised micelles successfully avoided macrophage uptake and extravasated via the EPR effect. The CCL micelles had a circulation profile resembling that of clinically applied PEG coated long-circulating liposomes, while their biodistribution profile indicated less RES uptake, presumably as a result of a denser PEG shell and a smaller size. The biodistribution kinetics of drug-loaded CCL micelles in tumour bearing mice was evaluated by encapsulation of a radiolabelled anticancer drug (¹⁴C-paclitaxel (¹⁴C-PTX)) in the ³H-labelled micelles. Thirty minutes after i.v. injection, both radiolabels had completely different distributions; for example

less than 5 % of the ^{14}C -PTX was in the blood versus 80 % of the ^3H -labelled CCL micelles. Despite the prolonged circulation of the CCL micelles themselves, the biodistribution of encapsulated PTX was similar to non crosslinked micelles and comparable to the commercial formulation (Taxol). This demonstrated that paclitaxel was rapidly released and/or extracted from the stabilised micelles.

The low internalisation rate of micelles (as described in chapter 6) creates the possibility to explore uptake via active targeting. Therefore, a route to synthesise a thiol-reactive PEG-macroinitiator was developed which initiated the polymerisation of HPMAM-Lac_n. A partially methacrylated PDP-block copolymer was mixed together with a rhodamine-labelled methacrylated block copolymer. After micelle formation, the methacrylate moieties were polymerised by potassium persulphate initiation. The resulting fluorescently labelled shell functionalised CCL micelles can be used for the attachment of various targeting ligands to promote cellular internalisation (**chapter 9**). In the current chapter (**chapter 10**), the various challenges when proceeding towards potential clinical applications of the polymeric micelles as drug delivery system are discussed and several possible improvements of the various steps encountered in the process from monomer synthesis to (drug-loaded) micelle formation are given.

In conclusion, the type of biodegradable thermosensitive micelle presented in this thesis can be used not only as a biocompatible nanosized drug carrier with a high drug-loading capacity, but also embeds a controlled degradation mechanism. Especially the core crosslinked micelles display favourable characteristics in terms of circulation kinetics and tumour accumulation. Together with the potential targetability, these unique characteristics make biodegradable thermosensitive micelles as described in this thesis very attractive as targeted drug delivery devices.

Discussion & perspectives

The results presented in this thesis demonstrate the high potential of biodegradable (crosslinked) polymeric micelles based on poly(methacrylamide-oligolactates) for targeted drug delivery purposes. The following favourable features (additionally to those mentioned in **chapter 1**) justifies further development towards phase I clinical evaluation:

- ❑ the encapsulation of a large variety of hydrophobic agents (photosensitisers (Si(sol)₂Pc, mTHPC), vitamin K, paclitaxel) is possible under mild conditions with high encapsulation efficiency and good loading capacity
- ❑ Si(sol)₂Pc can stably be encapsulated in the micellar core and is stably retained, even in the presence of serum
- ❑ the side chains of the hydrophobic block can be crosslinked via a facile procedure, which results in micelles with an excellent stability and long circulation properties
- ❑ the (core crosslinked) micelles are fully bioresorbable; the chemical composition and the crosslink density controls the micellar dissociation time under physiological conditions
- ❑ crosslinked micelles display a prolonged blood circulation with respect to non-stabilised micelles as the PEG-shell likely prevents the adsorption of proteins and aspecific uptake by non-targeted cells (MPS organs)
- ❑ the small size of the CCL micelles promotes their tumour accumulation via the EPR effect
- ❑ no direct toxic effects are observed

Ideally, the micelles protect the encapsulated drugs by providing a nanocontainer in the core that is completely segregated from the external environment. However, the biodistribution of paclitaxel-loaded micelles revealed that the drug rapidly diffuses out of the micellar core after intravenous administration. Before clinical application, especially the topics of stable drug encapsulation and controlled release properties of the thermosensitive micelles need to be addressed. Additionally, the effect of multiple administrations is to be evaluated, a large-scale GMP production procedure should be developed and the safety profile needs to be established.

Pharmaceutical technology

The use of thermosensitive micelles is very advantageous as a drug delivery system, since their formation only requires the heating of an ice-cold aqueous polymer solutions to above the (composition dependent) CMT. So far, the polymeric micelles described in this thesis have been prepared batch-wise on a small scale only (typically in the order of 0.5 - 3 mL). For the potential clinical application of these micelles, a preparation procedure should be developed to formulate loaded thermosensitive micelles on a larger scale and under GMP conditions. A prototype device for the preparation of drug-loaded micelles is shown in Figure 2 and will be evaluated in the near future. Initial experiments will comprise the formulation and characterisation of micellar dispersions up to 20 mL per batch, but it is anticipated to scale-up the procedure to approximately 220 mL. Preferably an FDA-approved excipient should be chosen as solvent for the hydrophobic drugs, such as ethanol, or when this is not possible the solvent has then to be removed by e.g. dialysis after the micelle formation step. Interestingly, it was noticed that the presence of $\text{Si}(\text{sol})_2\text{Pc}$ in the micellar core lowered the CMT of $\text{mPEG-}b\text{-p}(\text{HPMAM-Lac}_2)$ thus enabling to store loaded micelles in buffer (pH 5) in the fridge at 4 °C, which is below the CMT of the empty micelles (6 °C). Regarding the long-term stability, freeze drying may be a good strategy to achieve a proper shelf-life of the drug-loaded micelles as this method is often applied to obtain stable dry formulations of nanoparticulate systems ^{1, 16}.

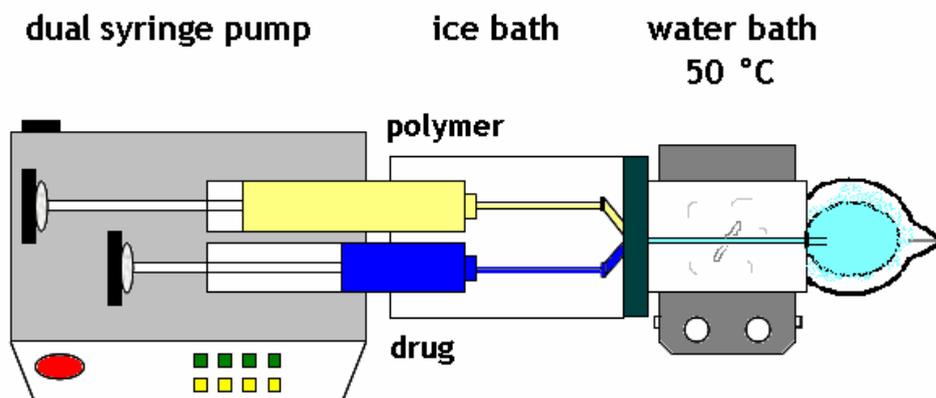


Figure 2 Device for the rapid heating of aqueous polymer-drug mixtures to prepare drug-loaded thermosensitive micelles

Stable encapsulation of hydrophobic drugs

The release of encapsulated drugs from mPEG-*b*-p(methacrylamide-oligolactates) micelles is likely retarded if the drug is highly compatible with the core-forming block. Either the partition of a drug between the micellar core and water, as predicted by the *n*-octanol/water partition coefficient (log P)⁶⁶, or the calculated solubility parameters²⁹ of the drug and the polymer indicate the extent of compatibility. Both aspects need to be optimised by adapting the polymer composition^{36, 38, 41}, for example by copolymerisation with aromatic-containing monomers to introduce π - π interactions between various drugs (obviously comprising aromatic groups) and this polymer⁷. An additional benefit is that this π - π stacking will also enhance the intermolecular polymer interactions and thus the micellar stability⁷.

Alternatively, a prolonged circulation of drug-loaded micelles is achievable by attaching the drug to the hydroxyl termini of the lactate grafts in the core via a degradable linker^{4, 5}. The coupling of a more hydrophobic moiety to the block copolymer will decrease its CMT, but this can be counteracted by changing the feed ratio of the different methacrylamide-oligolactate monomers. Possible premature micellar disassembly after *in vivo* administration is not considered as a drawback in this case. The reason for this is that various single polymer-drug conjugates have already demonstrated an enhanced tumour accumulation (via the EPR effect) and concomitantly, a better therapeutic effect was still observed as compared to free drug³³.

In case of crosslinked micelles, an option to decrease the burst release is to increase the crosslink density and/or to use triblock copolymers where only the middle block is crosslinkable^{39, 42}. Such an interfacial layer can act as a real steric barrier while not interfering with the drug loading capacity of the core.

Controlled drug release

Ideally, intact loaded micelles should accumulate to a high extent at the targeted tissue. Next, drug release is preferably induced by local triggers. Because of the slightly decreased pH value in tumour tissue and in intracellular lysosomes, thermosensitive block copolymers with acid-labile hydrophobic side chains are interesting. Either, the grafts can be coupled via cis-aconityl⁴⁵ or hydrazone²¹ linkages whereas the protection of bis-hydroxyl presenting methacrylamide with acetal, ketal³⁷, alkyloxybenzene⁸ or orthoester²³ groups is also attractive. In recent studies, these moieties were shown to be

selectively cleaved under acidic conditions (pH 5) with favourable degradation kinetics (< 3 hours) whereas no hydrolysis was observed at pH 7.4 in the same time frame ^{8, 23}. Furthermore, high concentrations of proteases and matrix metalloproteinases ³⁵ are present in tumours and inflamed tissues either intracellularly (endosomes and lysosomes) or in the extracellular space. Hence, enzymatically cleavable linkages (e.g. -GPLG- ^{11, 54, 71}) between the methacrylamide backbone and hydrophobic side chains may provide a local trigger to disintegrate micelles.

Since light-sensitive polymeric micelles dissociate rather rapidly upon illumination with light of a specific wavelength ²⁶⁻²⁸, it will be advantageous to combine this approach with photodynamic therapy and to encapsulate photosensitisers in such micelles. Ideally, the entrapped PS will be released only at the illuminated place and simultaneously generate cytotoxic radical oxygen species. This means that the micelles remain intact in non-illuminated areas, thereby confining the photodynamic therapeutic effect specifically to the illuminated areas.

The number average molecular weight of the current class of PEG-*b*-poly(methacrylamide-oligolactates) polymers comprises, including the PEG₅₀₀₀ block, approximately 25000 g/mol. Presumably, a longer (> 50 kDa) but fully degradable polymeric backbone will generate more stable micelles that can still be cleared by renal excretion despite its high molecular weight: long-term accumulation is prevented as the polymers are cleaved into smaller fractions upon degradation ^{12, 25}. An interesting biodegradable thermosensitive polymer was recently reported that consists of a polyphosphazenes backbone grafted with pNIPAAm oligomers ⁷². Further, elastin-like thermosensitive polymers are anticipated to be enzymatically degradable ⁵¹.

The thermosensitive polymers described in this thesis were synthesised via standard free radical polymerisation reactions, which implies the formation of low amounts of unwanted homopolymer. Better defined block copolymers (i.e. lacking the presence of homopolymers and having a lower polydispersity) are obtained by reversible addition fragmentation chain transfer (RAFT) ^{6, 43} or atom transfer radical polymerisation (ATRP) ⁵⁶. By using a PEG-RAFT ^{20, 61, 70} or PEG-ATRP macroinitiator ^{31, 73}, block copolymers with very narrowly distributed molecular weights are obtained that display very sharp phase transitions around their critical micelle temperatures ^{49, 69}. The resulting micelles will presumably have a better defined (i.e. less variation) controlled release profile as a result of more uniform properties of the composing block copolymers.

A library of well-defined monomers and polymers can be synthesised under microwave conditions^{18, 24} or by automated workstations (Chemspeed®). These techniques will generate polymers with a broad variety of (physico-) chemical properties. Accordingly, the polymer-drug compatibility and degradation kinetics (i.e. stimuli-sensitivity) can be optimised for each drug to precisely fine-tune the micellar dissociation time and accompanying release of the encapsulated compounds.

In chapter 8, it was found that the core crosslinked micelles displayed a high skin localisation after 48 hours, similar to long-circulating liposomes,^{9, 10}. The significant skin accumulation of doxorubicin containing PEGylated liposomes (~ 100 nm) is followed by gradual leakage of the drug, thereby causing dose-limiting toxicities such as palmar-plantar erythrodysesthesia and mucositis/stomatitis^{9, 10}. In contrast to liposomes, the CCL micelles contain a controlled destabilisation mechanism as they are subject to hydrolysis under physiological conditions. This gradual hydrolysis will increase the hydrophilicity of the micellar core, which is likely to affect and trigger drug release. When the building blocks of the micelles are properly selected, it is anticipated that all drug is already released from the micelles before extensive skin accumulation occurs, thereby preventing side effects.

Circulation

The effect of multiple administrations of our PEGylated CCL micelles should be examined since the repeated injections of PEGylated liposomes resulted in a so-called accelerated blood clearance (ABC)³⁴. The exact mechanism behind this phenomenon is still not elucidated but is reported to be correlated with anti-PEG antibody formation⁶⁷. In case the ABC-phenomenon is also observed for the CCL micelles, alternative (stealth) coatings need to be applied⁵³ or developed. Via the successive RAFT polymerisation^{47, 58} of HEMAm with methacrylamide-oligolactates, well-defined thermosensitive block copolymers such as pHEMAm-*b*-p(HEMAm-Lac_n) can be synthesised. It is interesting to evaluate whether pHEMAm (or pHPMAm) in analogy to PEG also provides sterical stabilisation to micelles and to explore how it affects the circulation upon repeated administration.

Active targeting

Attaching targeting ligands onto the micellar surface aims at the selective delivery of encapsulated drug at its therapeutic (intracellular)

target⁵⁰. The presence of possible immunogenic targeting ligands ((fragment(s) of) antibodies⁶³ or specific peptides such as cRGD³²) can be masked by applying smart multilayer coatings that expose the ligands only after a local trigger (reduction, pH-change, enzymatic degradation)^{44, 52, 59}. Further, currently, relatively new conjugation techniques are developed which may efficiently couple various ligands to micellar shells. 'Click' chemistry comprises very selective alkyne-azide coupling reactions^{6, 14} whereas functionalised RAFT-agents can be used to synthesise reactive, well-defined block copolymers that can subsequently be conjugated to various ligands¹⁹.

Toxicity profile

Although some polymeric micelles are already in clinical trials (see chapter 1), the long-term effects of used polymers are still unknown including possible systemic/local toxicity and immune reactions³⁰. The hydrophilic polymers applied in various micellar formulations such as PEG, pHPMAM and Pluronics are generally regarded as safe as they are biocompatible and excreted via the renal pathway up to a molecular weight of ~ 50 kDa. The (micellar assemblies of) hydrophobic thermosensitive polymers based on methacrylamide-oligolactates as described in this thesis are also expected to be fully bioresorbable as their degradation products (i.e. lactic acid, PEG and the hydrophilic pHEMAM or pHPMAM) are either bio-assimilated or eliminated from the body⁶⁵. However, before proceeding towards potential clinical applications, it is essential to establish a complete toxicity profile for these amphiphilic block copolymers, not only for intravenous but also for local and oral administration.

Applications

The attractiveness of the polymeric micelles as described in this thesis can additionally be enhanced by the entrapment of an imaging agent^{15, 55} which enables to report on the localisation of the drug-loaded carrier. Ideally, once observed that the nanoparticles have accumulated to a certain extent at their targeted tissue, an external trigger will selectively release the drug at its site of action. In the case of photodynamic therapy, illumination will result in PS activation, i.e. the production of ROS. Examples of traceable PDT therapeutics include PEG-conjugated fullerene that contained gadolinium ions⁴⁰, CdSe/ZnS quantum dots²² and gold nanoparticles modified with phthalocyanines⁶⁸. The (functionalised block co)polymers developed in our department are potentially

suitable as sheddable coatings / carriers for these hydrophobic (inorganic, magnetic or metallic) nanoparticles⁴⁸. A good starting point would be to encapsulate Si(Sol)₂Pc in CCL micelles and to evaluate the biodistribution and therapeutic efficacy of the photosensitiser. The real-time skin localisation of Si(sol)Pc₂ encapsulated in micelles can be assessed by multi-photon tomography⁵⁷ and gives information about the optimal time interval between administration and illumination.

Besides, the protocols to treat cancer are becoming more and more sophisticated as they combine various advanced strategies into one treatment. For example, a multidrug approach¹³, a combination of cytostatic agents with PDT⁶² and the additive effect of boron neutron capture therapy on PDT¹⁷ have been reported. So far, these combined techniques are not yet applied in micelles, but the possibilities should definitely be explored.

Recently, the oral administration of drug-loaded polymeric micelles has been investigated as a possible way to enhance the (bile-salt-independent) oral uptake of various hydrophobic drugs⁴⁶. In preliminary studies, vitamin K-loaded mPEG-*b*-p(HPMAm-Lac₂) micelles were administered to rats via a stomach tube. Each rat was given a dose of 1 mg which resulted in a C_{max} of ~ 2 µg/mL in the blood after 2 hours, indicating the significant uptake of vitamin K. Currently, studies are ongoing in cholestatic rats (i.e. animals with an interrupted bile duct). Although radioactively labelled polymers were reported to be recovered in the blood after oral administration of micelles⁴⁶, the exact uptake mechanisms are unknown yet. The transport of polymeric micelles may be by the internalisation of intact micelles through the intestinal wall or by uptake of single unimers. Upon administration of radioactive labelled core crosslinked micelles, the question might be answered whether integer micellar structures can be taken up. If the latter is the case, it would be very interesting to evaluate the oral uptake of Si(sol)₂Pc-loaded micelles as previously reported for PEG-PE micelles with encapsulated porphine⁶⁰.

Biodegradable thermosensitive polymers can almost unrestrainedly be designed and may be used as biodegradable coatings², as building blocks for hydrogels, for the purification of bioconjugates³ or for the encapsulation of any hydrophobic compound (e.g. contrast agents, dyes, etc.) in micelles. The hydrophobic interactions between thermosensitive polymers above their CP is used for the formulation of micelles, but it can also be applied for the self-assembly of hydrogels. These types of hydrogels are very attractive for hydrophilic drug or protein delivery and tissue engineering purposes. The strength of hydrogels based on the triblock copolymers p(HPMAm-Lac_n)-*b*-PEG-

b-p(HPMAm-Lac_n) was enhanced with increasing temperature and concentrations⁶⁴. Furthermore, the mechanical stability of the hydrogels could be improved by additional crosslinking, similar to the CCL micelles as described in chapter 7.

In conclusion, very sophisticated multifunctional micelles can be created not only in men's imagination but they are already realised by smart chemical approaches. Ultimately, block copolymers will be designed such to obtain, after self-assembly, the ideally stable long-circulating micelles, which stably chaperone drugs, selectively target diseased cells, release the drug upon tuneable disassembly and specifically induce tumour ablation.

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appendices

Nederlandse samenvatting

List of abbreviations

List of publications & abstracts

Curriculum Vitae

Dankwoord



Achtergrond

In de afgelopen jaren zijn colloïdale deeltjes steeds meer toegepast als geneesmiddeldragers en gecontroleerde afgiftesystemen. Veel gebruikte dragers zijn liposomen en diverse nanodeeltjes waaronder polymeren micellen die opgebouwd zijn uit amfifiele ketens. Dit zijn blok(co)polymeren die bestaan uit een hydrofiel (vaak poly(ethyleen glycol) (PEG)) deel en een hydrofoob (co)polymeer. In een waterige oplossing zijn de hydrofobe blokken niet oplosbaar en assembleren in kleine vetachtige bolletjes en vormen zo de kern van de micel. Daarbij richten de hydrofiele PEG ketens zich naar het water toe en vormen de mantel van de micel. De buitenkant is gehydrateerd en houdt op deze manier de micellen in oplossing. Het insluiten van hydrofobe farmaca in de micellaire kern is om diverse redenen aantrekkelijk. Na intraveneuze toediening kan de ingesloten actieve stof geen directe toxische effecten veroorzaken en is het geneesmiddel beschermd tegen afbraak en snelle klaring uit het bloed. Verder bevordert de micellaire formulering de systemische circulatie en accumuleren de farmacon-beladen micellen selectief in door ziekte aangetast weefsel als gevolg van het ‘verhoogde permeatie en retentie’ effect. Aansluitend vallen de micellen idealiter alleen uit elkaar in het doelgebied zodat het farmacon enkel daar een therapeutisch effect veroorzaakt en het gezonde weefsel onaangetast laat.

Het doel van het onderzoek, zoals beschreven in dit proefschrift, is het maken van micellen waarin diverse hydrofobe farmaca (onder andere lichtgevoelige stoffen) op een stabiele wijze kunnen worden ingesloten. Deze dragersystemen zouden vervolgens op een gecontroleerde manier uit elkaar moeten vallen met afgifte van de ingesloten farmaca als gevolg. Een ander doel was de effectiviteit van farmaca beladen micellen bij kankercellen (*in vitro*) te onderzoeken. Tevens diende alternatieve syntheseroutes te worden ontwikkeld om micellen te vormen die voorzien zijn van een fluorescent of radioactief label, dan wel waaraan een zogenaamd ‘targeting’ eenheid gekoppeld kan worden. Ten slotte is onderzoek verricht om de stabiliteit van micellen na injectie in de bloedbaan (*in vivo*) te verbeteren om aldus een lange circulatietijd te verkrijgen en ophoping in tumorweefsel te bevorderen.

Er zijn meerdere mogelijkheden om micellen lang in de bloedbaan te laten circuleren en uiteenlopende strategieën om de deeltjes gevoelig te maken voor (externe) stimuli (hoofdstuk 2a). De twee belangrijkste parameters voor een lange circulatietijd zijn een compacte hydrofiele mantel (waarvoor meestal PEG wordt gebruikt) en een kleine diameter. Verder is het vereist dat

de micellen lang genoeg stabiel zijn in de bloedbaan. Een methode om dit te verkrijgen is door (omkeerbare) verknopingen in de mantel aan te brengen, of in de tussenlaag tussen de kern en de mantel of de kern zelf. In het ideale geval worden op deze manier deeltjes gevormd die initieel bestand zijn tegen de invloed van eiwitten in het bloed. Met andere woorden, ze behouden hun intacte micellaire structuur en vertonen geen of weinig adsorptie van plasma-eiwitten. Echter, het ingesloten farmacon dient uiteindelijk wel vrij te komen op de gewenste plaats (bijvoorbeeld een tumor) en liefst op een gecontroleerde manier. Het gebruik van lokale dan wel externe stimuli kan onder andere een hydrofoob naar hydrofiel conversie van de micel vormende polymeren teweeg brengen (hoofdstuk 2b). De hydrofobe krachten die de polymeren in de kern bij elkaar houden, vallen als gevolg van deze transitie weg waardoor de micellen uit elkaar vallen met een gecontroleerde geneesmiddelafgifte als resultaat.

Temperatuurgevoelige polymeren micellen

Een speciaal type polymeren micellen bevat temperatuurgevoelige blok(co)polymeren, die bijvoorbeeld bestaan uit een hydrofiel PEG-deel en een gesubstitueerde (meth)acrylamide blok. Bij lage temperaturen zijn deze polymeren volledig in water oplosbaar, maar bij verwarming treedt boven de zogenaamde kritisch miceltemperatuur (CMT), een fasescheiding op van het temperatuurgevoelige blok (hydrofiel-naar-hydrofoob conversie). Het polymeer is dus niet meer volledig wateroplosbaar waarbij de temperatuurgevoelige blokken aggregeren en aldus een micellaire kern vormen terwijl het PEG-blok de mantel van het aldus gevormde deeltje creëert. Deze systemen zijn biodegradeerbaar omdat de hydrofobe zijketens via labiele verbindingen aan de methacrylamide keten zijn gekoppeld. Na micelvorming zullen deze zijketens onder fysiologische condities worden afgesplitst en daarmee de hydrofilititeit van het polymeer doen toenemen. Uiteindelijk zal de CMT van het polymeer stijgen boven de 37 °C en zijn de blok(co)polymeren volledig oplosbaar in water wat leidt tot het uit elkaar vallen van de micellen.

De bouwstenen (monomeren) voor deze temperatuurgevoelige micellen zijn melkzuur-derivaten (Lac_n , n staat voor het aantal melkzureenheden) van methacrylamide verbindingen. In deze studie is zowel *N*-(2-hydroxypropyl)methacrylamide als *N*-(2-hydroxyethyl)methacrylamide (HPMAm- Lac_n en HEMA- Lac_n , respectievelijk) gebruikt. Deze monomeren zijn gemaakt volgens een stapsgewijs syntheseprotocol op een tamelijke grote schaal (tot 20 gram eindproduct) en met een grote zuiverheid (> 99 % zuiver) (hoofdstuk 3). Polymeren gebaseerd op HEMA- Lac_2 hadden een fase-

overgangstemperatuur van 22 °C. Door inbouw van het meer hydrofobe HEMAm-Lac₄ werd de CMT verlaagd tot de gewenste temperatuur, die vanwege praktische redenen tussen de 0 en 15 °C ligt (hoofdstuk 4). Amfifiele blokcopolymeren opgebouwd uit PEG₅₀₀₀ en een temperatuurgevoelig deel bestaande uit 80 % HEMAm-Lac₂ and 20 % HEMAm-Lac₄ vormden micellen van circa 80 nm boven 6 °C. De melkzuurketens werden onder fysiologische condities (pH 7.4 bij 37 °C) afgesplitst en hierdoor vielen de micellen na circa 5 uur uit elkaar. Deze desintegratietijd is echter afhankelijk van het type methacrylamide polymeer dat gebruikt wordt. Het bleek dat bij gebruik van poly(HPMAm-Lac₂) als temperatuurgevoelig blok, de micellen onder fysiologische condities pas na een week uit elkaar vallen. Met andere woorden, de destabilisatietijd van de micellen is te sturen door de polymeren methacrylamideketen te variëren.

Fotodynamische therapie

Fotodynamische therapie (Engels: 'Photo Dynamic Therapy' (PDT)) is een opkomende behandelingsmethode voor lokale tumoren. Hierbij wordt een lichtgevoelig stof intraveneus toegediend en na enige tijd wordt alleen het te behandelen tumorweefsel met een laser belicht. De interactie tussen het licht en de lichtgevoelige stof leidt via chemische reacties tot de vorming van diverse zuurstofradicalen die uiteindelijk tumorcel dood veroorzaken. Het doel van PDT is een selectieve tumor behandeling. De verdeling van de lichtgevoelige stof over het lichaam, en dus ook de huid, leidt echter bij blootstelling aan daglicht al tot (ernstige) brandwonden. Een selectievere ophoping van lichtgevoelige stoffen in de tumor en niet in de rest van het lichaam is dus gewenst. Een nieuwe lichtgevoelige stof (een solketal gesubstitueerde phthalocyanine (Si(sol)₂Pc) liet in eerder onderzoek een zeer hoge tumorcel dood (zogenaamde fotocytotoxiciteit) zien. Deze stof is echter zeer hydrofoob en daardoor bijzonder slecht oplosbaar in water. Allereerst moest dus een geschikte formulering worden ontwikkeld alvorens bij proefdieren het fotodynamische effect op lokale tumoren kon worden geëvalueerd. De mPEG-*b*-p(HPMAm-Lac₂) micellen bleken het Si(sol)₂Pc zeer efficiënt in te sluiten. De micellen hadden een grootte van circa 75 nm en de eindconcentratie van Si(sol)₂Pc die bereikt kon worden was circa 2 mg/mL (hoofdstuk 5). Bij lage concentraties ($\leq 0.05 \mu\text{M}$ PS en 0.45 mg/mL polymeer) bleek de PS moleculair opgelost te zijn in de micellaire kern, terwijl bij hogere belading de PS in een geaggregeerde toestand aanwezig was. In een tweetal

kankercellijnen (zijnde B16F10 en 14C cellen) was het fotodynamische effect van $\text{Si(sol)}_2\text{Pc}$ beladen micellen (met de PS in moleculair opgeloste vorm) gelijk aan de vrije lichtgevoelige stof. Bij beide formuleringen ging in aanwezigheid van 10 % serum 50% van de 14C cellen dood bij een concentratie van 3.0 ± 0.2 nM $\text{Si(sol)}_2\text{Pc}$. De hoog beladen $\text{Si(sol)}_2\text{Pc}$ micellen vertoonden een zeer goede stabiliteit, zelfs in aanwezigheid van 50 % serum. De lichtgevoelige stof werd enkel vrijgegeven na desintegratie van de micellen, dat zichtbaar was na 5.5 uur in pH 8.7 bij 37 °C. Zowel de stabiliteit in serum als de degradatie gecontroleerde afgifte van het micellair geformuleerde $\text{Si(sol)}_2\text{Pc}$ betekent dat deze micellen erg interessant zijn voor toekomstige fotodynamische therapie toepassingen. Idealiter hopen de beladen micellen na intraveneuze injectie voornamelijk op in de tumor en niet in de rest van het lichaam, zodat de kans op bijwerkingen (zoals brandwonden) minimaal is.

Fluorescente labeling

Om de micellen te kunnen waarnemen bij microscopisch onderzoek met cellen, werd een fluorescent label (rhodamine methacrylate, RhodMA) ingebouwd in het p(HPMAm-Lac₂) blok (hoofdstuk 6). Het bleek lastig het niet gekoppelde label volledig te verwijderen. Na uitvoerige dialyse bevatte het polymeer volgens gel permeatie chromatografie (GPC, analyse gebaseerd op groottescheiding) analyse nog 0.22 % vrij label ten op zichte van de totale hoeveelheid aanwezig rhodamine. Cellulaire opname studies met RhodMA-gelabelde micellen in B16F10 of 14C cellen lieten zien dat slechts een zeer kleine fractie (0.1 %) van de micellen werd opgenomen na 6 uur incubatie. Bovendien bleken de sporen van vrij RhodMA te leiden tot behoorlijke intracellulaire fluorescentie zoals te zien was met confocale microscopie. De intracellulaire aanwezigheid van micellen kon echter niet worden gevisualiseerd, vermoedelijk als een gevolg van fluorescentie-uitdoving.

Micel stabilisering

Na injectie van polymeren micellen in de bloedbaan is de stabiliteit vaak onvoldoende aangezien ze soms vroegtijdig uit elkaar vallen dan wel voortijdig de ingesloten farmaca vrijgeven. Mogelijke oorzaken hiervoor zijn de (te) grote verdunning die optreedt na intraveneuze injectie of interacties van de micellen met diverse eiwitten die aanwezig zijn in het bloed. Stabiele, zogenaamde 'core crosslinked' (CCL) micellen werden verkregen door het verknoopen van de polymeerketens in de micellaire kern. Dit werd bereikt door

polymerisatie van methacrylaatgroepen die aan sommige van de melkzuurzijketens waren gekoppeld (hoofdstuk 7). Na toevoeging van een andere amfifiele stof (natrium dodecyl sulfaat) vielen niet-gestabiliseerde (NCL) micellen direct uiteen terwijl de CCL micellen intact bleven. De gestabiliseerde micellen bleken bovendien nog steeds volledig afbreekbaar te zijn onder fysiologische condities. Allereerst werden de niet-gemodificeerde melkzuurzijketens afgesplitst, wat leidde tot een geleidelijke toename in hydrofiliciteit van de micellaire kern en de niet-gestabiliseerde micellen vielen dan ook uit elkaar na 5 uur. Ondanks de hydrofiele kern bleven de CCL micellen door de covalente verknoppingen intact; pas na hydrolyse van esterbindingen in deze zogenaamde crosslinks desintegreerden de micellen. Na intraveneuze toediening van (niet) gestabiliseerde micellen in tumordragende muizen was er een groot verschil waarneembaar in circulatiekinetiek (hoofdstuk 8). Na 6 uur was nog meer dan 50 % van de CCL micellen in het bloed aanwezig terwijl de hoeveelheid van de NCL deeltjes op dat moment minder dan 10 procent was. De CCL micellen werden minimaal opgenomen door lever of de milt, wat aangeeft dat ze behoorlijk succesvol de natuurlijk afweermechanismen van het lichaam kunnen ontwijken. Even zo belangrijk was de relatief hoge tumorophoping van de CCL micellen (circa 6 % 48 uur na injectie). De kleine deeltjesgrootte en de verlengde circulatie zijn gunstig om maximaal te kunnen profiteren van het 'verhoogde permeatie en retentie' effect van tumorweefsel. De CCL micellen vertoonden een circulatieprofiel dat sterk lijkt op dat van klinisch toegepaste PEG gecoate liposomen, terwijl het biodistributie patroon beter was dan van deze liposomen, vermoedelijk door de kleinere diameter en de compactere PEG coating van de CCL structuren.

Vervolgens is de verdeling na intraveneuze injectie van een antikanker-farmacon (paclitaxel, PTX) beladen micellen bestudeerd. Al na dertig minuten bleek de lokalisatie van de micellen zelf en de paclitaxel totaal verschillend. Zo was minder dan 5 % van het PTX in het bloed aanwezig terwijl circa 80 % van de toegediende dosis van de micellen nog in de circulatie aanwezig was. Ondanks de lange circulatietijd van CCL micellen blijkt de biodistributie van de ingesloten paclitaxel overeen te komen met de PTX beladen NCL micellen en vergelijkbaar te zijn met de commerciële formulering (Taxol). Dit wijst erop dat paclitaxel snel werd afgegeven en/of geëxtraheerd uit de (gestabiliseerde) micellaire kern.

De lage cellulaire opname van de micellen maakt het mogelijk te onderzoeken of door koppeling van zogenaamde 'targeting ligands' actieve celinternalisatie bevorderd kan worden. Een syntheseroute is ontwikkeld om

een blokcopolymeer te verkrijgen met een reactief PEG-uiteinde. Deze reactieve groep kan in de toekomst gebruikt worden om diverse liganden aan de micellaire mantel te koppelen (hoofdstuk 9).

In hoofdstuk 10 zijn diverse mogelijke verbeterpunten in het gehele proces van monomeersynthese tot (farmacon beladen) micellen aangedragen evenals enige uitdagingen om deze micellen richting klinische toepassing te ontwikkelen. De belangrijkste aspecten zijn het verkrijgen van een stabielere farmaconinsluiting en het vaststellen van een compleet toxiciteitsprofiel van de polymeren. Het type biodegradeerbare temperatuurgevoelige micellen, zoals beschreven in dit proefschrift, heeft grote potentie als biocompatibele geneesmiddeldrager door de hoge beladingcapaciteit en tevens door het gecontroleerde desintegratiemechanisme. Vooral de ‘core crosslinked’ micellen vertonen zeer gunstige eigenschappen vanwege hun lange bloedsomloop en hoge tumorophoping. De combinatie met de mogelijkheid tot actieve targeting maakt dat deze biodegradeerbare temperatuurgevoelige micellen erg aantrekkelijk zijn als ‘targeted’ gecontroleerde farmaconafgiftesystemen.

List of abbreviations

ABCPA	4, 4'-azobis(4-cyanopentanoic acid)
ACN	acetonitril
AIBN	α , α' -azoisobutyronitrile
ATRP	atom transfer radical polymerisation
AUC	area under the curve
BSA	bovine serum albumin
CCL	core crosslinked
CDCl ₃	deuterated chloroform
CHCl ₃	chloroform
CLSM	confocal laser scanning microscopy
CMC	critical micelle concentration
CMT	critical micelle temperature
CP	cloud point
Cryo-TEM	cryo transmission electron microscopy
D ₂ O	deuterated water
DCC	<i>N,N</i> -dicyclohexyl carbodiimide
DCM	dichloromethane
DDS	drug delivery system
DLS	dynamic light scattering
DMA	dimethylacrylamide
DMEM	dulbecco's modification of eagle's medium
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
DMSO- <i>d</i> ₆	deuterated dimethyl sulfoxide
DNQ	2-diazo-1,2-naphthoquinone
DOX	doxorubicin
EDTA	ethylenediaminetetraacetic acid
EPR	enhanced permeation and retention effect
FBS	foetal bovine serum
FPEG	ferrocenylalkyl moiety
GOx	glucose oxidase
GPC	gel permeation chromatography
HEMA	2-hydroxyethylmethacrylate
HEMAm	<i>N</i> -(2-hydroxyethyl)methacrylamide
HEMAm-Lac _n	<i>N</i> -(2-hydroxyethyl)methacrylamide esterified with a lactic acid side chain of <i>n</i> units

appendices

HPLC	high performance liquid chromatography
HPMAm	<i>N</i> -(2-hydroxypropyl)methacrylamide
HPMAm-Lac ₂	HPMAm esterified with lactoyl lactate
IR	infrared
i.v.	intravenous(ly)
kDa	kilo dalton
KCps	number of counts measured by DLS at the indicated attenuator
LCST	lower critical solution temperature
MPS	mononuclear phagocyte system
M _n	number average molecular weight
MTD	maximum tolerated dose
mTHPC	meta(tetrahydrophenyl)chlorin
M _w	weight average molecular weight
MWCO	molecular weight cut off
M _{w(mic)}	weight average molecular weight of micelles
N _A	Avogadro's constant
N _{agg}	number of aggregation
nm	nanometer
NCL	non crosslinked
NIPAAm	<i>N</i> -isopropylacrylamide
NIR	near infrared
NMR	nuclear magnetic resonance spectroscopy
ρ _{mic}	density of a micelle
p2VP	poly(2-vinylpyridinium)
p4VP	poly(4-vinylpyridine)
pAAc	poly(acrylic acid)
pAsp	poly(aspartic acid)
PBA	poly(<i>tert</i> -butylacrylate)
PBLA	poly(β-benzyl L-aspartate)
PBLG	poly(γ-benzyl L-glutamate)
PBMA	poly(butyl methacrylate)
PBS	phosphate buffered saline
PCL	poly(ε-caprolactone)
PD	polydispersity
pDEA	poly(2-(diethylamino)ethyl methacrylate)
pDMAAm	poly(<i>N,N</i> -dimethylacrylamide)
pDMAEMA	poly(<i>N,N</i> -dimethylamino-2-ethyl methacrylate)

PDMS	polydimethylsiloxane
PDP	(2-pyridyldithio)-propionate
PEG	poly(ethylene glycol)
PEI	poly(ethylene imine)
PEVP	poly(<i>N</i> -ethyl-4-vinylpyridinium)
PFS	polyferrocenylsilane
pGMA	poly(glycerol monomethacrylate)
PHB	poly(3-hydroxybutyrate)
pHEMA	poly(2-hydroxyethyl methacrylate)
pHEMAM	poly(<i>N</i> -(2-hydroxyethyl)methacrylamide)
pHPMAM	poly(<i>N</i> -(2-hydroxypropyl)methacrylamide)
PiPrOx	poly(2-isopropyl-2-oxazoline)
PIC	polyion complex
PLA	poly(lactic acid)
PLGA	poly(DL-lactic-co-glycolic acid)
pLys	poly(L-lysine)
PM	polymeric micelles
PMA	poly(methyl acrylate)
PMAa	poly(methacrylic acid)
pMEMA	poly(2-(<i>N</i> -morpholino)ethyl methacrylate)
pMMA	poly(methyl methacrylate)
pNIPAAm	poly(<i>N</i> -isopropylacrylamide)
PPO	poly(propylene glycol)
PPS	poly(propylene sulphide)
PS	photosensitiser
PSt	polystyrene
PStS	polystyrene sulphonate
pTMC	poly(trimethylene carbonate)
PTX	paclitaxel
PVP	poly(<i>N</i> -vinyl-2-pyrrolidone)
QD	quantum dot
RAFT	reversible addition fragmentation chain transfer
RES	reticulo-endothelial system
R_h	radius of hydration
R_g	radius of gyration
RhodMA	rhodamine methacrylate
RI	refractive index
RITC	rhodamine isothiocyanate

appendices

RT	room temperature
SANS	small angle neutron scattering
SDS	sodium dodecyl sulphate
SLS	static light scattering
Si(sol) ₂ Pc	axially di-solketal substituted silicon phthalocyanine
SnOct ₂	stannous 2-ethyl hexanoate
SPDP	<i>N</i> -succinimidyl 3-(2-pyridyldithio) propionate
t	time
t _{1/2}	half-life
T	temperature
TAIC	trichloroacetyl isocyanate
TBAF	tetra- <i>N</i> -butylammonium fluoride
THF	tetrahydrofuran
TNBSA	2,4,6-trinitrobenzenesulfonic acid
UPLC	ultra performance liquid chromatography
UV	ultraviolet
vis	visible
XTT	sodium 3'-(1-(phenylaminocarbonyl)-3,4-tetrazolium)-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate
Z _{Ave}	average particle size as determined with DLS

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CJF Rijcken, JW Hofman, F van Zeeland, CF van Nostrum and WE Hennink, Formulation of Si(sol)₂Pc in polymeric micelles: a characterization and *in vitro* PDT efficacy study *submitted for publication*

CJF Rijcken, F van Zeeland, CF van Nostrum and WE Hennink, *In vitro* cellular internalisation of rhodamine labeled polymeric micelles *manuscript in preparation*

CJF Rijcken, CJ Snel, RM Schiffelers, CF van Nostrum and WE Hennink, Hydrolysable core crosslinked thermosensitive polymeric micelles: synthesis, characterisation and *in vivo* studies *submitted for publication*

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Awards

- best plenary lecture Dutch Polymer Days, February 2006
- academic cafe - soapbox session, June 2006
- 2nd place PhD competition FIGON days, September 2006
- 1 st place poster presentation, GPEN, October 2006

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08-2001 till 07-2003 master Pharmacy, Utrecht University (NL) (including Honours Programme), licensed pharmacist

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06-2000 till 08-2000 summer internship Procter & Gamble, Corporate Group, Egham (UK)
08-2000 till 11-2000 internship OctoPlus BV, Leiden (NL); Feasibility of an expert system for parenteral protein formulations
11-2000 till 12-2000 internship Yamanouchi, Meppel (NL); Supply chain management of clinical trial material
01-2001 till 07-2001 internship / research project Organon, Oss (NL); The effect of the hydrophobicity of the active compound on the distribution over granule fractions
11-2002 till 2-2003 internship Community Pharmacy, Hessen Pharmacy, The Bilt (NL)
03-2003 till 05-2003 internship Hospital Pharmacy, Rijnstate Hospital, Arnhem (NL)
05-2003 till 08-2003 internship Pharmacy Animal Sciences, Utrecht (NL)
09-2003 till 09-2007 PhD student - Department of Pharmaceutics, University Utrecht (NL)
promotor: Prof. Dr. Ir. WE Hennink
project title: "Thermosensitive biodegradable polymeric micelles for drug delivery"
The results of the research are described in this thesis.

Dankwoord

'A little voice inside my head said, "Don't look back. You can never look back". Those days are gone forever, I should just let them go...⁸. Sometimes you wonder if this fight is worthwhile¹⁸. All my work and endless measures, never seem to get me very far. Walk a mile just to move an inch, now even though I'm trying so damn hard¹.'

Mijn liefste maatje heeft me grootgebracht met de zin 'Niemand komt je iets brengen, je zult er zelf voor moeten knokken'. Immers, thuis in onze kelder aan de Vrouwenraetslaan werd er al consequent hard gewerkt^{4, 5, 11}. Na het succesvol overwinnen van diverse uitdagingen genoten we dan daarna wel altijd heerlijk van het goede Bourgondische leven door te gaan uiteten dan wel uit te rusten in de Oostenrijkse bergen^{6, 12}. Van pa leerde ik dat de beste levenshouding bestaat uit gerechte schouders met de borst vooruit en daarbij de oren en oogjes open te houden¹⁰. Verder had hij altijd een motiverende houding; bij een 8 als rapportcijfer was zijn reactie 'Waarom is het geen negen?' Allerbeste pa, ik merk dat ik tegenwoordig steeds meer op jou lijk²¹ en probeer zoveel mogelijk kansen te grijpen. Mijn grote dank en respect voor jullie beide², ik ben nog elke dag blij voor de inspiratie die jullie me geven om te vechten voor de toekomst en de idealen⁷. Hopelijk kunnen we nog vaak, in een zo optimaal mogelijke gezondheid, samen genieten van de mooie dingen in het leven¹⁵.

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'Ook al is het leven glashelder, dan nog steeds worden we allemaal geconfronteerd met een reeks grootse kansen, op schitterende wijze vermomd als onoplosbare problemen' (*John W Gardner*). Dit boekje is het resultaat ervan dat ik 4 jaar geleden als aio op het project 'polymeric micelles with transient stability' kon starten ¹⁶. Wim, je enorm brede kennis leidde altijd via interessante discussies tot creatieve ideeën en oplossingen. Erg aangenaam was je duidelijke en supersnelle communicatie; al was het dinsdagavond laat of zondagmiddag, altijd kwam er binnen een uur reactie terug. Rene, wat kun jij creatief met woorden omgaan en verhelderende figuren creëren! Fijn dat je deur altijd open stond en ik waardeer het dat je de hoogste versnelling hebt gebruikt om alle manuscripten op het einde na te lopen. Mijn hartelijke dank voor jullie kennis en support de afgelopen jaren; hopelijk kan ik jullie advies ook nog inwinnen gedurende het (opstarten van het) valorisatietraject.

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Emotion or the reason, now which one do you obey ²⁰? This world cannot bring me down ¹.' Lieve Birgit, dank voor de spontane heerlijke uitjes vol sportieve, culturele en smakelijke momenten op de Canarische eilanden. Je bent een mooi oprecht, optimistisch en ondernemend type, onze levensgesprekken hebben me altijd geraakt. Oh ja, mijn spaarpot voor Gran Melua stroomt langzaam vol... ☺. Martin, ik citeer jouw eigen woorden: 'Het handboek van het leven bestaat niet, omdat iedereen anders is... sterker nog: je bent zelf de schrijver'. In mijn boek heb ik daarom genoteerd dat we ondanks je nieuwe baan in Amsterdam wel weer naar het theater toe gaan, er mooie fietsroutes in het verschiep liggen en er over het leven gefilosofeerd mag worden! Lieve Ursula, jaja, kaboutertje is nu echt klaar!! Het is tijd om samen met Charlie aan die peperkoekenbrug naar Delft te gaan bouwen ☺. Of gaan we eerst eens muziek maken, bijpraten, fietsen, koffiedrinken bij Cobus etcetc.? Leonie, dank voor de vele msn-gesprekken vol humor die het werken tot laat in de avond/nacht ook nog erg gezellig maakte, het tig-gangen diner kent nu zeker geen excuus meer! Succes met je zoektocht naar een volgend slachtoffer om diep in de nacht lastig te gaan vallen ☺. Bedankt Mariëlle dat je de cover van dit proefschrift hebt willen ontwerpen en vormgeven! Binnen afzienbare tijd staat de picknick klaar in het park, ook voor Bieke! Christina, een betere buurvrouw kan een mens zich niet wensen. Naast de dagelijkse krant waren de culinaire hapjes en vrolijke praatjes een aangename afleiding tijdens de lange uurtjes achter de pc. Maartje, hoe relativerend was destijds je sms 'De zon schijnt in Amsterdam en het bier smaakt goed, promoveren kan altijd nog'. Ik had die erg gezellige middagen inderdaad absoluut niet willen missen, hopelijk volgen er nog vele! Alvast heel veel succes met het afronden van je eigen promotie. Maaïke en Lorraine, zo goed dat de nacho's smaakten als ik aan kon schuiven na een dag ploeteren op het lab! Binnenkort in de return dan wel biertjes drinken in de stad? Ingrid en Erik, reuze bedankt voor de vreselijk lieve kaartjes en smsjes, graag kom ik spoedig nog een keer de stoomoven mee uitproberen! Bianca, ik kijk nu al uit naar een spontane terrasafpraak dan wel een filmavond! Marjolein, het theaterseizoen is bij deze door mij officieel geopend, maar een elk ander uitje lijkt me ook leuk! Miriam, wanneer gaan we gezellig bijpraten en wandelen aan het Haagse strand? Niels, na goed contact toen je nog in de Bilt woonde, heb je inmiddels in Zuid-Afrika een eigen leven opgebouwd. Tot binnenkort en veel succes met je eigen promotie! Rogier, mijn benen kriebelen om het retourtje Enschede-Utrecht in z'n volledigheid een keer te fietsen!

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en laten we dan nu maar goed gaan genieten!

Muziek

- 1 3 Doors Down, Duck and run, *The better life* **2000**
- 2 Anouk, One word, *Hotel New York* **2004**
- 3 Billy Joel, Pianoman, *Pianoman* **1973**
- 4 Chicane, Saltwater, *Behind the sun* **2000**
- 5 Chicane ft Bryan Adams, Don't give up, *Behind the sun* **2000**
- 6 Delerium ft Sarah McLaughan, Silence, **2004**
- 7 Dj Tiesto, Just be, *Just be* **2004**
- 8 Don Henley, Boys of summer, *Building the perfect beast* **1984**
- 9 Gnarl's Barkley, Crazy, *St. Elsewhere* **2006**
- 10 Ilse de Lange, Flying solo, *World of hurt* **1998**
- 11 Kane, Hold on to the world, *So glad you made it* **2001**
- 12 Lucie Silvas, Breath in, *Breath in* **2004**
- 13 Madonna, Holiday, *Madonna* **1983**
- 14 Madonna, Jump, *Confessions on a dancefloor* **2005**
- 15 Michael Buble, Home, *It's time* **2005**
- 16 Morcheeba, Wonders never cease, *The antidote* **2005**
- 17 Reamonn, Supergirl, *Tuesday* **2001**
- 18 Roxette, Listen to your heart, *Look sharp* **1988**
- 19 Sash ft Tina Cousins, Mysterious times, **1998**
- 20 Spooks, Things I've seen, *S.I.O.S. Volume One* **2000**
- 21 Stef Bos, Papa, *Is dit nu later* **1990**

