Dextran-based microspheres

as

controlled delivery systems for proteins

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Cover: Schematic representation of the encapsulation and release of a protein molecule from dex-HEMA microspheres.

Dextran-based microspheres as controlled delivery systems for proteins

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Contents

Chapter 1	Introduction	7
Chapter 2	Preclinical and clinical *in vitro in vivo* correlation of an hGH dextran microsphere formulation	27
Chapter 3	Modeling the release of proteins from degrading crosslinked dextran microspheres using kinetic Monte Carlo simulations	51
Chapter 4	Effect of polymerization conditions on the network properties of dex-HEMA microspheres and macrogels	73
Chapter 5	Effect of excipients on the encapsulation efficiency and release of human growth hormone from dextran microspheres	89
Chapter 6	The *in vitro* degradation behaviour of microspheres based on crosslinked dextran	107
Chapter 7	Synthesis and characterization of dextrans substituted with hydrophilic polymerizable side groups and their application in protein releasing microspheres – an introductory study	129
Chapter 8	Summary and Perspectives	145
Appendix A	Achtergrond en samenvatting	159
Appendix B	List of abbreviations and list of symbols	167
Appendix C	Curriculum vitae en publicatielijst	171
Appendix D	Dankwoord	175

Chapter 1

Introduction

1. Introduction

During the past two decades, recombinant DNA and hybridoma technologies have resulted in the commercial availability of a significant number of pharmaceutically active proteins. However, the therapeutic application of these proteins was hampered for a long time because of formulation and delivery problems. Compared to low molecular weight drugs, proteins are large molecules with a complex three-dimensional structure that is very susceptible to chemical and physical degradation[1,2]. Disruption of the three-dimensional structure or chemical modifications of proteins can lead to loss of the biological activity or to immunogenicity[3]. Protein formulation is therefore a most challenging task. Another issue hampering the therapeutic applicability of proteins is that due to their high molecular weight and ionic charge, they have a low permeability across biological membranes. As a result, after oral administration absorption of proteins is poor and as a consequence their bioavailability is low. Moreover, when applied orally, enzymes such as gastric, pancreatic or brush border proteases present in the gastrointestinal tract easily degrade proteins. Therefore, parenteral administration of protein based drugs by e.g. subcutaneous, intramuscular or intravenous injection of these proteins is the preferred route. However, many pharmaceutical proteins are rapidly eliminated in vivo, which results in very short half-lives. To obtain a sustained therapeutic effect, proteins are usually administered by repeated injections or continuous infusion. Regular invasive dosing is an important problem in terms of patient compliance and comfort and often requires costly hospitalization. To circumvent these problems, delivery systems are needed to ensure that proteins access their target at the right time and for a prolonged period of time.

2. Sustained release systems

In recent years, long-acting formulations have been designed to avoid frequent invasive dosing and to provide patient compliance as well as comfort. The duration of action of a protein can be extended by chemical modification of the protein structure, e.g., by pegylation or acylation or by designing specific drug delivery systems[1]. By protein pegylation or acylation, the protein is chemically attached to poly(ethylene glycol) or fatty acids thereby improving the circulation time in blood[4]. Successful examples of pegylated proteins are pegylated α-interferons (PEG-Intron®, Pegasys®). However, chemical modification generally compromises the intrinsic activity of the protein and is therefore not a universal option for all proteins. Moreover, due to the introduction of additional product heterogeneity, the clinical and pharmaceutical documentation required for drug

approval could be even more demanding than for non-modified proteins[1]. Therefore, polymeric drug delivery systems are widely studied. Proteins are encapsulated in a polymeric matrix and gradually released over time as a result of diffusion, swelling and degradation of the matrix.

Sustained release systems based on poly (D,L-lactic-co-glycolic acid) (PLGA) have been extensively studied for this application because of their ease of fabrication, their biodegradability, good biocompatibility proven by the successful use for several decades in biodegradable surgical sutures[5,6,7]. This has resulted in FDA approval of several controlled release products based on these polymers for the delivery of peptides (see Table 1).

However, the FDA approval of these PLGA-based systems and the success in controlled release of small peptides was not accompanied by a correspondingly large number of marketed products for controlled release of proteins. Until now, only one protein containing PLGA-based controlled release microsphere systems gained FDA approval (Nutropin Depot; FDA approved in 1999 (Genentech, USA)). However, in 2004 the production of this product was discontinued due to manufacturing resource issues[9]. The lack of marketed protein delivery systems based on PLGA may be related to a number of drawbacks associated with the use of PLGA.

Table 1 *Overview of FDA approved PLGA based controlled release microsphere formulations*[5, 8].

Drug	Trade name	Company	Formulation	Application
Leuprolide acetate	Lupron Depot®	Takeda-Abbott	3-months depot	Prostate cancer, endimetriosis
Octreotide acetate	Sandostatin LAR®	Novartis Pharma	1-month depot	Growth hormone suppression
Tritorelin	Decapeptyl Depot®	Debiopharma	1-month depot	Prostate cancer
Somatropin	Nutropin Depot®	Genentech-Alkermes	1-month depot	Growth hormone deficiency

One major drawback is the limited possibility to modify the release. Very often, burst release is observed followed by non-zero order release kinetics and ending up in incomplete release. Several studies have shown that incomplete release due to aggregation/degradation often occurs[10,11]. Degradation of the polymer results in acidification of the matrix which was shown to induce protein aggregation and degradation[12,13]. Moreover, the hydrophobic character of these polymers requires the use of organic solvents for the preparation of the protein-loaded matrices. Similar problems were also observed for microsphere systems based on other hydrophobic biodegradable polymers such as poly(orthoesters) (POE) and poly(anhydrides)[14,15]. The hydrophobic character of these polymers requires protein unfriendly formulation techniques such as solvent removal or hot melt

encapsulation[16,19]. To overcome these problems, more protein friendly polymeric systems are currently under investigation.

3. Hydrogels

Hydrogels consist of crosslinked hydrophilic polymers that can absorb and retain large amounts of water. The presence of either chemical crosslinks (covalent) or physical crosslinks (ionic interactions, entanglements, hydrogen bridges, hydrophobic interactions and crystallites) renders a network structure that prevents the polymers from dissolution in an aqueous environment[20-23]. In Table 2 examples of chemically and physically crosslinked hydrogel systems are shown. Because of their rubbery structure and high water content, hydrogels are generally very compatible with proteins and living tissue. Therefore, the last two decades, a wide range of polymer compositions have been investigated to design hydrogels for biomedical and pharmaceutical applications[20-23].

Table 2 *Examples of chemically and physically crosslinked hydrogel systems*[21,22].

Physically crosslinked hydrogels		*Chemically crosslinked hydrogels*	
Polymer	Crosslinking process	Polymer	Crosslinking process
- PVA	⇒ Crystallization	- PVA, PEG, PAA	⇒ Radiation,
- p(HEMA-g-oligolactates), dextran-g-oligolactates	⇒ Stereocomplex formation	- HEMA+EGDMA, Gelatine-, dextran-, (hydroxyethyl meth)acrylates	⇒ Radical polymerization
- PEO-PPO-PEO block copolymers, PEGT/PBT co-polymers	⇒ Hydrophobic interactions	- PEG+ diisocyanate, Collagen + glutaraldehyde, PEG acrylates with DTT	⇒ Chemical reaction of complementary groups
- p(MAA-g-EG)	⇒ Hydrogen bonding		
- sodium alginate + polylysine	⇒ Ionic interactions	- PEG-Q_a+ Poly(lysine-co-phenylalanine) + transglutaminase	⇒ Enzymatic reaction
- coiled-coil peptides attached to pHPMA	⇒ Peptide/protein interactions		

PVA= poly (vinyl alcohol), HEMA=hydroxyethyl methacrylate, PEO-PPO-PEO= Poly(ethyleneoxide)-b-poly(propyleneoxide), PEGT/PBT = Poly(ethylene glycol) terephtalate poly(buthylene terephtalate) (MAA-g-EG)=Poly (methacrylic acid-g-ethylene glycol), PAA=poly(acrylic acid), DTT= dithiotreitol, PEG-Q_a=tetrahydroxy PEG functionalized with glutaminyl groups.

3.1 Biodegradable hydrogels

The first hydrogels studied as matrix for the controlled release of proteins were based on poly(acrylamide)[24,25], poly(vinyl)alcohol[26] and poly(hydroxyethyl methacrylate)[21,27]. However, these hydrogels are not biodegradable. An important disadvantage of non-degradable systems is that surgery is required to remove the drug-depleted systems from the patient. Ideally, hydrogels degrade *in vivo* either enzymatically or non-enzymatically yielding biocompatible and non-toxic degradation products along with the progressive release of the entrapped protein. Enzymatically degrading hydrogels are degraded by a matching enzyme, which is encapsulated in the hydrogel or present in the vicinity of the hydrogel at the site of application. Chemically (or non-enzymatically) degrading hydrogels mainly degrade by hydrolysis of labile bonds resulting in a decreased amount of effective crosslinks or entanglements present in the swollen polymer matrix. The degradation rate of hydrogels depends on the type of bond that has to be hydrolyzed and the crosslink density of the gel. Generally, the more hydrolytically labile the bond is, the shorter the degradation time. Furthermore, the higher the crosslink density, the more bonds have to be hydrolyzed before the gel dissolves and thus the longer the degradation time becomes. In biodegradable hydrogels protein release is governed by degradation (e.g., hydrolysis of crosslinks) and therefore the advantageous properties (e.g. good biocompatibility with proteins and living tissue) are combined with the flexibility to control the release by their degradation kinetics[20,29-32].

3.2. Release of proteins from hydrogels

Protein transport in hydrogels occurs primarily within the water filled pores between the polymer chains. Therefore, average pore size, pore size distribution and pore interconnections are important parameters of a hydrogel matrix[21,33,34]. The most important parameters used to characterize the network structure of hydrogels are the polymer volume fraction in the swollen state, the molecular weight of the polymer strand between to neighboring crosslinks and the corresponding mesh size[21,33,34]. The molecular weight of the strand between two consecutive (chemical or physical) crosslinks is a measure for the degree of crosslinking. The correlated distance between two adjacent crosslinks (defined as mesh size) provides the space between macromolecular chains available for protein diffusion.

Regarding their release mechanism, hydrogel matrices can be divided into three categories: (1) diffusion-controlled systems, (2) swelling-controlled systems and (3) degradation controlled systems[35]. In diffusion-controlled systems, the hydrodynamic radius of the protein is smaller than the mesh size of the network. The release rate is governed by diffusion and gradually decreases in time, due to a decreasing concentration gradient in the hydrogel. Protein release from these

hydrogels follows Fick's law of diffusion, which implies that the initial drug release is proportional to the square root of time[38,39]. The release rate of proteins (expressed in mg protein released per unit of time) from these matrices decreases with (1) increasing polymer volume fraction, (2) increasing solute hydrodynamic diameter and (3) decreasing loading of the protein in the matrix[36]. Protein release from hydrogel systems based on non-degradable polymers is usually governed by diffusion. However, to avoid toxic peak concentration zero order release profiles are preferred.

In swelling-controlled protein delivery systems and in degradation-controlled systems, the protein drugs are initially entrapped in the pores of the network. In other words, the initial mesh size of the hydrogel is smaller than the hydrodynamic radius of the protein. The release of entrapped proteins is then controlled by swelling of the hydrogel, by degradation of the polymeric matrix, or both. For swelling-controlled systems, as swelling continues, the mesh size increases in time until the equilibrium state is reached. At this state retractive forces acting on the polymers chains are in equilibrium with swelling forces. In degradation-controlled systems, the number of (physical or chemical) crosslinks is decreases with time, which results in an increasing mesh size. Both in swelling-controlled and in degradation-controlled release systems, the protein can be released from the matrix when the mesh size has become larger (because of hydrolysis of crosslinks or swelling) than the hydrodynamic diameter of the protein. This can result in zero-order release profiles in which protein is released immediately or after a delay time during which no protein is released.

Many models have been developed to describe the release of drugs from various drug delivery systems[36-43]. Mathematical modeling is a modern and widely used tool for predicting diffusion behavior and the drug release rates from these systems by the use of an appropriate model. Moreover, modeling can deepen the understanding of the physical chemical processes involved in protein release, thereby reducing the number of experiments needed and facilitating the development of new drug delivery systems. Over the last few decades, simple empirical, semi-empirical or mechanistic models have been developed to describe drug release from polymeric matrices. Many empirical models assume a single zero-order process describing the overall drug release rate and do not give insight in the underlying mechanisms. An example of such models is the swelling-controlled model developed by Hopfenburg[44] and Cooney[45]. Mechanistic models proposed to describe the release process of drug from hydrogel matrices[37,42] are based on the description of the underlying physical processes (e.g. mass transfer, hydrolysis of crosslinks, etc.) involved in drug release kinetics. In these models, protein release is often described by time dependent diffusion coefficient, obtained from either polymer degradation kinetics or swelling kinetics. Good agreement is obtained between in vitro release data and model predictions.

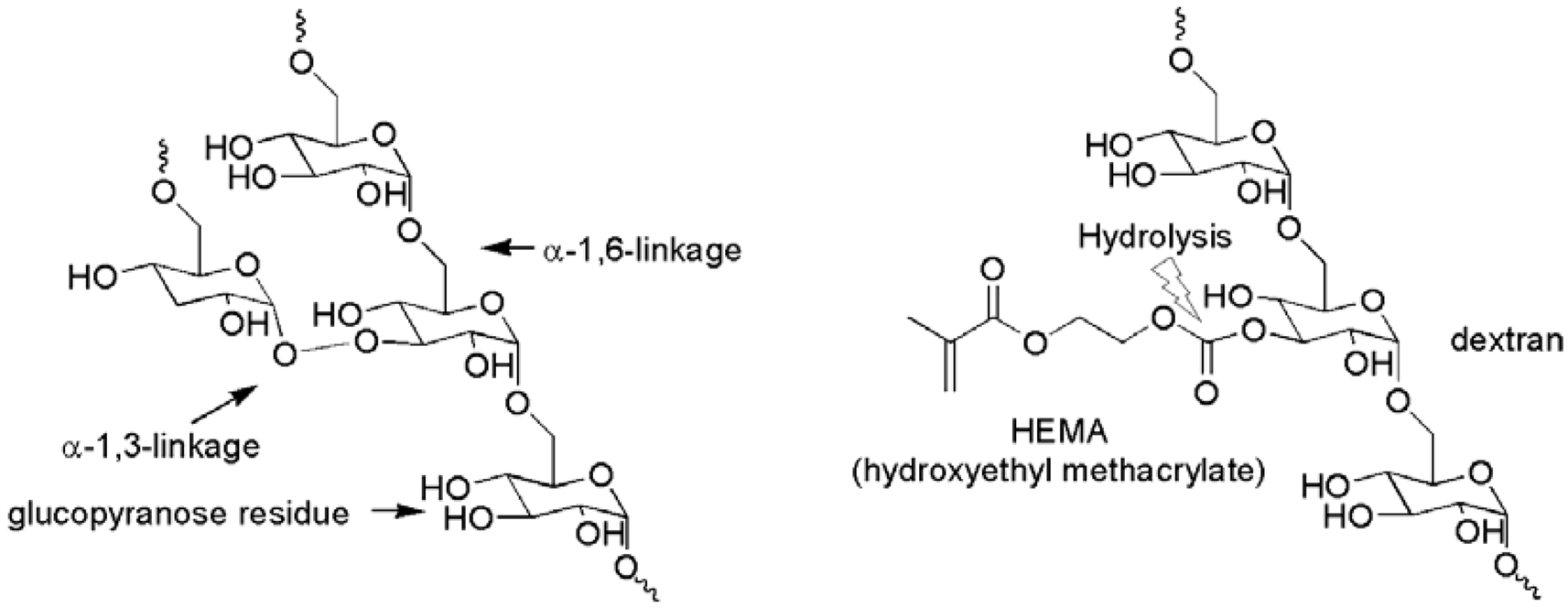
Nevertheless, most of these models do not fully cover the observed trends in the data (e.g. decreasing release rate with increasing polymer volume fraction, solute hydrodynamic diameter, concentration of the protein)[40].

A special class of mechanistic models describing polymer degradation and release of proteins at the microscopic level is based on Monte Carlo simulations. In Monte Carlo based models degradation of the polymer and protein release is simulated as a series of random events. Important results were obtained with these models in modeling the degradation and drug release from PLGA based systems[46-51]. In hydrogels, these models are used to study for example water transport or diffusion in porous media[52,53]. So far, models based on Monte Carlo simulations have not been applied to investigate the drug release from biodegradable hydrogels. An important advantage of this type of simulations is the possibility to obtain a stochastic description for the state of a small domain inside hydrogel.

4. Dextran-based hydrogels

4.1 Dextran

Due to its high water solubility, relative stability, large number of hydroxyl groups for conjugation and good biocompatibility, dextran is widely investigated as polymeric carrier in both hydrogels and macromolecular prodrugs[54]. Dextran is a water-soluble bacterial *exo*-polysaccharide consisting mainly of α-1,6-linked D-glucopyranose residues and a low percentage of 1,3 branches (Fig. 1a) and is produced in a sucrose rich environment by *Lactobacillus*, *Leuconostoc* or *Streptococcus species*[55]. Low molecular weight dextran (40-100 kDa) is obtained by partial acid hydrolysis of high molecular weight dextran (about 10^3 kDa), followed by fractionation[55]. These low molecular weight fractions are relatively inert and non-toxic and are used for blood flow enhancement and/or plasma volume expansion[54-56]. Dextran with a molecular weight of <40 kDa is excreted by the kidneys and has a plasma half-life of 8 hours[57]. High molecular weight dextran is not excreted by the kidneys, but is finally degraded by the reticuluoendothelial system[57].



a *b*

Figure 1. *Dextran derived from Leuconostoc mesenteroides (a) and dextran derivatized with hydroxyethyl methacrylate (dex-HEMA) (b).*

4.2. Macroscopic dextran-based hydrogels

Dextrans are ideal polymers to form hydrogels for pharmaceutical purposes because of their low toxicity. In recent years, different dextran hydrogels have been developed. For example, dextran hydrogels were obtained by chemical crosslinking with diisocyanates, glutaraldehyde and epichlorohydrin[58-60]. Physically crosslinked dextran gels were formed by ionic gelation using potassium chloride[61]. However, these gels were less suitable for protein delivery since either the preparation procedures required highly reactive crosslinkers or high pH[58-60] or the obtained gels had limited stability in water[61].

Edman et al. prepared dextran hydrogels by radical polymerization of dextran derivatized with glycidyl acrylate[62]. However, as the preparation of the polymerizable dextran was performed in an aqueous solution, the degree of acrylate substitution of the dextran molecules was difficult to control due to hydrolysis of the glycidyl acrylate before and after the reaction. To obtain stable hydrogels, acrylated dextran was polymerized with N, N'-methylene bisacrylamide.

Van Dijk-Wolthuis et al. developed an alternative method to prepare polymerizable dextran. In their method, dextran is reacted with glycidyl methacrylate (GMA) in dimethyl sulfoxide (DMSO) using 4-(N,N-dimethylamino)pyridine (DMAP) as a catalyst yielding dex-MA[63,64]. With this method good control over the degree of methacrylate substitution and an almost quantitative incorporation of the GMA was obtained. Proteins could be incorporated in the gel by adding them to the polymer solution before formation of the hydrogel.

In general, hydrogels based on dex-MA were biocompatible, but essentially not degradable without the addition of an enzyme (dextranase)[65-67]. Franssen et al. investigated the release of proteins and dextranase-induced degradation of these hydrogels. The release and degradation

kinetics were dependent on the network properties and the amount of incorporated dextranase[67-69]. However, it was found that the degradation was limited to low degrees of methacrylate substitution (DS<7; the number of methacrylates per 100 glycopyranose residues). This was attributed to the reduced accessibility of the dextran chain for the enzyme as a result of the increased degree of substitution[69].

Biodegradable dextran hydrogels were obtained when dextran was derivatized with hydroxyethyl methacrylate (HEMA, Fig. 1b) or with HEMA containing an oligolactate spacer (HEMA-lactate)[70,71]. With the introduction of hydrolytically sensitive carbonate ester groups in the side groups that were linked to the dextran, these gels degrade under physiological conditions. The release of proteins is controlled by the presence and length of the hydrolyzable spacer, the DS and the initial water content of the gel. At low DS and high water content the release was controlled by diffusion. At high DS and relatively low water content the release was controlled by degradation and could be tailored from days to months. Moreover, high protein encapsulation efficiencies were obtained with these systems[35,72].

4.3. Dextran based microspheres

A limitation of macroscopic hydrogel systems is that their application requires surgery, which is inconvenient for patients and expensive. Therefore, a method was developed to prepare an injectable microsphere formulation based on dex-HEMA[73]. The method is based on phase separation between an aqueous solution of dex-HEMA and an aqueous solution of PEG. This phase separation was used to prepare an emulsion in which an aqueous dex-HEMA solution is emulsified in an aqueous PEG solution (Fig. 2). After emulsification, the microspheres are obtained by polymerization of the dextran conjugated hydroxyethyl methacrylate groups. Proteins are encapsulated when added to the dex-HEMA phase prior to polymerization and released under physiological conditions upon hydrolysis of the crosslinks. An important advantage of using the dex-HEMA/PEG system is the all-in-water preparation procedure, which avoids the use of organic solvents. In previous studies, it was demonstrated that high encapsulation efficiencies and zero order release with low burst release could be obtained[35, 72, 73]. Similar to macrogels, the release rate is dependent on the crosslink density of the dextran network, which in turn can be tailored by the initial water content of the microspheres and the DS.

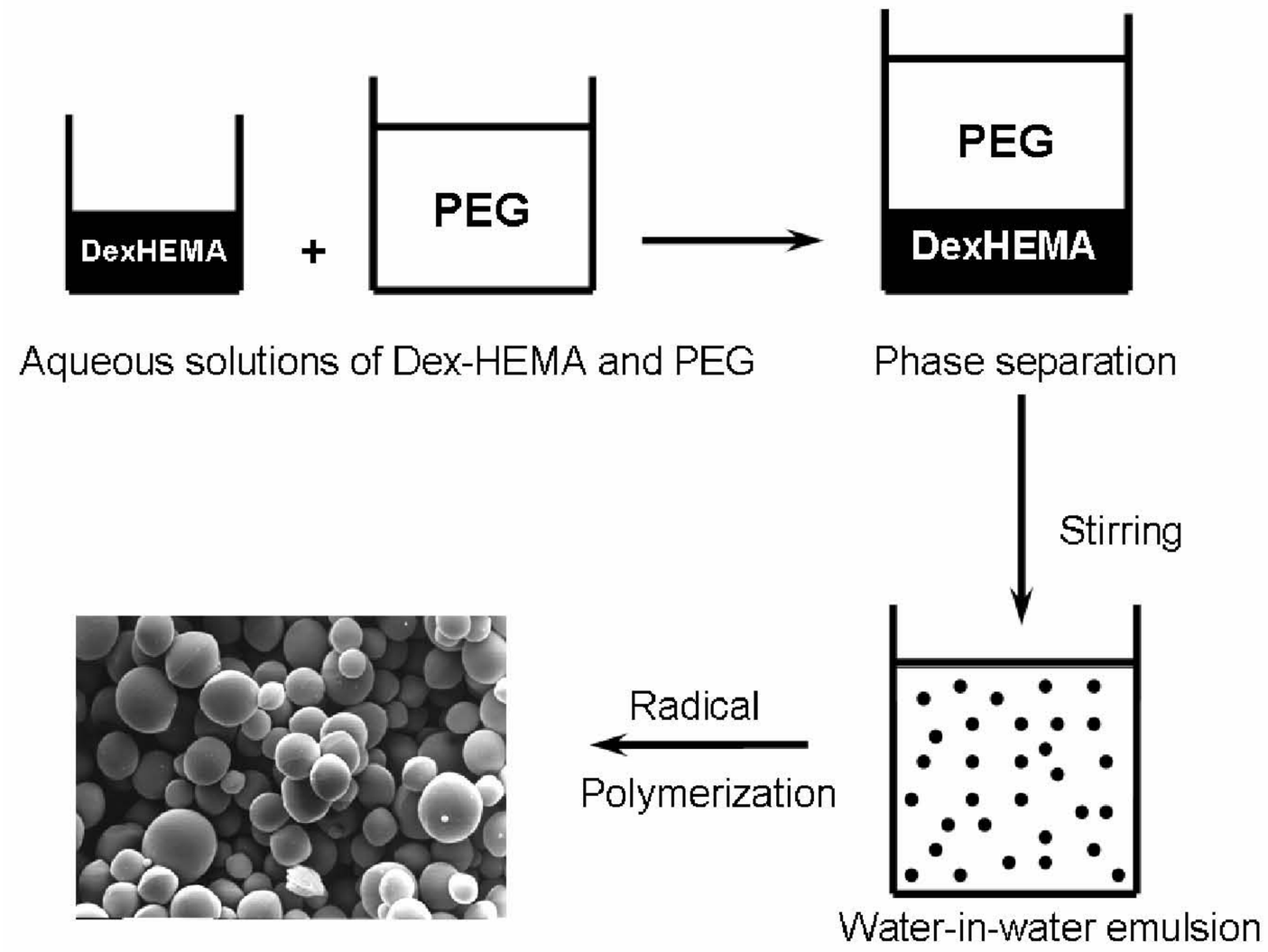


Figure 2. *Schematic representation of the microsphere preparation process.*

4.4. Biocompatibility and therapeutic efficacy

The design and development of biodegradable microspheres containing bioactive proteins for therapeutic application require a fundamental understanding of the release and biodegradation characteristics *in vivo* as well as insight into the cellular and tissue responses which are important factors concerning the biocompatibility of the microspheres[7,74]. According to the ISO norms[76], biomaterials should be evaluated at three levels: (1) the biological safety of the various ingredients used to manufacture the basic materials, (2) the biological safety of potential leachable substances or degradation products, and (3) the biological safety of the final biomaterial. The cytotoxicity of dextran, methacrylated dextrans, dextran based hydrogel discs and microspheres and their leachable substances was studied by measuring the cell proliferation inhibition index (CPII) on human fibroblasts *in vitro*[77]. Methacrylated dextrans only slightly inhibited the cell growth. Moreover, implantation studies in rats carried out by Cadée et al. showed that hydrogels and microspheres are well tolerated[78]. In another study, the therapeutic efficacy of IL2-loaded dex-HEMA microspheres was shown[79]. Local administration of rhIL-2 in mice at the site of the tumor with low doses is highly effective as was demonstrated in animal tumour models and human cancer patients[80-86]. However, repeated administration was necessary[85,86]. De Groot et al. showed that a single injection of IL-2-loaded dex-HEMA microspheres resulted in similar therapeutic effects as obtained with IL-2 injections on 5 consecutive days[79]. Interestingly, it was shown that cured mice were immune to

the tumour. Histopathological observations revealed that microsphere deposits are surrounded by macrophages infiltrates digesting the microspheres in minute granules. Apparently, the uptake of IL-2-loaded dex-HEMA microspheres by macrophages resulted in an effective immune reaction as also observed with consecutive IL-2 injections[79].

In case of subcutaneous injection of protein-loaded polymer microspheres, the protein uptake into the systemic circulation depends not only on the release rate, but also on the diffusion of the protein through the subcutaneous tissue[87,88] or on transport via the lymphatic system[89,90]. Many release systems lack a good correlation between the *in vitro* and *in vivo* release[91-94]. Very often the importance of release conditions *in vitro* (performed in glass or plastic tubes or flasks at 37 °C with or without agitation)[95,96] or protein instability in release media[93,94,96] has been neglected. Moreover, the degradation *in vivo* may significantly differ from the situation *in vitro*[97]. Size, shape and physical properties of the biomaterial may have an important effect on the degradation of the systems and the resultant release profile *in vivo*.

A poor *in vitro-in vivo* correlation (IVIVC) makes the development of sustained delivery systems to clinically applicable products difficult[98]. Once an *in vitro-in vivo* correlation has been established, dissolution tests may serve as a guide to formulation development. Changes in the production site or manufacturing process that result in a different *in vitro* release profile are then predictive for the *in vivo* behavior. A good *in vitro-in vivo* correlation allows not only prediction of clinical pharmacokinetics with only *in vitro* data, but it also gives more robustness to the protein delivery system.

5. Human growth hormone

Human growth hormone (hGH) is a small 22 kDa protein that is used to treat short stature caused by growth hormone deficiency, Turner's syndrome, or chronic renal failure[99-102]. This protein has also been applied in growth hormone replacement therapy in adults, in diabetic wound healing and in the treatment of skin burns to improve the regenerative capacity of the skin[103,104]. HGH is a protein drug with a short half-life after intravenous administration[90,91]. Therefore, to obtain therapeutic effects and maximize growth the patients are treated with 3 to 7 subcutaneous injections per week. However, efficacy and patient compliance could be significantly improved if the daily injections could be reduced to weekly or monthly injections. In the 90s different clinical studies have shown that continuous subcutaneous infusion of hGH (via external) pumps provided comparable growth rates of hGH deficient patients to daily injections[106-109]. One way to obtain

continuous exposure of hGH is the use of sustained release formulations. Therefore, during the last decade the design of sustained release hGH formulations has gained much attention. This has resulted in the development of PLGA based hGH-releasing system (Nutropin Depot®). In 1999, this sustained-release system requiring only two administrations per month was approved by the US FDA for the treatment of hGH deficiency in children and adults[110,111]. A major challenge in the development of this drug delivery system was to maintain the stability of hGH during formulation and release. As hGH is very susceptible to organic solvent induced aggregation, Tracy et al. developed a cryogenic spray drying process in which hGH is complexed with zinc-ions prior to processing[112,113]. However, clinical studies showed that the growth rate of children treated with this formulation was lower than the growth rate of children treated with daily hGH injections. Moreover, a higher incidence of anti-hGH antibody response was found[113]. In 2004, Nutropin Depot was withdrawn from the market. Following Nutropin Depot, also other long acting hGH formulations have been developed by either entrapment of hGH in a polymeric matrix based on physically or chemically crosslinked hydrogels (poloxamer[116] and multiacrylated PEG crosslinked with dithiols[117]) or by chemical modification of hGH with PEG[118]. Despite the experience gained in formulating hGH in microspheres, until now, no new long acting hGH formulation has been FDA approved.

6. Conclusions

In previous studies the feasibility of dex-HEMA based microspheres as controlled release system for a wide variety of model proteins and an active protein (IL-2) was investigated. However, to make this controlled delivery system suitable for clinical applications, additional research is necessary. The major objectives of this thesis are to obtain better insight into the possibilities and limitations of dextran-based microspheres as sustained delivery systems by studying the protein release and polymer degradation processes.

In **Chapter 2** a suitable hGH-loaded dex-HEMA microsphere formulation is designed. The IVIVC of the hGH release from this formulation was studied in a growth hormone deficient mouse model and in healthy human volunteers.

In **Chapter 3** a numerical model based on Monte Carlo simulations is proposed which accurately describes the experimental release curves of proteins of variable size from dex-HEMA microspheres with different crosslink densities. In addition, the experimental observations and the

computed release curves combined with confocal microscopy experiments are used to deepen the understanding of the processes determining the release from dex-HEMA based microspheres.

Chapter 4 is focused on the effect of polymerization conditions on the network properties of dex-HEMA microspheres prepared under different conditions. The network properties (crosslink density, pore size) were derived from the mechanical properties as obtained by the micromanipulation technique. For comparison and verification, the rheological characteristics of dex-HEMA macroscopic hydrogels were also determined.

In **Chapter 5** the possibility is studied to modulate the release of hGH from dex-HEMA microspheres by using excipients that either induce precipitation of the protein or change the polymerization kinetics of the dextran bound HEMA-groups.

In **Chapter 6** the nature and the quantity of both the soluble and insoluble degradation products of dex-HEMA based microspheres with different crosslink densities are studied *in vitro*.

In **Chapter 7**, the synthesis of a new class of polymerizable dextrans is described. The solubility of the degradation products and their potential application in microspheres is studied.

A summary of the results and conclusions is given in **Chapter 8**. Furthermore, the perspectives are discussed for dextran-based microspheres that have to be taken into account when using these microspheres as drug delivery systems for therapeutic applications.

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Chapter 2

Preclinical and clinical *in vitro in vivo* correlation of an hGH dextran microsphere formulation

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Submitted

Abstract

The aim of this study is to investigate the *in vitro in vivo* correlation of a sustained release formulation for human growth hormone (hGH) based on hydroxy ethyl methacrylated dextran (dex-HEMA) microspheres in both Pit-1 deficient Snell dwarf mice and in healthy human volunteers. A microsphere formulation with an hGH release time ~7 days, a high loading recovery (≥90 %) and a low burst release (≤10%) was developed. In a pharmacodynamic study, subcutaneous single dose administration of these hGH containing microspheres in Snell dwarf mice resulted in a dose dependent and significant increase in body lengths and weights during the protein release period (~10-15 days). Importantly, a good correlation between *in vitro* hGH release and in vivo effects was obtained. This indicates the released hGH has mainly retained its bioactivity. A pharmacokinetic study of hGH loaded dex-HEMA microspheres in healthy volunteers showed an increase in hGH serum concentrations starting from day 2 with peak serum hGH concentration between 1-2.5 ng/ml, 7-8 days after injection. These hGH serum concentrations were in good agreement with the hGH serum concentrations calculated from the *in vitro* release data, which indicates again a good *in vitro in vivo* correlation. Moreover, administration of this hGH dex-HEMA microsphere formulation resulted in an increased serum concentration of the biomarkers insulin-like growth factor-I (IGF-I) and IGF binding protein-3 (IGFBP-3). This again indicates that bioactive hGH was released from the microspheres. A good *in vitro in vivo* correlation is important to predict the effect of a controlled drug delivery product in a clinical situation and is therefore an important advantage of dex-HEMA microspheres as a parenteral protein delivery system.

1. Introduction

Since the introduction of recombinant human Growth Hormone (hGH) in 1985, the treatment of patients suffering from pediatric growth hormone deficiencies has improved considerably. However, hGH replacement therapy still suffers from the burden of daily injections, which negatively affects patient compliance and comfort. The availability of injectable systems, delivering hGH with a controlled rate over a period of one week to one month would be a significant improvement of the current daily treatment.

Several parenteral sustained release systems for the controlled delivery of growth hormone have been investigated[1-5]. The best known hGH sustained delivery system consists of polylactide-co-glycolide acid (PLGA) based microspheres, containing up to 22.5 mg hGH per injection, which was designed for monthly and bi-weekly treatment of growth hormone deficient patients[6-9].

In general, PLGA microsphere systems have several drawbacks, such as a high burst release of the active ingredient, the use of organic solvents for the microsphere preparation and acidification of the PLGA matrix during release of the active ingredient which may lead to protein aggregation[10-13]. High burst release results in the loss of significant amounts of hGH during a short period of time and reduces the efficacy of the formulation. Importantly, the release of aggregated protein may result in an immunogenic response[14]. Especially for proteins these issues will limit successful application of PLGA as controlled release matrix.

Hydrophilic matrices such as hydrogels are generally gentler for sensitive proteins than hydrophobic matrices such as PLGA[15-18]. We have developed hydrogel microspheres, consisting of degradable networks of crosslinked dextran[19]. Therapeutic proteins can be physically entrapped in the network and released by degradation of the network upon administration. Typical release profiles of proteins from these hydrogels range from zero-order to sigmoidal with a duration of typically one to four weeks[19,20]. Details on the preparation and properties of dex-HEMA microspheres have been described previously[20-24].

At present, no information is available about the *in vitro in vivo* correlation (IVIVC) of protein-loaded dex-HEMA microsphere formulations. Once a correlation is established, *in vitro* release tests can serve as a guide to formulation development.

In this study, an hGH-loaded dex-HEMA microsphere formulation was designed which releases the protein for 7 days. The IVIVC of this formulation in Pit-1 deficient Snell dwarf mice and in healthy human volunteers was investigated. In dwarf mice, increase in body length and in body weight were measured after a single subcutaneous injection of hGH loaded dex-HEMA microspheres and compared with data from daily subcutaneously injected solutions of hGH in

saline during a period of four weeks. In a clinical setting, hGH serum concentrations were compared to the calculated theoretical serum concentrations, based on the *in vitro* release profile of hGH after a single subcutaneous injection with hGH loaded dextran microspheres. To investigate the biological activity of released hGH serum levels of IGF-1 and IGFBP-3, two biomarkers for hGH, were also monitored.

2. Materials and methods

2.1. Materials

Poly(ethylene glycol) (PEG) 10 kDa and potassium peroxodisulfate (KPS) were obtained from Merck, Darmstadt, Germany. *N,N,N',N'*-tetramethylethylenediamine (TEMED), sucrose and carboxymethylcellulose were purchased from Fluka (Buchs, Switzerland). Hydroxyethyl methacrylate derivatized dextrans (dex-HEMA) with a degree of HEMA substitution (DS, number HEMA groups per 100 glucose units) of 12, 16 and 20 were synthesized and characterized according to Van Dijk-Wolthuis *et al.*[25] and obtained from Polymer Service Center Groningen (PSCG, Groningen, the Netherlands). HGH-190 (Somatogen®), which is a truncated form (lacking one phenylalanine residue) of the natural human growth hormone) was kindly supplied by Biotechna Sicor Inc. (Irvine, CA, USA).

2.2. Preparation of the microspheres

2.2.1. Formulation development

Different batches dex-HEMA microspheres were prepared at 5 g scale with various DS, KPS concentrations and protein load (Table 1). The preparation procedure was based on the water-in-water emulsion technique as described elsewhere[19,21]. In detail, for microspheres with an initial water content of 50 % and a protein load of 3% w/w (weight hGH/weight dex-HEMA), 61 mg of dex-HEMA was dissolved in 0.6 ml hGH solution (3.2 mg/ml hGH in 10 mM phosphate buffer pH 7.4 containing mannitol and glycine) and the resulting solution was freeze dried (Lyostar, FTS Systems, Inc. Stone Ridge, USA). Next, the obtained cake was hydrated in 4.9 g of a 27 % w/w PEG solution in 25 mM phosphate buffer pH 7.0. The mixture was vortexed for 1 minute and subsequently 100 µl of a TEMED solution (20% (v/v), pH neutralized with 4 M hydrochloric acid) was added, followed by vortexing for 20 s. Then, 180 µl KPS (concentration 50 mg/ml in PBS) was added, the emulsion was shaken gently and allowed to polymerize for 1 hour at room temperature. The formed microspheres were washed 5 times with 5 ml 25 mM phosphate buffer pH 7.4.

2.2.2. Animal trial material (ATM)

For the animal trial, two different batches of hGH-loaded microspheres (dex-HEMA DS16, initial water content of 50% and target protein loading of 10 % hGH w/w dry weight (ATM 1) or initial protein loading of 10 % hGH w/w dry weight (ATM 2)) and placebo microspheres (dex-HEMA DS16 initial water content of 50%) were prepared. The microspheres were prepared at 30 g scale (corresponding with 0.4 g dry weight of microspheres). Apart from scale and amounts of raw materials and reagents adjustments, the preparation procedure of the microspheres was the same as described in the previous section. Only, the preparation procedure of the microspheres of ATM2 slightly deviated from the standard preparation procedure. These microspheres were obtained by homogenization of the dex-HEMA/PEG emulsion for four minutes at 10,000 psi with an Avestin C5 homogenizer (ATA scientific, Lucas Heights, Australia), prior to polymerization. The microspheres were washed with water by five centrifugation (3500 rpm, 20 min) and washing steps (90 ml water). Finally, the microspheres were resuspended in 5.5 ml 25 mM phosphate buffer pH 7.4 also containing 3% (w/v) carboxy methyl cellulose (CMC) to prevent sedimentation of the microspheres and freeze-dried.

2.2.3. Clinical trial material (CTM)

The hGH loaded dex-HEMA microspheres for the study in volunteers were prepared at a 1kg process scale (12 g dex-HEMA). Except for scale adjustment of amounts of raw materials and reagents, microspheres (dex-HEMA DS16, 50% initial water content, 10% hGH w/w dry weight) were prepared essentially according to the procedure described above. Briefly, the PEG/dex-HEMA/buffer mixture was emulsified for 55 minutes at 1500 rpm in an autoclavable 2-L jacketed Bioreactor unit equipped with baffles and a three bladed impeller (4.5 cm in diameter; Applikon B.V., Schiedam, the Netherlands). Next, 4 ml TEMED solution (20 % (v/v), pH neutralized with 4 M hydrochloric acid) was added and stirring was continued for 5 minutes. The stirring rate was set to 500 rpm and 2.7 ml of 50 mg/ml KPS-solution was added. The stirring was stopped after 5 minutes and the emulsion was incubated at room temperature for 60 minutes to polymerize the dextran grafted HEMA groups. Removal of PEG, KPS and TEMED was performed by crossflow filtration with a FiberFlo hollow fiber capsule filter (0.22 μm: Minntech, Mineappolis, USA) using 5.5 L 5mM phosphate buffer pH 7.4 containing 3% w/w sucrose. The microspheres were suspended in 450 ml 5 mM phosphate buffer pH 7.4 containing 3% w/w sucrose and concentrated over the filter to 500 ml. The resulting microsphere suspension was pumped into 20 ml vials (7.3 g per vial) and freeze-dried (Lyostar, FTS Systems, Inc.Stone Ridge, USA). The hGH content of the microspheres was determined by amino acid analysis (Ansynth, Roosendaal, The Netherlands).

Briefly, about 1 g of microsphere suspension (10 mg microspheres/g) was incubated with HCl (6 M) by incubation at 110°C for 22 h to hydrolyze the matrix and the protein. The sample solution was mixed with internal standard solution (Sigma, USA) and analyzed for amino acid concentration with an amino acid analyzer (Biochrom 20, Pharmacia, Uppsala, Sweden), using a weakly acidic cation exchange column (Bio20 Lithium, 250 x 4.6 mm, Ultropac 8 Li-form, Pharmacia, Uppsala, Sweden).

To ensure the sterility of both the animal and clinical trial material, the microspheres were produced aseptically. Prior to use, all solutions were pumped through a sterile Millipak 200 filter into the vials. All equipment was autoclaved before use (30 minutes 121°C, Sauter autoclave (Sauter AG, Sulgen, Switzerland) and vials were heated for 4 hours at 250°C. Average particle diameters and size distributions of the microspheres were measured with a Malvern Mastersizer 2000 (Malvern instruments Ltd, Worcestershire, United Kingdom) with water as diluent.

2.3. In vitro release characteristics of hGH microspheres

Freeze dried microspheres (40 mg) were hydrated in 5 ml 25 mM phosphate buffer pH 7.4 at 37°C. At different time points, the microspheres were centrifuged for 5 min at 3200 g; 3 ml supernatant was removed and replaced by 3 ml of the same buffer. The hGH concentration in the samples was determined by GPC (see next section). The burst release is defined as the percentage of the encapsulated amount hGH released after 7 h of incubation. The recovery is defined as percentage of the total amount of released hGH (after 4-20 days; dependent on the formulation) divided by the encapsulated amount hGH. The encapsulated amount of hGH in the microspheres was determined by summation of the total amount released from the microspheres and the amount of hGH present in the microspheres after the release period (after 4-20 days; dependent on the formulation) determined with nitrogen analysis (see nitrogen analysis).

2.4. Analyses

2.4.1. Size exclusion chromatography

High performance size exclusion chromatography (HP-SEC) was used to determine the hGH concentration in the release samples. The HPLC system consisted of a pump series 200, a vacuum degasser series 200, an autosampler series 200, a column LC 101 oven, and a UV detector series 200 (all Perkin Elmer Instruments, Norwalk, USA), thermostated (35 ^{0}C) Thosohaas TSKgel G300SWXL column and a Thosohaas TSKgel SWXL guardcolumn (Montgomeryville, PA, USA). The flow rate was 0.7 ml/min and a mobile phase containing phosphate buffer (64 mM, pH 7.0) and

3 % (v/v) isopropanol was used. The chromatograms were recorded and analyzed with the Totalchrom data acquisition system (Perkin Elmer Instruments, Norwalk, USA).

2.4.2. Nitrogen analysis

The amount of protein present in the microspheres (corrected for the nitrogen content in placebo microspheres) at the end of the release period (after 4-20 days; dependent on the formulation) was determined by nitrogen analysis. The samples (500 µl) were injected using an auto injector (ELS 2100) into a nickel boat, which was subsequently transported into the furnace tube TN 3000. The analyzer was equipped with a cold trap TX/TS module and a UV detector (both thermo Euroglass corp. Delft, Holland). The argon flow was set at 340 ml/min, the oxygen flow was set at 15 ml/min and the ozonator was set at 110 ml/min. The temperature of the furnaces was set at 1000 ^{0}C, while temperature of the NOx scrubber was set at 320 ^{0}C. A calibration curve was obtained injecting 500 µl of an aqueous glycine solution (0.1-10 µg/ml). The hGH-190 concentration was calculated by assuming it contains 16.7 % w/w nitrogen[26]. The data were recorded and analyzed with the data acquisition system of Thermo Euroglass DIN 38409 (Thermo Euroglass corp. Delft, Holland).

2.5. Animal trial

2.5.1. Animal experiments

Pit-1 deficient Snell dwarf mice were bred and kept under standardized laboratory conditions (24 °C, relative humidity 60%). Dwarfs were bred by mating heterozygous females (+/dw) to dwarf males (dw/dw), rendered fertile by treatment with 1 mg L-thyroxine and 50 mg prolactin 5 days a week for at least 4 weeks. After weaning at 4-5 weeks, the dwarfs were maintained on a diet of commercial food pellets, supplemented with a mixture of milk powder, wheat embryo, and raw egg (1:2:2) suspended in 10% glucose in water[27]. All animals were weighed and their length was measured weekly under ether anesthesia[28]. Since growth patterns of male and female dwarfs turned out to be similar, groups contained both males and females (age 6-8 weeks).

2.5.2. Study procedure and assay methods

The dwarf mice were selected to obtain equal initial means and standard deviations for length and weight and an equal distribution over both sexes and age at the start of the experiment. The mice were divided over 5 groups each consisting of 5 animals. Group 1 received 0.1 ml phosphate buffered saline (PBS for 5 days a week for 4 weeks, once daily); Group 2, hGH-190, 8.3 µg dissolved in 0.1 ml saline for 5 days a week for 4 weeks, once daily (total dose of hGH

administered 166 μg); Group 3 received only at the start of the experiment 0.1 ml of a hGH loaded dex-HEMA microsphere (ATM1; releasing 232 μg hGH) suspension in saline; Group 4 received only at the start of the experiment 0.1 ml of a hGH loaded dex-HEMA microsphere (ATM2; releasing 156 μg hGH) suspension in saline.

Weekly, body length and weight were measured. Local reactions of the injected microspheres were assessed during the course of the experiment and just before the animals were killed by decapitation, 4 weeks after the start of the experiment, by two independent researchers. Four weeks after the start of the experiment and 2 hours after the last injection of animals from groups 1 and 2 all animals were killed by decapitation under ether anaesthesia. Blood was collected, and the sera were pooled per group. To investigate the formation of hGH antibodies, sera were incubated overnight at 4 ºC with ^{125}I-labeled-hGH (obtained as described by Salacinski et al.[29] and purified by chromatography on Sephadex G-50 followed by Sephadex G-100[30] at different dilutions. Separation of bound and free hormone was performed by adding 100μl Sac-Cel anti-rat IgG coated cellulose suspension (Immunodiagnostic Systems, Boldon UK). After 30 minutes of complexation at room temperature, 0.5 ml distilled water was added, and the samples were centrifuged at 10,000 g for 4 minutes. Radioactivity in the pellets was counted in a Packard γ-counter.

2.6. Clinical trial

2.6.1. Subjects

Subjects were enrolled at the Clinic of Cardiology in Tartu, Estonia, for a single-dose pharmacokinetic study in healthy elderly volunteers. Institutional Review Board approval was obtained and patient signed informed consent forms were obtained before screening. Healthy men between 50 and 80 years were recruited for this study. All subjects passed pre-study physical examination, laboratory tests and ECG. Entry criteria included body mass index between 22 and 27 kg/m^2, peak growth hormone level < 3 μg/L and IGF-1 level < 280 μg/l and absence of indications of major diseases. Subjects were excluded if they had a history of diabetes mellitus, or active thyroid function abnormalities, or active hepatic, renal, metabolic or endocrine diseases.

2.6.2. Study treatment

After a 2-day baseline period, 10 volunteers received one dose of hGH loaded dex-HEMA microspheres formulation (Age: 62 ± 7 yrs, Body weight: 82 ± 13 kg, BMI: 27 ± 3 kg/m^2, peak growth hormone level < 3 μg/L and IGF-1 level < 280 μg/l) equivalent to 14 mg hGH. Each subject received the above treatments by subcutaneous injection in the abdomen. The dose was

administered in volumes of approximately 1.1 ml (75 mg microspheres/ml) by 4 subcutaneous injections, each at a different abdominal site.

2.6.3. Study procedure and assay methods

Subjects were screened for eligibility within 3 weeks prior to the institutionalization period. After fulfilling all inclusion and exclusion criteria subjects were institutionalized from the evening before day -2 up to the morning after the day study medication has been administered (day 2). Hereafter subjects returned to the institute on day 3, 5, 7, 8, 9, 10, 11, 12, 14, 24 and 38. Subjects returned for a last follow-up visit to the institute on day 90 (± 3 days). Growth hormone serum levels were measured during screening, at baseline and after treatment up to day 38. From day 3 to day 38 after drug administration, 3 blood samples were taken at 30-min intervals, on each assessment day. Endogenous growth hormone is released from the pituitary in a pulsatile fashion (short peak every two hours[31], and a single growth hormone sample might coincide with an endogenous growth hormone peak, thus masking the growth hormone levels due to the hGH loaded dex-HEMA microsphere treatment. To minimize this risk, 3 samples were taken as described, and the lowest growth hormone value of each triplet sample was taken to account for endogenous hGH spikes. On the basis of this minimum-out-of-three approach, mean growth hormone profiles for all treatment groups were obtained.

The collected blood samples for the determination of growth hormone, IGF-1 and IGFBP-3 were collected using 4 ml clot-activator tubes, and later centrifuged for 10 min at 1100-1300 g to collect the serum. Serum was stored at -20 ^{o}C until analysis. Samples were labeled by a bar code, which contained the subject number, sample number, study day and study code. Analyses were performed by HTI Laboriteenuste AS (Hiiu 44, Tallinn, Estonia). Serum concentrations of hGH, IGF-1 and IGFBP-3 were determined by a 2-site chemiluminescent enzyme immunometric assay standard kit (Immulite, Diagnostic Products Corporation, Los Angeles, USA) using an IMMULITE 2000 Analyzer (Diagnostic Products Corporation, Los Angeles, USA).

2.6.4. In vitro in vivo correlation

The serum hGH concentration as function of time was calculated with Modelmaker 4 (AP Benson, Oxfordshire, UK) from the in vitro release data, using a one-compartment linear model[32]. In this model, blood is considered as a single compartment and the serum concentration as function of time is calculated from the absorption of subcutaneous hGH into the blood and the hGH elimination from the blood. Furthermore, it is assumed that equilibrium between the hGH serum concentration and that in extravascular tissues is rapidly formed, and that the excretion and

absorption follow first order kinetics. In Fig. 1 a schematic overview is given of the calculations in Modelmaker 4.

Briefly, from the in vitro release data, the amount of hGH released subcutaneously per unit of time was calculated. From these data, following first order reaction kinetics, the rate of hGH absorption (mg hGH/unit of time) into the blood was calculated, using the absorption rate constant derived from absorption half life ($t_{1/2,a}$=0.7 h) obtained by Jorgenson et al.[33] for subcutaneous administration of hGH and a bioavailability of 75 %[34] using:

$$\frac{dA_1}{dt} = R(t) - B \cdot k_a \cdot A_1 \qquad (1)$$

Where, A_1 is the amount of hGH present subcutaneously, $R(t)$ is the amount of hGH released from the microspheres per unit of time, k_a is the absorption rate constant, B is the bioavailability of hGH. Again following first order reactions kinetics, the amount of hGH in the serum as function of time was calculated from the absorption rate data using the elimination rate constant (derived from elimination half life $t_{1/2,el}$=12 min[33]) according to:

$$\frac{dA_2}{dt} = B \cdot k_a \cdot A_1 - k_{el} \cdot A_2 \qquad (2)$$

Here A_2 is the amount of hGH present in serum and k_{el} is the elimination rate constant. Finally, the serum hGH concentration followed from dividing this amount by the average distribution volume (V_d) of the elderly male human volunteers (70 ml/kg body weight). The in vitro in vivo correlation was evaluated by comparing the calculated serum concentration time data to the measured serum concentration time data.

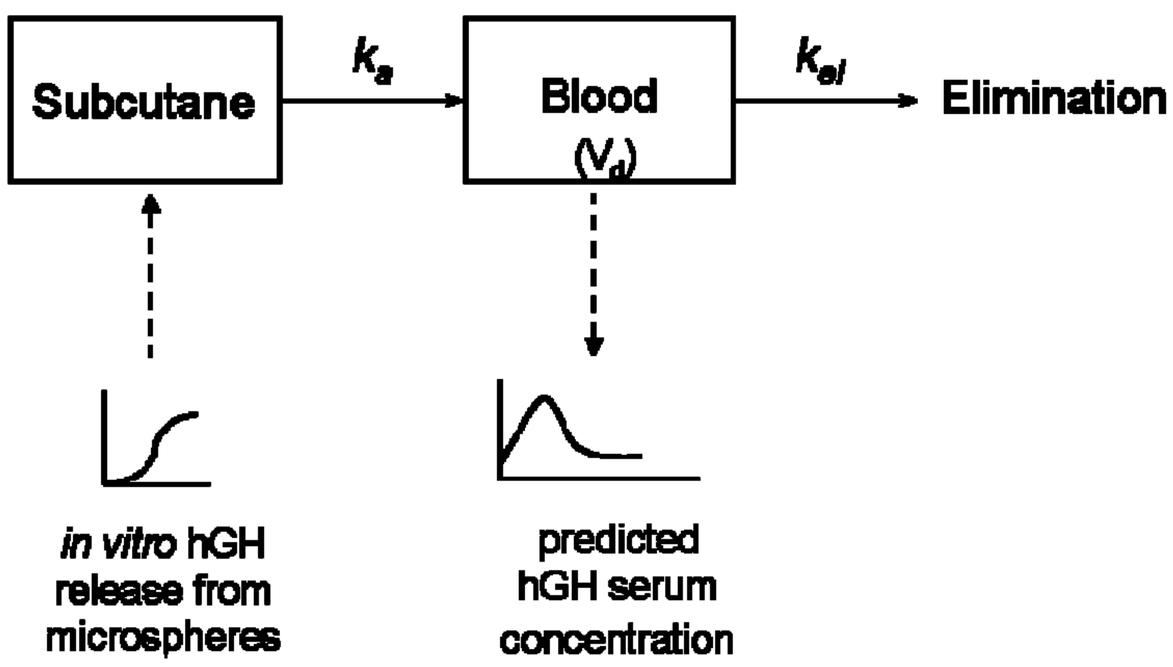

Figure 1. *Schematic overview of model used to predict the in vivo serum concentration of hGH.*

3. Results

3.1. Formulation development

Fig. 2 shows some typical cumulative release curves of hGH from these dex-HEMA microspheres. The results of all formulations are summarized in Table 1. Fig. 2 shows that hGH was released with almost zero order kinetics from dex-HEMA microspheres for 5-15 days, dependent on the formulation. This figure also shows that the time to release more than 90 % of hGH increased with an increasing DS and decreasing protein loading, which is consistent with previous observations[19,35]. The results in Table 1 show that the time to release 90 % of hGH decreased with increasing KPS concentration (e.g. compare formulation 8,9). Furthermore, the burst release ranged between 8 and 40 % of the loading and increased with increasing protein loading (e.g. compare formulation 1,3,4). The recovery of hGH ranged respectively between 71 and 100 % and no obvious effects of KPS concentration, loading and DS on recovery were found.

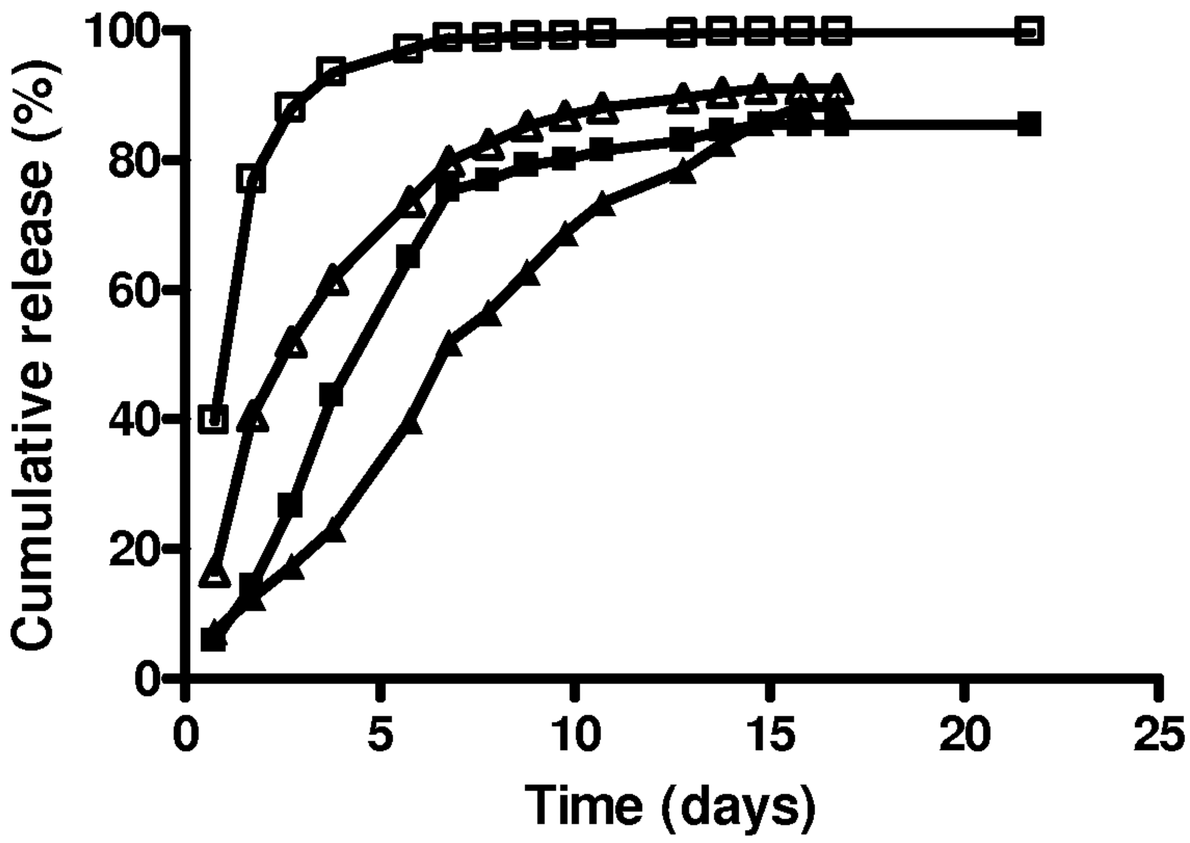

Figure 2. *Cumulative release of hGH from DS 12 (■, □), and 20 (▲, Δ) microspheres .The initial water content was 50 % and the protein load was 3 % (open symbols) and 20 % (closed symbols). The values are the average of 2 independent measurements and deviated less than 10 %.*

In Table 1, the formulations are scored for the preferred characteristics (time to release 90%> 7 days, burst release<10%, recovery>90%). Only formulation 6 meets all the requirements. Therefore, the processing parameters identified for the preparation of this formulation were used for the preparation of microspheres for both the animal trial (ATM) and the clinical trial (CTM) microsphere batches.

Table 1. *Characteristics of the different hGH containing dex-HEMA formulations.*

Formulation	DS	Initial protein loading (% w/w)	KPS concentration (mg/ml)	Recovery (%)		Time to release 90% (days)		Burst (%)	
				*≥ 90 %**		*≥ 7days**		*≤ 10 %**	
1	12	3	50	87	-	5.5	-	11	-
2	12	10	13	96	+	3.7	-	32	-
3	12	16	50	97	+	4.3	-	28	-
4	12	20	50	100	+	3.1	-	40	-
5	16	3	50	84	-	6.9	-	8	+
6	*16*	*10*	*50*	*91*	+	*7.7*	+	*9*	+
7	16	20	13	94	+	6.6	-	11	-
8	20	3	13	72	-	9.9	+	10	-
9	20	3	50	86	-	12.4	+	12	-
10	20	20	50	95	+	7.6	+	14	-

NOTE: + and – indicate whether the formulation requirements (*) are met (+) and or not (-). The values are the mean of 2 independent measurements that deviated less than 10 %.

3.2. Formulation and in vitro release characteristics of animal trial material (ATM) and clinical trial material (CTM)

In Table 2, the characteristics of the dex-HEMA microspheres prepared for the animal trial and for the clinical trial are presented. The homogenization of the emulsion resulted in substantially smaller particles than without homogenization (compare the median volume diameter of ATM1 and ATM2; d(0.5)=19.7 μm and d(0.5)=4.6 μm, respectively). For the preparation of the CTM batch, no homogenization was used, which resulted in the formation of hGH loaded dex-HEMA microspheres with a (d(0.5)=18.0 μm).

Table 2. *Characteristics of ATM and CTM hGH-loaded dex-HEMA microsphere batches.*

Formulation	Median volume-based diameter (d(0.5)) (μm)	Recovery (%)	Time to release 90 % (days)	Burst (%)
hGH loaded dex-HEMA microspheres (ATM1)	19.7	98	9.0	8
hGH loaded dex-HEMA microspheres (ATM2)	4.6	66	8.8	10
hGH loaded dex-HEMA microspheres (CTM)	18.0	100	8.5	1

The values are the average of two independent measurements that deviated less than 10 %.

In Fig. 3, the cumulative release profiles of hGH from the ATM1 and ATM2 batches are shown. For both batches, near zero-order release of hGH was observed for 9 days. Both ATM1 and ATM2 showed a small burst release of 8% and 10 % respectively (Table 2). A recovery of 66 % and 100% was obtained for the homogenized (ATM2) and the not homogenized batch (ATM1; Table 2), respectively. Fig. 4 shows the release curve of the clinical trial material (CTM). In contrast to the microspheres prepared at 30 g scale, microspheres produced in the bioreactor at a 1 kg scale had a more sigmoidal release profile with a delay of approximately three days.

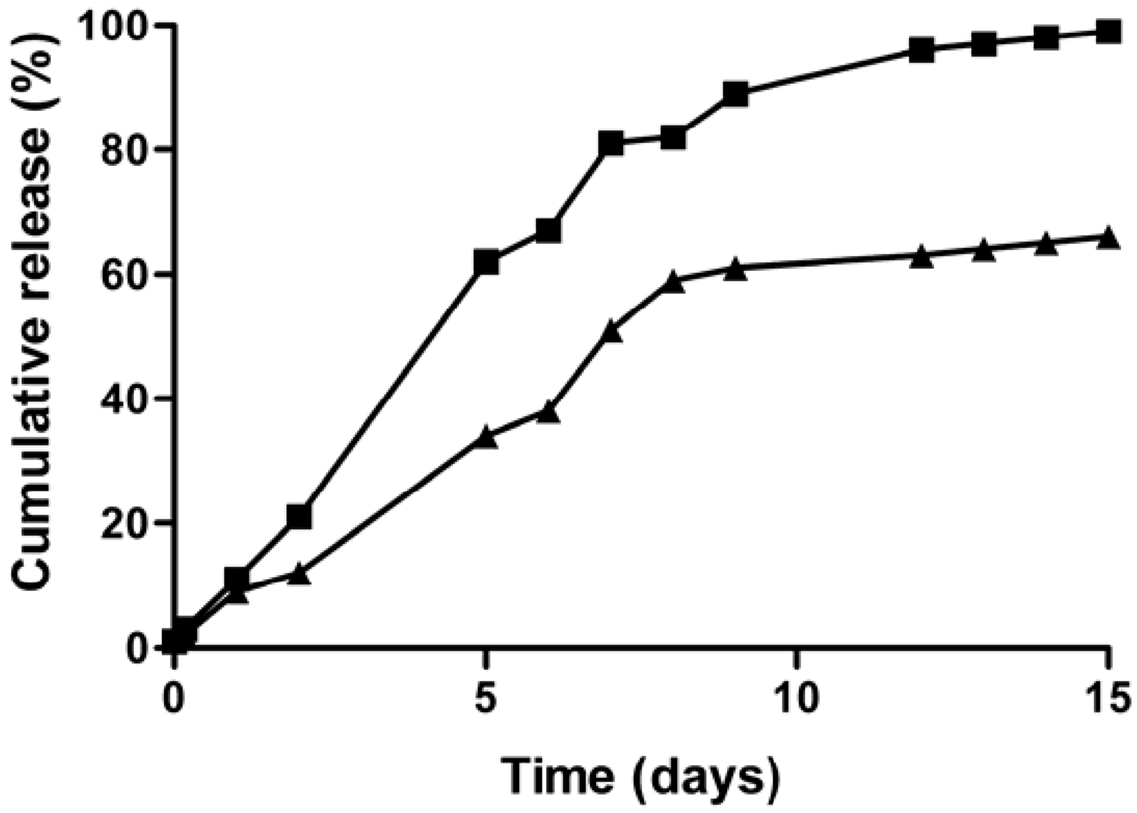

Figure 3. *Cumulative release of hGH from dex-HEMA microsphere (DS 16, initial water content 50 %, initial protein loading 10 % w/w) batches prepared without homogenization (ATM1, Table 2) (■) and with homogenization of the emulsion prior to polymerization (ATM2, Table 2) (▲). ATM1 and ATM2 were used in the animal study. The values are the average of 2 independent measurements and deviated less than 10 %.*

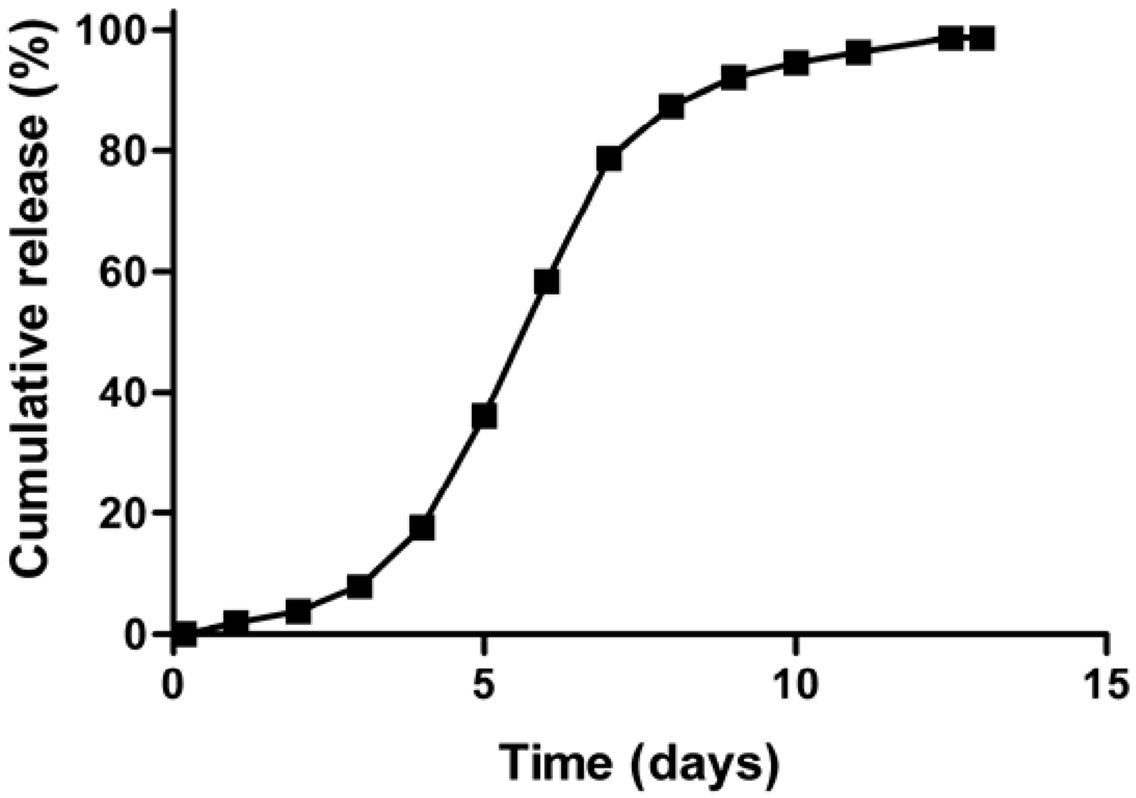

Figure 4. *Cumulative release of hGH from dex-HEMA microspheres (DS 16, initial water content 50 %, initial protein loading) used in the clinical study (CTM, Table 2). The values are the average of 2 independent measurements and deviated less than 10 %.*

3.3. Dwarf mice study

In Figs. 5 and 6, the growth curves of dwarf mice injected with an hGH solution, hGH-loaded microspheres (ATM1, ATM2), and PBS are shown. These figures show that after repeated injection of an hGH solution as well as a single subcutaneous injection of hGH loaded dex-HEMA microspheres, both weight and length of the mice steadily increases in time (over a period of 28 days for mice injected with single subcutaneous injections and over a period of 14 days for mice injected with ATM1,2).

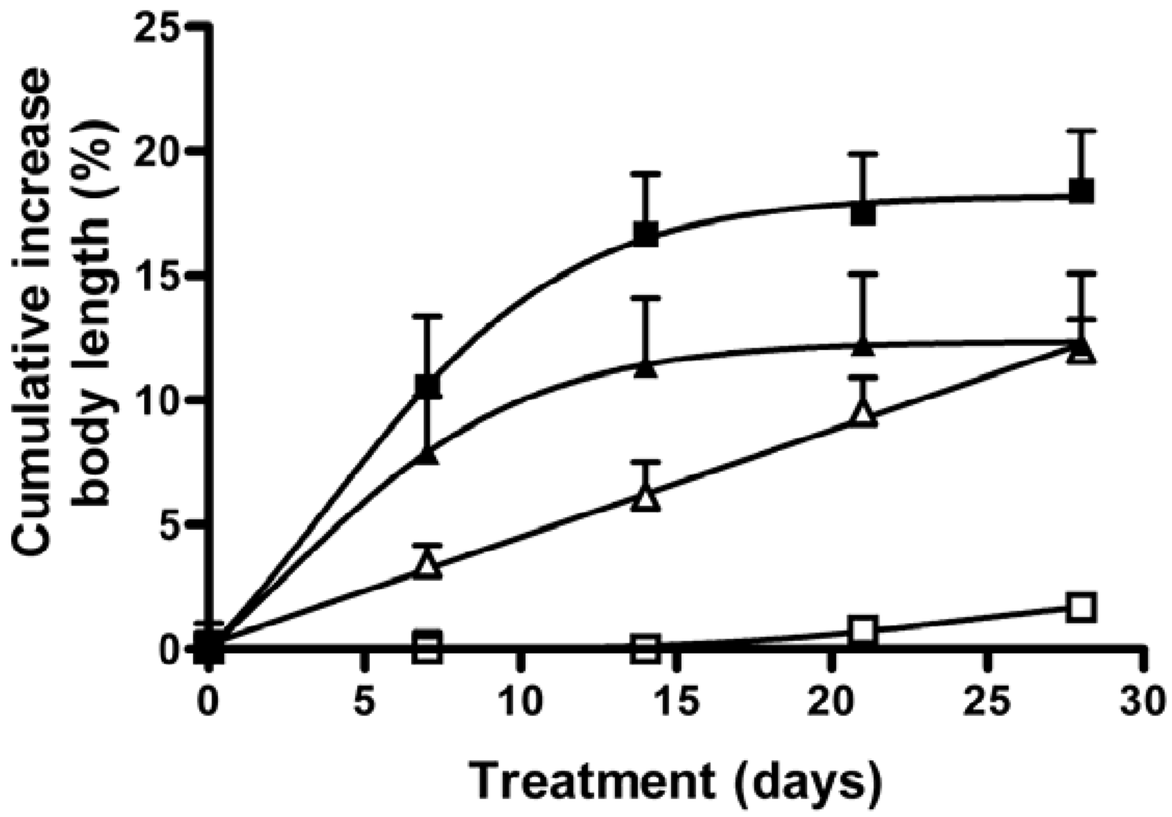

Figure 5. *Cumulative % increase in body length of dwarf mice during 4 weeks after sc injection of saline (□), hGH 8.3 μg/per day for 28 days (Δ), ATM1 microspheres (■) and ATM2 microspheres (▲). Mean ± S.E. (n = 5). Curves are significantly different (P<0.05).*

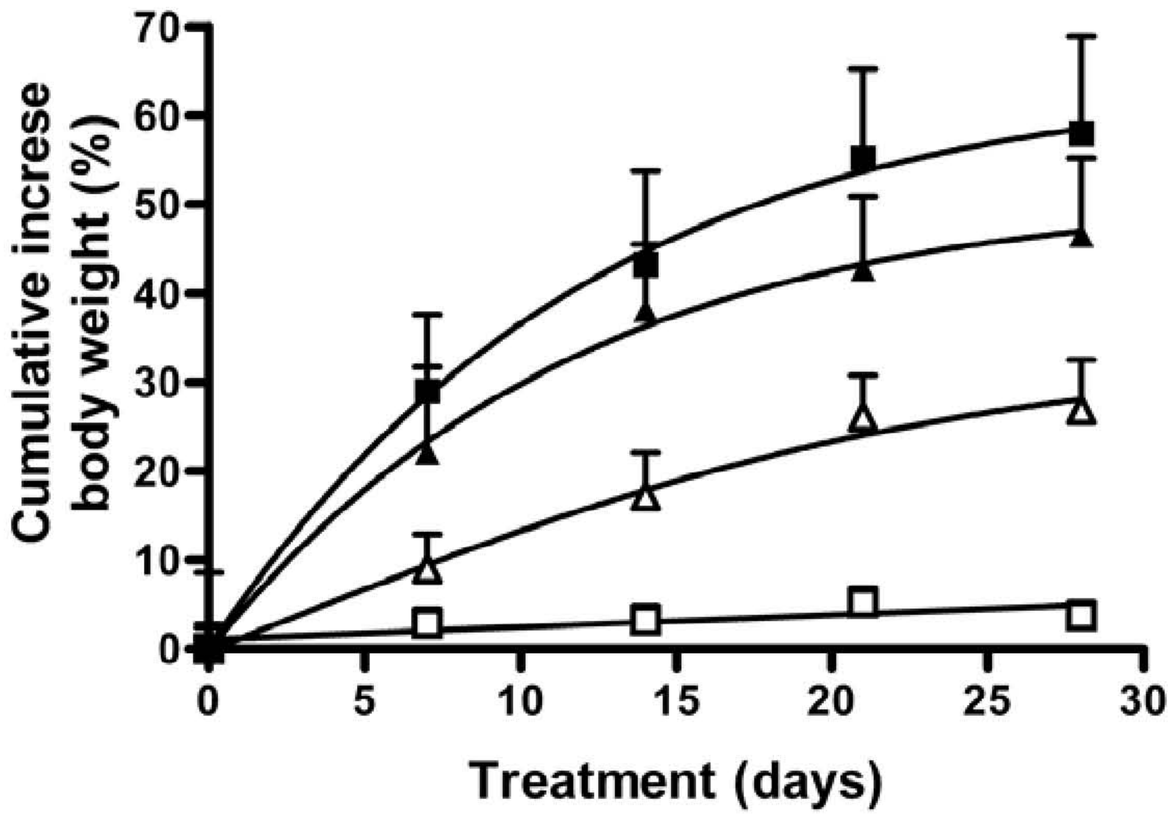

Figure 6. *Cumulative % increase in body weight of dwarf mice during 4 weeks after sc injection of saline (□), hGH 8.3 μg/per day for 28 days (Δ), microspheres of ATM1 (■) and of ATM2(▲). Mean ± S.E. (n = 5). Curves are significant different (P<0.05).*

In Fig. 7 the cumulative *in vitro* release of hGH (ATM1, ATM2) and the cumulative administered amount of hGH (control) are plotted against the observed pharmacodynamic effect (increase in body length). The figure shows that the pharmacodynamic effect almost perfectly follows the *in vitro* release of hGH. Importantly, this figure demonstrates that the growth per μg of hGH is comparable both for hGH loaded dex-HEMA microspheres and for the repeated injection of an hGH solution.

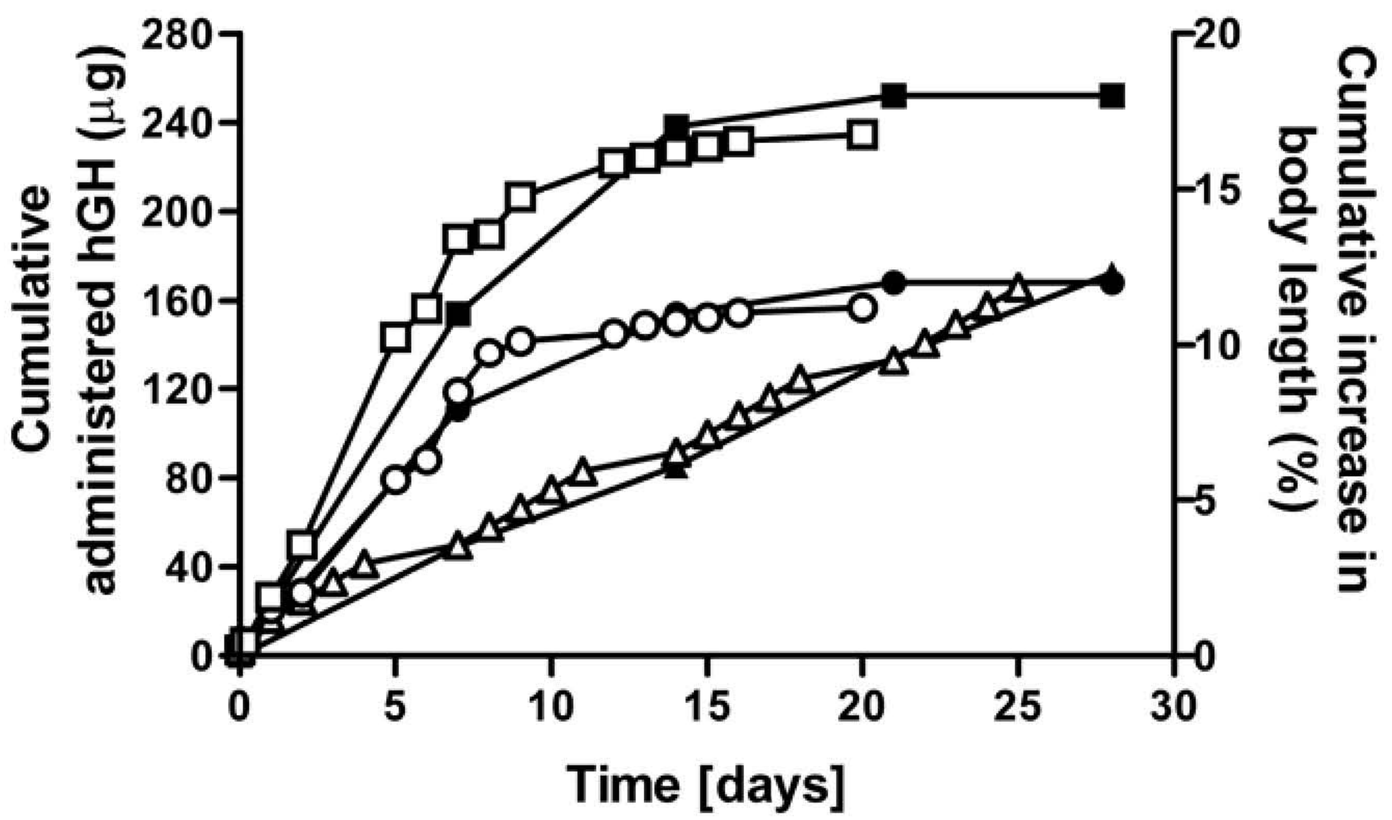

Figure 7. *In vitro in vivo correlation between cumulatively administered/released hGH (open symbols) and cumulative increase in body length (closed symbols) for hGH 8.3 μg/per day (Δ,▲), microspheres of ATM1 (□,■) and microspheres ATM2 (○,●).*

Although hGH-190 is active in Snell dwarf mice, it is a foreign protein for mice, and therefore can induce the formation of antibodies and give rise to local site effects. Fig. 8 indeed shows that four weeks after administration of hGH, hGH-specific antibodies were detected in the sera of the mice. In this figure the binding of radioactively labeled hGH (^{125}I-hGH) to formed hGH antibodies present in the pooled sera of mice is shown. HGH-specific antibodies were found four weeks after the start of treatment with hGH. The ED_{50} for the binding of ^{125}I-hGH to antibodies in the serum was obtained at serum dilutions of approximately 200x, 800x and 1200x for free hGH, hGH released from microspheres from ATM1 and ATM2 respectively. Apparently, both free hGH and hGH released from the microspheres evoked an immune response in these mice. Mice that had received the smaller ATM2 microspheres showed higher antibody levels than mice injected with the larger ATM1 microspheres. Moreover, a higher incidence of palpable local injection site reactions for ATM2 was found; one out of 5 mice developed an injection site reaction after injection with ATM1, while ATM2 caused injection site nodule formation in all mice.

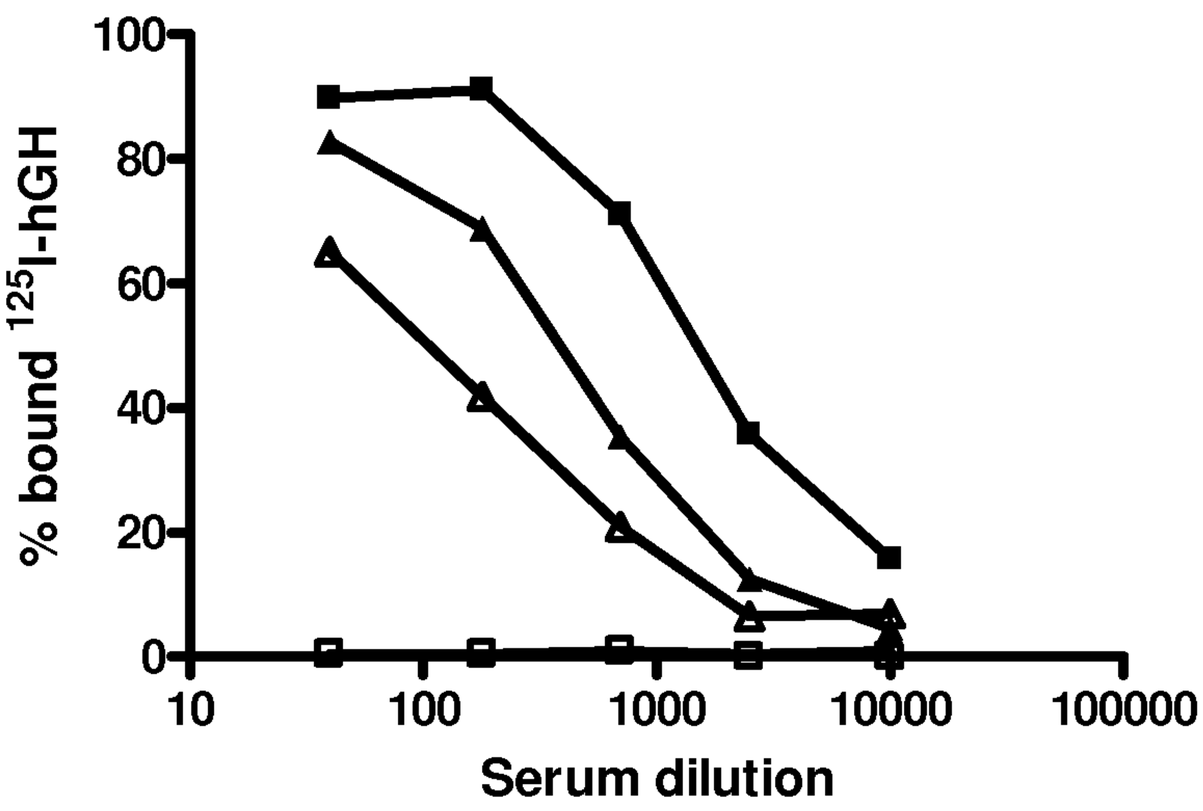

Figure 8. *Binding of ^{125}I-hGH to hGH- antibodies present is sera of Snell dwarf mice having received a single injection microspheres of ATM1 (■) and microspheres of ATM2 (▲) and daily injection of saline (□) or daily injection of a solution containing 8.3 μg hGH-190 (Δ).*

3.4. Clinical Study

Fig. 9 shows the serum hGH concentration in male elderly volunteers after subcutaneous administration of dex-HEMA microspheres. A short-lived rise in serum growth hormone concentration was observed directly after administration. Hereafter, hGH concentrations started to rise from day 2, peaking and leveling off approximately on days 7 and 8 after injection. The peak hGH serum concentrations were approximately between 1.0 and 2.5 ng/ml all subjects.

Also shown in this figure are the serum concentration time data calculated from the in vitro release curve of hGH from the dex-HEMA microspheres of the clinical trial material using the model described in the materials and methods (Fig. 4). The calculated serum concentration is in good agreement with the measured average serum hGH concentration.

The serum concentrations of biomarkers IGF-1 and IGFBP-3 are shown in Figs. 10 and 11. The average serum profiles followed the average serum growth hormone profile well.

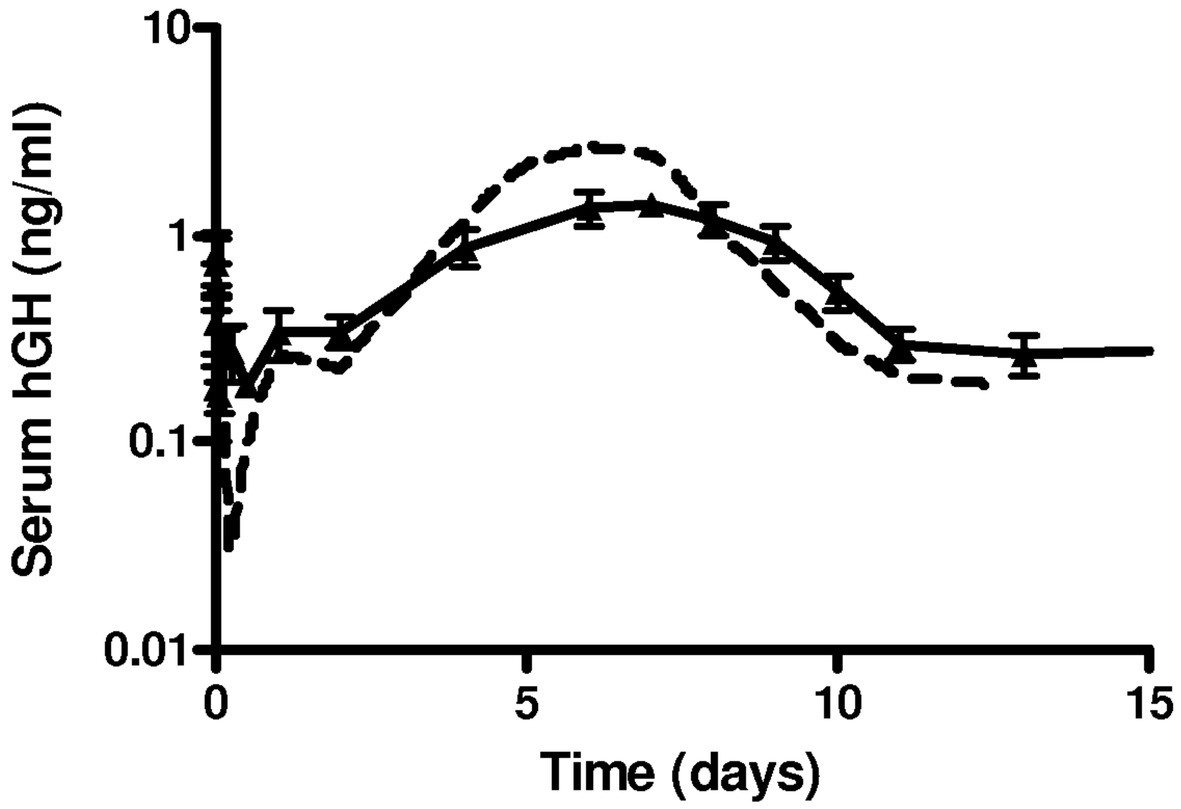

Figure 9. *Measured hGH concentration in serum of human volunteers after administration of CTM microspheres), (▲) and hGH concentration in serum (dotted line) calculated from the in vitro release of hGH using the model as described in the materials and methods. Mean ± S.E. (n = 10).*

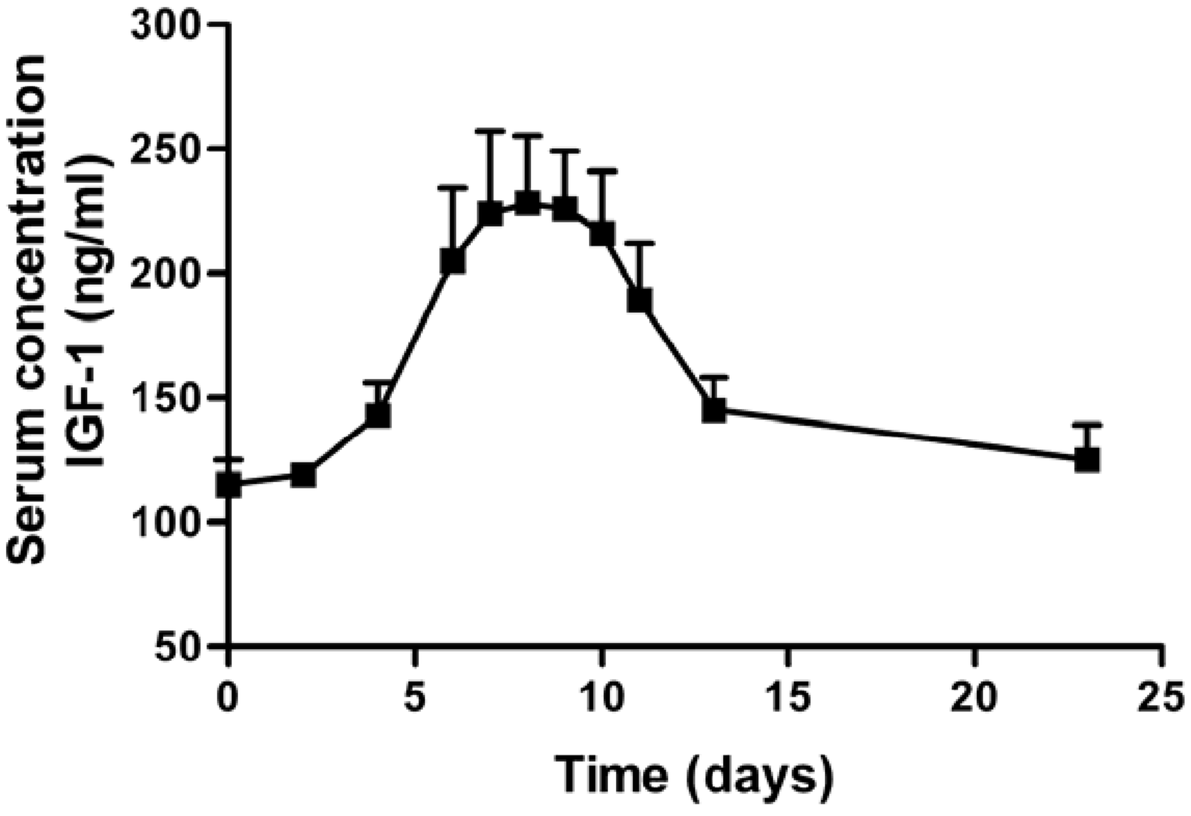

Figure 10. *Serum concentration IGF-1 after administration of CTM microspheres. Mean ± S.E. (n = 10).*

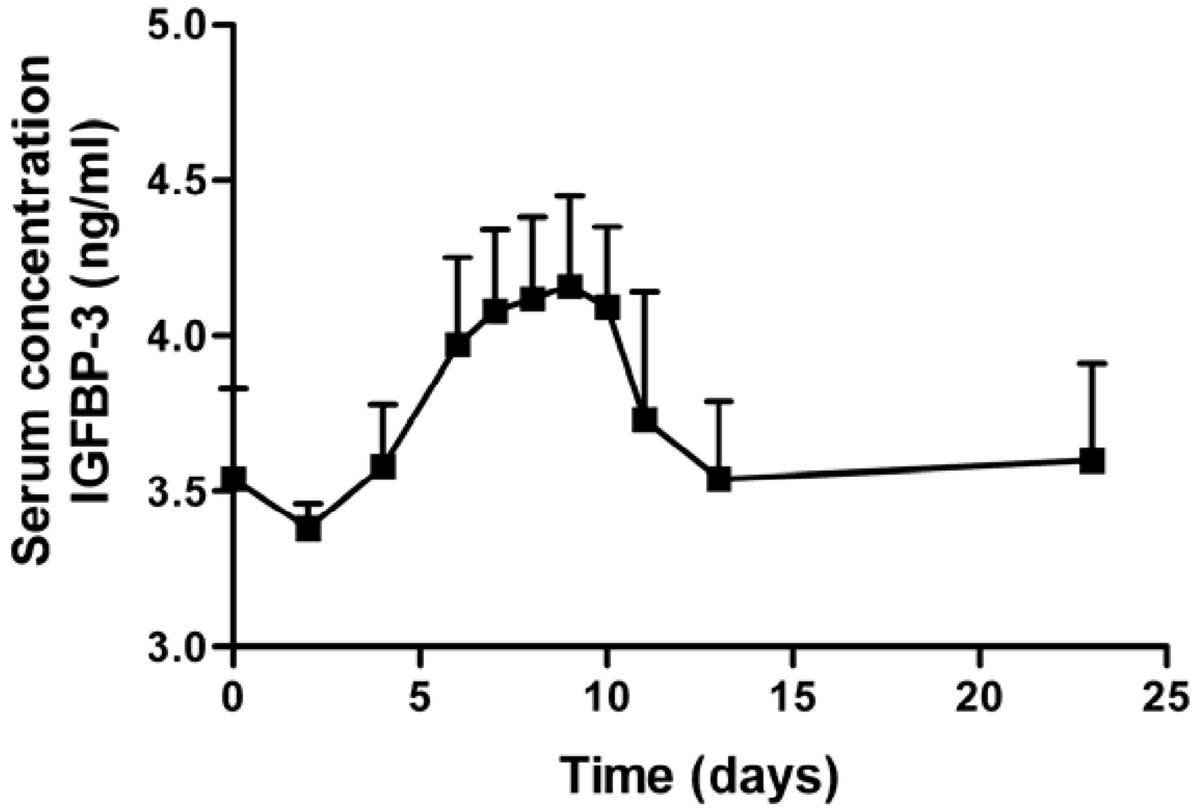

Figure 11. *Serum concentration IGFBP-3 after administration of CTM microspheres. Mean ± S.E. (n = 10).*

4. Discussion and Conclusion

To save protein, the primary screening for a suitable protein loaded microsphere formulation is generally performed with microsphere formulations prepared at small-scale. However, to obtain enough material for in vivo studies, preparation at larger scales is necessary. Differences between process parameters (such as stirring speed, process time) at small scale and at larger scales sometimes induce differences in microsphere characteristics. In this study, microsphere characteristics were initially not affected by preparation at 5 g scale or 30 g scale (ATM1). However, introduction of an extra homogenization step in the emulsification of dex-HEMA and PEG prior to the polymerization of the dex-HEMA at 30 g scale (ATM2) resulted in smaller microspheres (d(0.5)=4.6 μm ATM2; d(0.5)=19.7 μm ATM1; Table 2) with a low recovery of hGH (66%; Table 2). As previously observed, higher shear forces (here induced by homogenization with a turrax) applied during emulsification finally results in smaller microspheres[21]. The low recovery of hGH from the microspheres prepared with the extra homogenization step (ATM2, Table 2) is likely also caused by the higher shear forces which might induce irreversible denaturation of the protein resulting in the formation of aggregates, which are subsequently encapsulated in the microspheres. Importantly, the clinical material (CTM batch, table 2) showed quantitative recovery of hGH.

The microspheres produced in the bioreactor at a 1 kg scale (CTM batch) had a more sigmoidal release profile with a delay of approximately three days (Fig. 4), while microspheres prepared at 30 g scale (ATM1,2) had a zero-order release profile (Fig. 3). This difference is attributed to the differences in process time (one hour emulsification time for the production of the

CTM material; one and five minutes emulsification time for respectively ATM1 and ATM2). It is known that PEG and dextran can cause precipitation of hGH[5, 36]. An emulsification time of one hour might result in the formation of large precipitates, which were subsequently encapsulated in the microspheres. Previously, it was shown that protein clusters entrapped in dex-HEMA microspheres, might cause a delay in the release[35].

The pharmacodynamic effects of the hGH-loaded dex-HEMA microspheres (ATM) were assessed in Snell dwarf mice. Figs. 5-7 clearly demonstrate that administration of hGH containing microspheres induces a dose dependent increase in both body length and weight and that there is a good correlation between the amount of released hGH and the pharmacodynamic effects as the increase in body length almost perfectly follows the in vitro release curves. Furthermore, hGH released from dex-HEMA microspheres has mainly retained its bioactivity as there is no difference between growth response to a certain amount hGH administered by single daily injection of a solution of hGH and to the same amount of hGH released from either small microspheres (ATM2) or large microspheres (ATM1).

The microsphere formulations induced formation of antibodies in the mice. The highest antibody levels were observed after administration of small microspheres (batch ATM2). In addition, a higher incidence of palpable local injection site reactions was observed after the administration microspheres from this batch than after the administration large microspheres (batch ATM1). These observations might be ascribed to the size of the microspheres (d(0.5)=4.6 μm ATM2; d(0.5)=19.7 μm ATM1). Microspheres smaller than 10 microns in diameter can be phagocytosed[37,38]. The observed higher antibody response after administration of ATM2 might therefore indicate that these microspheres were phagocytosed by antigen presenting cells. In addition, for ATM2 a recovery of 66 % was obtained (Table 2), these microspheres might contain more hGH aggregates, which might be the cause of the higher antibody response. Importantly, antibody formation does not affect the growth pattern, which suggests that the antibodies were not neutralizing.

Fig. 9 shows that the shape of the serum hGH curve follows the cumulative *in vitro* release profile well, demonstrating a good *in vitro in vivo* correlation after subcutaneous administration of hGH loaded dex-HEMA microspheres to elderly male human volunteers. The delay observed in the *in vitro* release curve is also observed in the average serum hGH concentration in the first three days. The curve of the calculated hGH serum concentration was in good agreement with the measured hGH serum concentration (Fig. 9*)*. The short-lived rise in serum human growth hormone serum concentration is probably caused by a small amount of non-encapsulated hGH in the hGH loaded dex-HEMA microsphere formulation. It should be noted that Fig.4 does not show burst

release as the microspheres were washed to remove the carboxy methyl cellulose before starting the *in vitro* release and therefore, small amounts hGH retrieved in the wash fractions were not included in the vitro release profile. The short elimination half-life of hGH (12 min[33]) parallels the rapid disappearance of this observation.

Figs. 10 and 11 show that the serum concentration of the biomarkers IGF-1 and IGFBP-3 followed the hGH serum concentrations. Generally, growth hormone exposure induces hepatic IGF-I production by increasing transcription of IGF-I mRNA[39]. Since this is not a rapid process, the serum levels of IGF-1 do not follow the pulse in hGH serum levels, but instead the serum levels were paralleled by a delayed (~36 h) increase in IGF-1 and IGFBP-3 that leveled out to correlate to average hGH levels. IGFBP-3 is the major carrier of IGFs in serum. More than 90% of IGF-1 and IGF-2 is bound to IGFBP-3[40,41]. IGFBP-3 may provide a storage pool for IGF-1 and may be involved in the transport of IGF-1 to tissues. Serum concentrations of IGFBP-3 are fairly constant throughout the day and are controlled by hGH and IGF-1 levels[42]. The average serum profiles IGF-1 and IGFBP-3 followed the average serum growth hormone profile well, which suggests the preservation of bioactivity of released hGH from microspheres. Moreover, IGF-1 is considered the main driver for growth promotion[43,44,]. Generally the hGH dose applied in this study (40 μg/kg/day) may not be considered optimal for growth stimulation (50-100 μg/kg/day). Nevertheless, here a 6-day period with average IGF-1 levels of more than 200 ng/ml was observed, which may be sufficient for growth promotion[45].

In conclusion, a strong correlation between *in vitro* release and pharmacodynamic or pharmacokinetic effects is important to predict the effect of a controlled drug delivery formulation in a clinical situation. It allows prediction of clinical pharmacokinetics with *in vitro* data, and it gives more robustness to the protein delivery system. The good *in vitro in vivo* correlation is therefore an important advantage of dex-HEMA based microspheres as a parenteral protein delivery system.

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Chapter 3

Modeling the release of proteins from degrading crosslinked dextran microspheres using kinetic Monte Carlo simulations

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Abstract

To optimize and predict the release of proteins from biodegradable microspheres based on crosslinked dextran, a fundamental understanding of the mechanisms controlling their release is necessary. For that purpose, a mathematical model has been developed to describe the release of proteins from these hydrogel-based microspheres. A kinetic Monte Carlo scheme for the degradation of a small domain inside the microsphere was developed. The results from this were used in a second kinetic Monte Carlo scheme to model the diffusion and the subsequent release of proteins. The only processes included in this model are diffusion and degradation. The general effects of diffusion, crosslink density, protein loading, and clustering of proteins on the release were investigated. The model crosslink density (X_{model}) and the model diffusivity (D_{model}) were fitted to experimental release data of BSA monomer from hydroxyethyl methacrylated dextran (dex-HEMA) microspheres. By using the experimental release curves of liposomes and BSA monomer, it was found that *(1)* the model crosslink density (X_{model}) scales with the hydrodynamic diameter (d_h) as $d_h^{1.64}$ and *(2)* the diffusivity of the protein (D_{model}) scales approximately with $1/d_h$ (Stokes-Einstein). Using these scaling relations, quantitative predictions of the release curves of BSA dimer, immunoglobulin G and human growth hormone were possible. In conclusion, this model may play an important role in the optimization, understanding and prediction of the release of various proteins from degradable hydrogels.

1. Introduction

Biodegradable hydrogels in the form of macroscopic (in situ forming) systems and injectable microparticles are attractive systems for the controlled release of pharmaceutically active proteins[1-6]. It has been shown that hydrogels have a good compatibility with proteins and living tissue[7-11]. Moreover, a unique property of hydrogels is the possibility to tailor the release by changing the crosslink density[12-15].

Biodegradable dextran-based microspheres are prepared in an all-aqueous environment by crosslinking dextran derivatized with hydroxyethyl methacrylate (dex-HEMA) droplets in an emulsion with poly(ethylene glycol) (PEG)[14-16]. Under physiological conditions, the carbonate esters linking the poly-HEMA to the dextran are hydrolyzed, which results in an increased mesh size in the hydrogel network. The encapsulated protein will be released when the mesh size exceeds its hydrodynamic diameter. In previous studies it was shown that the release of entrapped colloidal particles (proteins, liposomes) from dex-HEMA microspheres could be tailored from days to months by varying the degree of substitution (DS, the number of hydroxyl ethyl methacrylates per 100 glucose units) of dextran and the initial water content of the microspheres[14-17].

To optimize the protein release characteristics of these systems, a fundamental understanding of the degradation mechanism, diffusion of the proteins through the matrix, as well as formulation parameters controlling the initial burst, the release rate, the lag time and the release time is required. The development of an appropriate model can be very helpful in the understanding and optimization of the release curves of these systems. Moreover, as experiments can be time- and labor consuming, computer simulations may provide an attractive alternative to deepen the understanding of the release process.

In dex-HEMA based systems, the protein is surrounded by crosslinked dextran. For this system, protein release is controlled by the opening of domains inside the microsphere as a result of hydrolysis of crosslinks. Many models have been developed to describe the release of drugs from various drug delivery systems[18-24]. Most related to dextran-based microspheres are the PLA-b-PEG-b-PLA gels developed by West et al.[25]. For these systems, Mason et al.[26] developed scaling laws that relate bulk-degradation to the changing transport properties of proteins during degradation. The predicted release curves agreed qualitatively with experimental observations. However, quantitative predictions were not possible, suggesting that not all important chemical and physical processes were taken into account properly.

Models describing the network changes at a microscopic level may result in better quantitative agreement between experiments and model. Most models describing polymer

degradation and release of proteins at microscopic level are based on Monte Carlo simulations[27-31]. An important advantage of this type of simulations is the possibility to obtain a stochastic description of the state of a small domain inside the microsphere. Moreover, the effects of diffusion and degradation can be studied separately.

Important progress was made in the understanding of the degradation and release from bioerodible systems with models based on Monte Carlo simulations. Following the pioneering work of Zygourakis[27-28], Göpferich[29-31] and co-worker constructed a model in which the erosion of polymer 'pixels' (i.e. small domains inside a microsphere) was considered as a random event. This erosion was combined with the diffusion of degradation products. Good agreement was obtained between this model and available experimental data. To investigate the drug release from bioerodible spherical particles, a new mathematical model was developed by Siepmann et al.[32], in which a stochastic process described local erosion. Release curves were obtained by solving Fick's second law numerically with a diffusivity related to local erosion. At present, no microscopic models are available describing the release of proteins by hydrolysis of crosslinks and diffusion of proteins in matrices based on dextran, PLA-b-PEG-b-PLA and other related systems[25].

The aim of this study is to describe the release of proteins of variable size from dex-HEMA microspheres with different crosslink densities. For that purpose, a numerical model based on Monte Carlo simulations was developed which accurately describes the diffusion and degradation processes inside the microspheres, while neglecting swelling effects of the microspheres. After validation of the model with experimental release data of bovine serum albumin (BSA) monomer from dex-HEMA microspheres, scaling laws for the model diffusivity and model crosslink density with the hydrodynamic diameter of the protein were obtained. This was used to predict release curves for different crosslink densities and encapsulated proteins (hGH, IgG) and liposomes, for which experimental release curves were taken from earlier work of our group[14,17,33]. The experimental observations and the computed release curves combined with confocal microscopy experiments were used to obtain a better understanding of the processes determining the release from dex-HEMA based microspheres.

2. Theory

2.1. Hydrolysis of crosslinks and opening of lattice sites

In the experimental situation, prior to polymerization, the protein molecules are dissolved in a dex-HEMA solution. After polymerization, these molecules are entrapped inside the network if the initial mesh size of the network is smaller than their hydrodynamic diameter (Fig. 1).

The microsphere is modeled as a cubic three-dimensional lattice. For simplicity reasons, the lattice is modeled as a cubic object instead of a spherical object, as diffusion inside a spherical object and a cubic one only differ by a constant geometric factor. Each lattice site represents a small domain of the microsphere containing a certain number of crosslinks (Fig. 1). At time *t=0* protein molecules are randomly distributed over the lattice sites. Two proteins can never occupy the same lattice site (excluded volume principle). No proteins are present at the surface lattice sites. Furthermore, when a protein molecule occupies a certain lattice site, no crosslinks are present at that site.

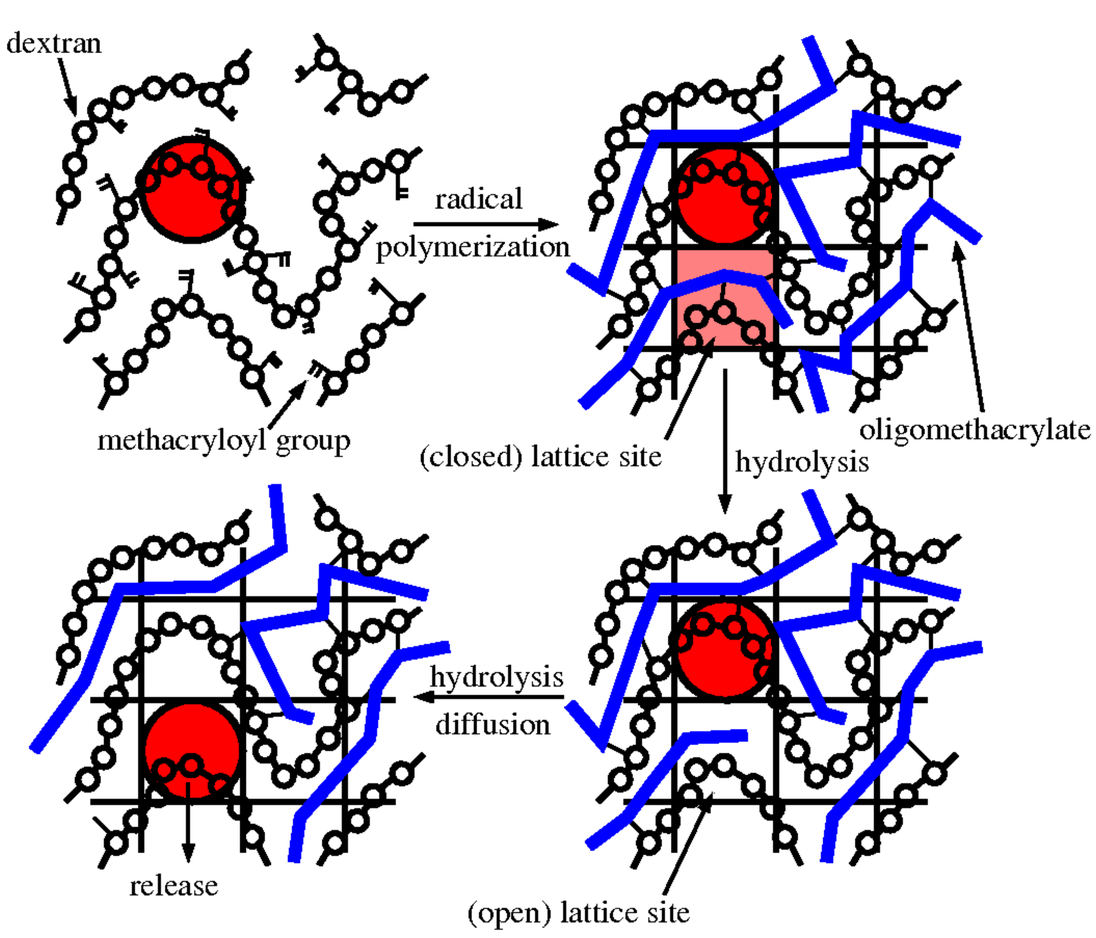


Figure 1. *Schematic two-dimensional representation of the confinement of a protein molecule and its subsequent release from dex-HEMA microspheres. Key features of the release are the opening of a neighboring lattice site through hydrolysis of crosslinks and the subsequent diffusion of a protein molecule to this lattice site.*

In the experiments, once a protein is entrapped inside the network (Fig. 1) the protein can only be released when *(1)* holes in the network are created through hydrolysis of the surrounding crosslinks, or, *(2)* swelling of the system creates a larger mesh size. Generally, swelling can occur directly after preparation (equilibrium swelling) and as a result of hydrolysis of crosslinks. It has been demonstrated before that after preparation, dex-HEMA microspheres immediately reach their

equilibrium swelling[34]. It is expected that the swelling as a result of hydrolysis of crosslinks is not contributing to the opening of lattice sites, as the remaining crosslinks will keep the proteins entrapped in the network. Therefore, swelling is neglected in this model.

In principle, the opening of a small domain inside the microsphere is determined by microscopic parameters such as the average distance between two crosslinks, the size and shape of the protein, and the topology of the crosslinked network. This information can be used to construct a geometric model based on the caging of a certain object[35,36]. However, the precise details of the microscopic parameters controlling this confinement are unknown. Therefore, in this model, a coarse-grained approach was used to model the opening of a small domain inside the microsphere (represented by a lattice site). In this approach, a single lattice site is modeled as N_{model} crosslinks arranged on a line (Fig. 2a). The lattice site is considered open when at least X_{model} neighboring crosslinks are hydrolyzed. Periodic boundaries are applied to identify neighboring crosslinks near the edges.

The opening of lattice sites around a protein by hydrolysis of crosslinks is related to two experimental parameters: *(1)* the experimental crosslink density and *(2)* the hydrodynamic diameter (d_h) of the protein. The actual crosslink density is determined by the degree of HEMA substitution (DS) of dextran and the water content of the microspheres. For larger proteins *or* higher crosslink densities, more crosslinks need to be hydrolyzed to open a lattice site. This means that when N_{model} is fixed, X_{model} increases with protein size and crosslink density of the gel. It is important to note that different combinations of the protein size and actual crosslink density can in principle result in similar release curves. This implies that the experimental situation can be described by a model crosslink density X_{model} which depends on *both* the hydrodynamic diameter *and* the degree of HEMA substitution (DS), while keeping the maximum number of modeled crosslinks (N_{model}) fixed. Keeping N_{model} fixed for all simulations is allowed, as variations in the actual crosslink density (which in turn at fixed water content is proportional to the degree of HEMA substitution) or hydrodynamic diameter (d_h) are already accounted for in X_{model}. In that situation, it is assumed that X_{model} is directly proportional to the actual crosslink density (DS). The precise interplay between X_{model} and d_h will be discussed in section 4.3.

Generally, the hydrolysis of crosslinks can be considered as independent events with rate constant k. Therefore, the degradation of the polymer network can be described by a Poisson process. The time evolution of a Poisson process can be followed by using the conventional kinetic Monte Carlo (KMC) scheme (see section 3.1.1).

2.2. Protein diffusion inside a microsphere

At *t=0* protein molecules are positioned at lattice sites. To model diffusion, it is assumed that a protein molecule can jump to a neighboring lattice site provided that it is "open", i.e. X_{model} cross-links are hydrolyzed so that the protein can enter this new lattice site (see section 2.1). Proteins jump to neighboring lattice sites with a certain rate: D_{model}. Attempted jumps to a "closed" lattice site are rejected, e.g. the protein returns to its original position. Proteins that leave the cubic three-dimensional lattice are considered as released. In this model it is assumed that jumps of protein molecules to neighboring lattice sites are uncorrelated. It is also assumed that hydrolysis of crosslinks and attempted jumps to neighboring lattice sites are uncorrelated processes as well. This means that it is possible to determine which lattice sites are open at time *t* before the start of the simulation.

3. Materials and methods

3.1 Modeling section

The modeling of the release of proteins from dex-HEMA based microspheres is split into two parts. The first part consists of modeling the hydrolysis of crosslinks and opening of lattice sites. This will provide a release curve for the opening of a single lattice site. This curve is used in the second part of the model, which describes the diffusion of proteins in the degrading polymer matrix and their subsequent release.

3.1.1. Hydrolysis of crosslinks and opening of lattice sites

Assuming that the average size of a microsphere is 10 μm and protein size is 10 nm (e.g. IgG, Table 1), a minimum lattice size of 1000x1000x1000 would be necessary to model each crosslink as a single lattice site. Simulations for these lattice dimensions are extremely time consuming. Therefore, the simulations were performed for a 50x50x50 lattice in which each lattice site represents either a protein or a certain number of crosslinks. Lower lattice dimensions will result in insufficient resolution. Simulations of slightly larger lattice dimensions do not change our results significantly. Therefore, the use of a 50x50x50 lattice is a compromise between resolution (large lattices) and computational resources (small lattices).

Each lattice site represents a certain number of crosslinks (here: N_{model}=2000), which is fixed as discussed in section 2.1.1. To open a lattice site, X_{model} out of N_{model} crosslinks have to be hydrolyzed, see Fig 2. The value of N_{model}=2000 is chosen in such a way that *(1)* the simulations are

able to describe a wide range of protein sizes and DS's, and *(2)* for the largest particle in this study, $X_{model}=N_{model}$. In this paragraph, the kinetic Monte Carlo scheme is described (see also Fig. 2b). In general, in the KMC algorithm the following steps are executed:

1. Set the time τ at $\tau=0$.
2. Construct a list of possible events (i.e. list of crosslinks that can be hydrolyzed). Suppose that there are K events with rate constants $r_1, r_2, r_3, \ldots r_K$ (in units of events per unit of time).
3. Select one of these events (l) with a probability

$$p_l = \frac{r_l}{\sum_{j=1}^{K} r_j} \tag{1}$$

4. Execute this particular event and advance the time for the next event (i+1) by

$$\tau_{i+1} = \tau_i + \frac{|\ln(u)|}{\sum_{j=1}^{K} r_j} \tag{2}$$

in which u is a uniformly distributed random number between 0 and 1.

Check whether X_{model} neighboring crosslinks are broken in such a way that the lattice site is open (Fig. 2a). If this is the case, record the time τ, otherwise return to step 2.

Repeat this process M time. Reasonable statistics were obtained with $M=10^6$.

7. Construct the cumulative distribution of τ by computing

$$P(t) = \frac{\sum_{i=1}^{M} \theta(t-\tau_i)}{M} \tag{3}$$

in which $\theta(t)$ is the usual Heaviside step function ($\theta(t)=1$ for $t>0$ and $\theta(t)=0$ otherwise). Essentially, $P(t)$ is the probability that X_{model} neighboring crosslinks out of N_{model} are hydrolyzed at time t. As τ can be written as a sum of exponentially distributed random variables, it is expected that the time derivative of $P(t)$ is Gaussian[37,38].

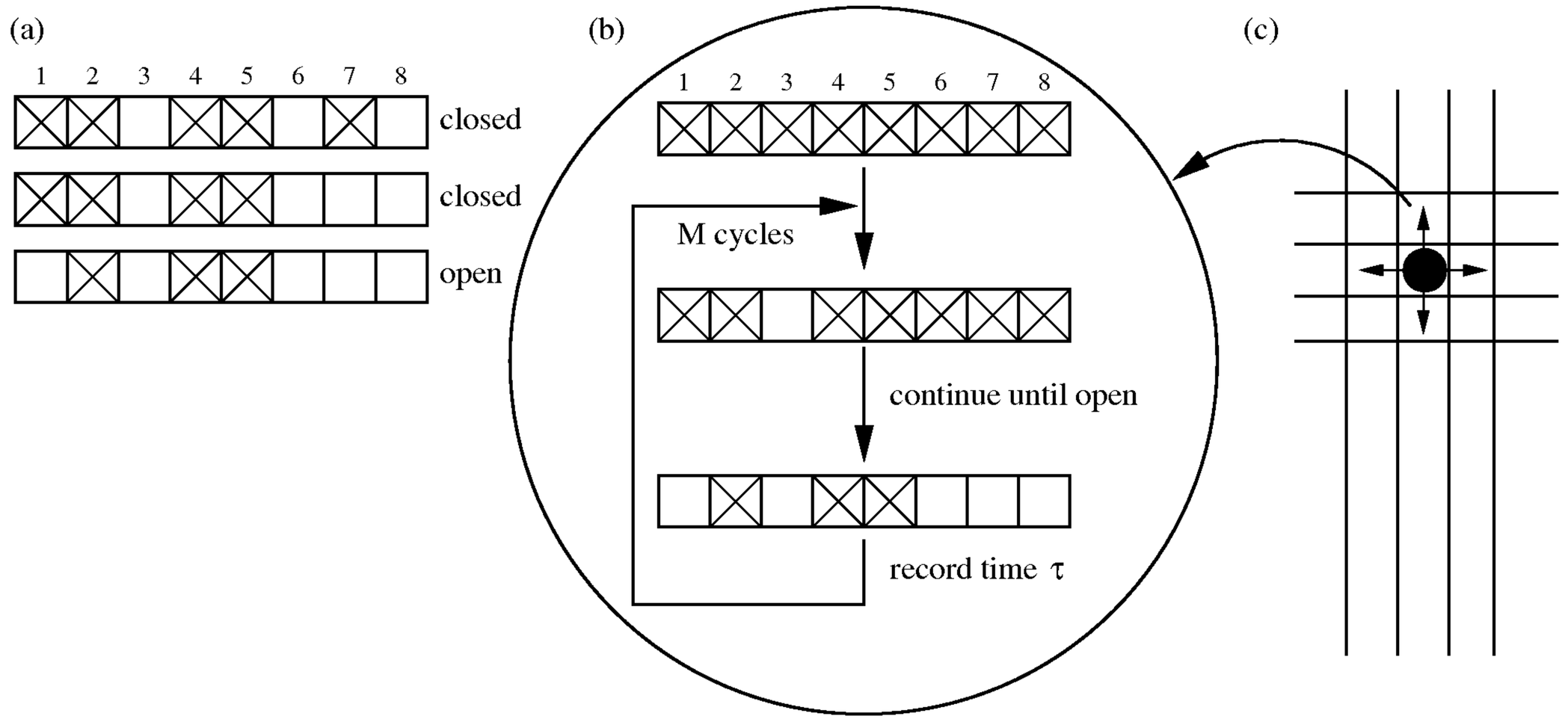


Figure 2. *Schematic representation of the model used in the simulations. (a) Example for the opening of a single lattice site with N_{model}=8 and X_{model}=4. The lattice site is considered open if four neighboring crosslinks are hydrolyzed. Periodic boundary conditions are applied. Intact crosslinks are indicated with a cross; hydrolyzed crosslinks are indicated with empty squares. (b) Schematic representation of the kinetic Monte Carlo scheme to compute P(t) for a single lattice site, see also Eq. 3. (N_{model}=8, X_{model}=4). (c) Diffusion of a protein in the degrading matrix. A protein can jump to a neighboring lattice site (jump rate: Dmodel) provided that it is open. The time τ_i at which lattice site i is open is computed by solving $P(\tau_i)$=u, in which u is a uniformly distributed random number between 0 and 1. This is done for each lattice site at the start of the simulation.*

3.1.2. Protein diffusion inside a microsphere

At t=0, P protein molecules are distributed randomly over the lattice. Each lattice site can be either open or closed at a certain time t with a probability given by Eq. 3. A lattice site occupied with a protein molecule does not contain crosslinks. The time τ_i at which lattice site i is opened can be calculated by solving $P(\tau_i)=u$, in which u is a uniformly distributed random number between 0 and 1. This equation was solved for each lattice site at the beginning of the simulation. The function $P(t)$ is uniquely determined by X_{model} and N_{model} and was computed using the method outlined in the previous section.

After the start of the simulation, protein molecules (or other entrapped colloidal particles such as liposomes) can diffuse from lattice site to lattice site with diffusivity D_{model} (here defined as a jump rate, in units of particle jumps per unit of time) using the same KMC scheme as described in the previous section. Jumps to new lattice sites are accepted when the new lattice site is open *and* not occupied by another protein molecule, see Fig. 2c. Proteins that diffuse out of the lattice are marked as released. The simulation stops when all proteins have been released from the cubic lattice. The approximate CPU time for a typical simulation of protein release takes less than 5 minutes. Clusters of proteins were modeled as a group of monomeric proteins, in which each protein occupies a lattice site. For example, a cluster of 10x10x10 proteins means a group of 1000

protein molecules occupying 1000 neighboring lattice sites. Once sufficient crosslinks are hydrolyzed, the protein molecules can move independently from each other to other open lattice sites. A cluster is not an aggregate, as the proteins are neither physically, nor chemically linked.

3.1.3. Comparison with in vitro release experiments

To compare the computed release curves with previously reported experimental release curves of BSA (see section 3.2), IgG[14], hGH[33] and liposomes[17], the time in the simulations was converted to days using the hydrolysis rate constant k of dex-HEMA in solution (Table 1). The release curves for BSA monomer, BSA dimer, hGH and the liposomes were normalized (maximum release was set at 1). This was not possible for the curves of IgG as complete release was not obtained, and therefore the release curves were used as reported in ref. 14.

For the simulations, the protein loading of the microspheres, in the experiments given in weight of protein/weight of dex-HEMA, was converted into protein volume/microsphere volume using an average microsphere size of 10 μm, a water content of 50%, the density of dextran (1.61 g/cm^3)[40] and the hydrodynamic diameters (d_h) and the molecular weights of the proteins (Table 1). The loading of microspheres with liposomes was arbitrarily set at 10% (volume of liposomes/volume of microsphere).

The relation between d_h and X_{model} and D_{model} respectively was determined in the following way. Initially, the experimental release curves of BSA monomer were fitted to the computed release curves to obtain the model crosslink density (X_{model}) and the model diffusivity (D_{model}) for BSA monomer. The experimental release curves of BSA dimer and liposomes were used to investigate the scaling of X_{model} and D_{model} with the hydrodynamic diameter (d_h) of the protein. Using these scaling relations, X_{model} and D_{model} were calculated for IgG, hGH and liposomes (see Table 1) and their release curves were computed. Confocal microscopy analysis of fluorescently labeled protein in some systems indicated the presence of protein clusters. The size of these clusters is of the order of the size of a microsphere. For these systems, the release curves were computed assuming that the proteins were initially arranged in clusters of similar size (10x10x10 compared to 50x50x50 for the whole lattice).

Table 1. *Overview of parameters used in experiments and simulations.*

Protein	Experiments				Simulations		
	d_h[a] (nm)	DS	pH	Hydrolysis rate constant[b] (s^{-1})	*P* (% v/v)	D_{model}	X_{model}
BSA							
- monomer (Mw= 67 kDa)	7	12	7.2	$3.24 \cdot 10^{-6}$	8	$1.0 \cdot 10^{-3}$	15
- dimer	14	12				$5.3 \cdot 10^{-4}$	43
IgG[14] (Mw= 150 kDa)	11	3	7.0	$2.06 \cdot 10^{-6}$	6	$6.6 \cdot 10^{-4}$	9
		6					15
		8					20
		12					29
hGH[33] (Mw= 22 kDa)	4	16	7.4	$5.09 \cdot 10^{-6}$	5	$1.8 \cdot 10^{-3}$	8
Liposomes[6]	180	8	7.2	$3.24 \cdot 10^{-6}$	10	$4.0 \cdot 10^{-5}$	2000

**[1] For IgG and BSA monomer d_h was taken from ref. 41. For hGH and BSA dimer d_h was derived using GPC data of refs.33, 34.*

**[2] The loading of microspheres with liposomes was not given in ref. 6. Arbitrarily, a loading of 10% was assumed. In principle, any loading is justified since at low diffusivity the loading does not affect the release curve.*

**[3] The rate constants were derived from the pH dependence of the hydrolysis rate constant k as previously reported by Van Dijk-Wolthuis[40].*

3.2. Experimental section

3.2.1. Materials

Poly(ethylene glycol) (PEG) 10,000 g/mol and potassium peroxodisulfate (KPS) were obtained from Merck (Darmstadt, Germany). Dextran 40.000, *N,N,N',N'*-tetramethyl ethylene diamine (TEMED), fluorescein isothiocyanate-bovine serum albumin (FITC-BSA) were purchased from Fluka (Buchs, Switzerland). Rhodamine B isothiocyanate was obtained from Aldrich (Milwaukee, USA). Bovine immunoglobulin G (IgG, fraction II) was purchased from ICN Biomedicals (Zoetermeer, the Netherlands). Hydroxyethyl methacrylate derivatized dextrans (dex-HEMA) with a degree of HEMA substitution 8 (8 HEMA groups per 100 glucose units) were synthesized according van Dijk-Wolthuis et al.[41] and obtained from Polymer Service Centre Groningen (PSCG, Groningen, the Netherlands).

3.2.2. Preparation of rhodamine labeled IgG

A solution of 50 mg/ml IgG in phosphate buffer (10 mM) was incubated with rhodamine (ratio 1:5 mol/mol) for 1 day at room temperature under stirring. The IgG was separated from the rhodamine by diafiltration over 10 kDa filters.

3.2.3. Preparation of microspheres

Dextran microspheres were prepared by a water-in-water emulsion technique[16]. First, a dex-HEMA solution containing protein was prepared. This solution was obtained by dissolving 61 mg dex-HEMA in 488 mg buffer and 61 µl of a solution of 50 mg/ml rhodamine labeled IgG or in 427 mg buffer and 120 µl of a solution of 50 mg/ml FITC-labeled BSA or BSA. Second, 610 mg of dex-HEMA/protein solution was added to a PEG solution (3.75 g, 35% w/w). All solutions were prepared in 10 mM phosphate buffered saline pH 7.0. An emulsion was obtained by vortexing this mixture in a 15 ml tube (1 minute, 14000 min^{-1}). Then, 100 µl TEMED solution (20% v/v, pH neutralized with 4 M hydrochloric acid) was added. The emulsion was vortexed for another minute, and 180 µl of 50 mg/ml KPS-solution was added and polymerization was allowed to proceed for 60 minutes at room temperature, without stirring. Multiple centrifugation and washing steps were performed to purify the crosslinked dextran particles.

3.2.4. Confocal laser scanning microscopy

The microspheres were mixed with FluorSave (Calbiochem, San Diego, CA, USA) before visualization. Confocal microscopy analysis was performed with a Leica TCS-SP confocal laser scanning microscope equipped with a 488 nm Argon laser used to detect FITC labeled BSA, 568 nm Krypton laser used to detect rhodamine labeled IgG. Laser power and photomultiplier settings were kept identical for all the samples.

3.2.5. In vitro release experiments BSA

The BSA loaded microspheres were resuspended in 5 ml 25 mM phosphate buffer pH=7.0. Periodically, the microspheres were centrifuged for 7 min at 3200x g, 3 ml supernatant was removed and replaced by 3 ml 25 mM phosphate buffer pH=7.4. The BSA monomer and dimer concentration was determined using size exclusion chromatography (SEC)[42].

4. Results and Discussion

4.1. Hydrolysis of crosslinks and opening of lattice sites

In Fig. 3 the distributions *P(t)* for different X_{model} (model crosslink density; number of neighboring crosslinks that need to be broken to open a lattice site) are plotted. As expected, as X_{model} increases, it will take on average more time to open a lattice site. All curves have an S-shape. The time derivative of *P(t)* is the probability distribution of the release time τ. The release time τ was nearly Gaussian distributed in this model. This is as expected, since τ equals the sum of exponentially distributed random variables (Erlang distribution).

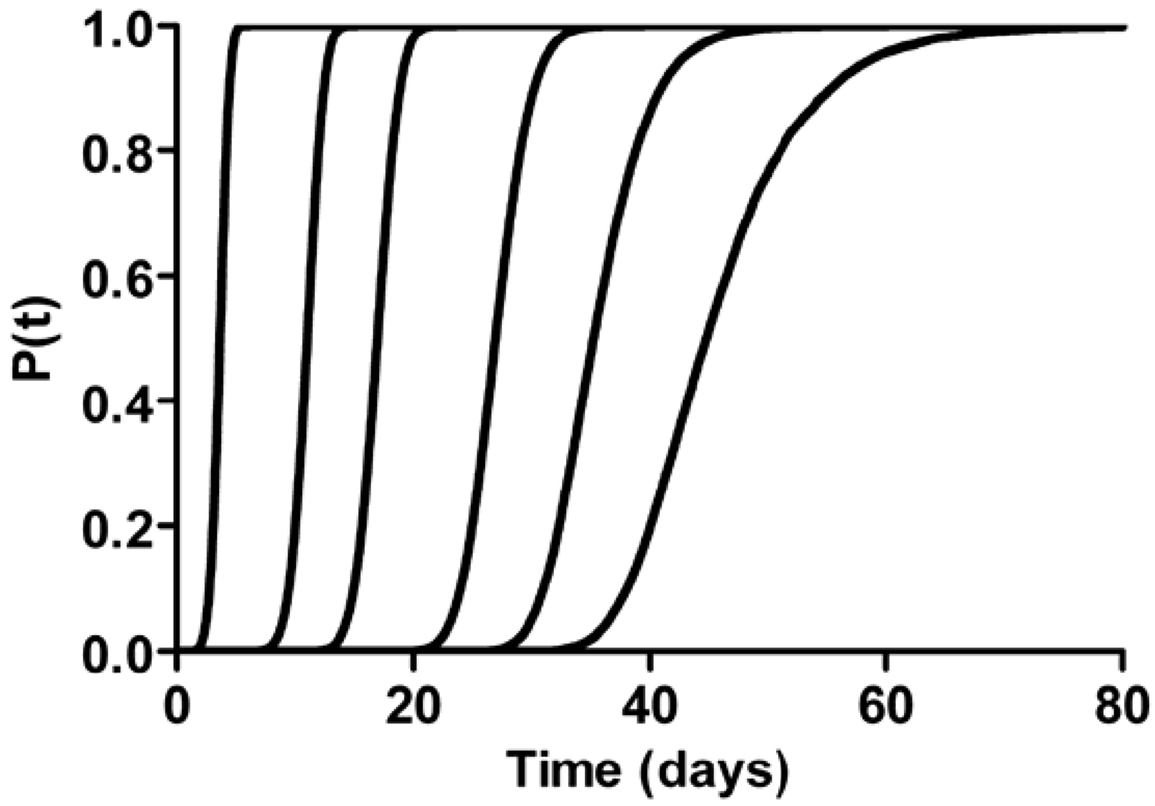


Figure 3. *Probability P(t) that a lattice site is open at time t for N_{model}=2000 and different model crosslink densities (X_{model}) as a function of time. Curves from left to right are for X_{model}=10, 40, 100, 400, 1100 and 2000. To convert the time in units of 1/k to time in days, a hydrolysis rate constant of $2.06 \cdot 10^{-6}$ s^{-1} was used (Table 1).*

4.2. Modeling release

4.2.1. Diffusion

The effect of diffusivity on the computed release curves was investigated. Fig. 4 shows the computed cumulative release curves for a variable D_{model}, at a fixed protein loading of 10% and model crosslink density X_{model}=10. For the whole range of tested model diffusivities, a biphasic release curve was observed. During the first phase, no protein was released. The duration of this phase, which is called the lag time, was almost independent of diffusivity (see Fig. 4b, lag time is around 4 days). In the second phase, the protein was released with a diffusion dependent release rate. During the first phase, crosslinks are hydrolyzed and some lattice sites are opened. However, not enough neighboring lattice sites are opened to release the protein from the matrix. In the second

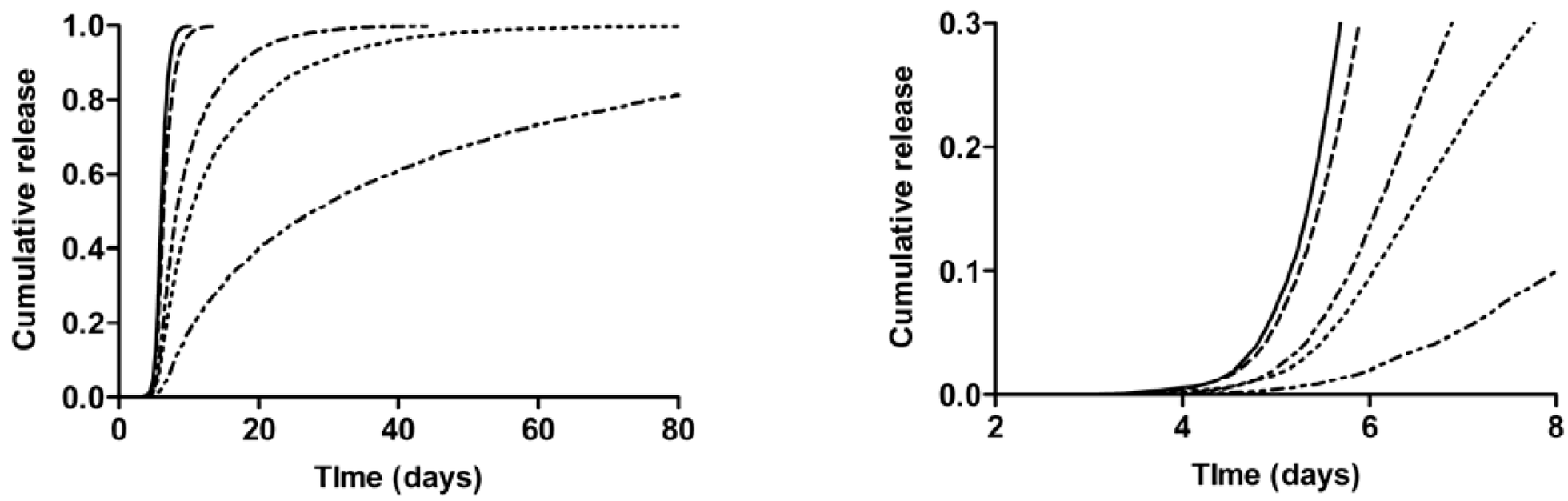
phase, when sufficient neighboring lattice sites are opened, protein molecules can jump from one lattice site to another and can consequently be released from the matrix.

Fig. 4 also clearly shows that the release kinetics after the lag time strongly depended on D_{model}. When D_{model} is increased from 10^{-4} to 10^{-2}, the release rate increases and the total release time (time to release all protein molecules) decreases. Assuming that the diffusivity of a protein is related to its size (Stokes-Einstein equation), for large proteins diffusion becomes the rate determining process, resulting in a substantially decreased release rate and also longer total release time. Note that for larger proteins the lag time will also increase because X_{model} increases.

4.2.2 Model crosslink density, protein loading and clusters

The effect of X_{model} on the release of proteins with different sizes, and thus different D_{model}, from the microspheres is shown in Fig. 5. In these simulations, again biphasic release curves were found. In this figure it is shown that the lag time of the first phase depends on X_{model} (~ 4 and 7 days for X_{model}=16 and 30, respectively). The release rate in the second phase was nearly independent of X_{model}, but strongly dependent on D_{model} (see also Fig. 4).

The effect of protein loading is also shown in Fig. 5. At higher protein loading the lag time is slightly shorter (~0.5 day shorter, comparing X_{model}=16 and 30 at D_{model}=$1 \cdot 10^{-2}$). If the protein molecules are initially homogeneously distributed over the lattice, more protein molecules are present close to the surface of the microspheres at higher protein loading. Since less lattice sites need to be opened to release proteins close to the surface, the release from microspheres with a higher loading starts consequently earlier. This effect becomes less pronounced at smaller D_{model} (larger proteins).



(a) *(b)*

Figure 4. *(a) Computed release curves of proteins from microspheres as function of time for a range of D_{model}. From left to right: D_{model}=$5 \cdot 10^{-1}$; $1 \cdot 10^{-1}$; $1 \cdot 10^{-2}$; $1 \cdot 10^{-3}$; $1 \cdot 10^{-4}$ (Note: loading= 10%, X_{model}=10, protein molecules are homogeneously distributed over the lattice, hydrolysis rate constant=$2.06 \cdot 10^{-6}$ s^{-1}). (b) Release during the first 8 days.*

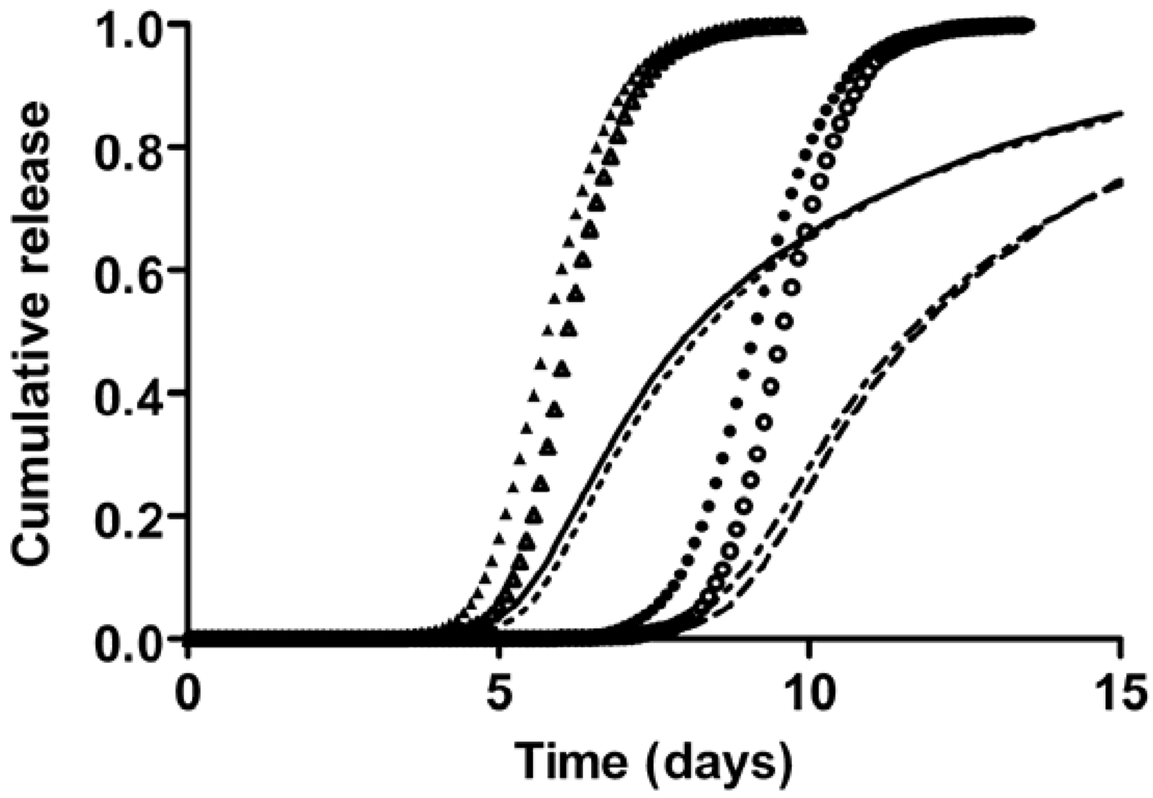


Figure 5. *Computed release curves of proteins from microspheres as function of time.* X_{model}*=16 (▲,Δ or ---, —), 30 (●,○ or − - − , − −), loading 25% (closed symbols, — or − - −) and 4% (open symbols, --- or − −),* D_{model}*=1·10*$^{-2}$ *(symbols) and 1·10*$^{-3}$ *(lines) (hydrolysis rate constant=2.06·10*$^{-6}$ s^{-1}*).*

Tuinier et al.[43] showed that phase separation of lysozyme in a dextran solution occurred at low dextran concentrations (<15% w/w) and low lysozyme concentrations. Likely, phase separation will also occur for other proteins in the highly concentrated dex-HEMA phase (50% w/w). As a result, after polymerization, clusters of protein are imprisoned in the network of the microspheres. With the currently developed model, the effect of clusters of proteins on the release curves was marginal. After the lag time (which was almost independent of the presence of clusters) the release rate is slightly higher for proteins homogeneously distributed over the microspheres than for proteins originally present in clusters (data not shown). This can be explained by the restricted mobility of proteins inside a cluster. Before proteins inside a cluster are able to move, first proteins at a neighboring lattice sites should have jumped at other (unoccupied) lattice sites. Only for large X_{model} and at high loading the effect of clusters becomes important. At X_{model}=30, D_{model}=10^{-3} and loading=25% v/v, the release curve of proteins from microspheres with clusters was shifted and the time to release 50% of the entrapped protein was increased from 8 to 9.5 days. It is important to note that of the effect of X_{model} on the release is much larger than the effects of loading and clusters.

4.3 Validation and prediction

As no length scales were introduced for the lattice or a single lattice site, the model crosslink density (X_{model}) and the model diffusivity (D_{model}) can not be converted directly into experimental diffusion and crosslink densities. Therefore, to compare the experimental release curves with the simulations, the experimental crosslink density should be translated into the model crosslink density (X_{model}). Moreover, a D_{model} should be fitted, representing the diffusion of proteins

inside a microsphere. The release of BSA monomer obtained from dex-HEMA microspheres (DS 12) was fitted with the computed release curves. Fig. 6a shows a confocal microscopy image of microspheres loaded with FITC-BSA. This suggests that BSA is homogeneously distributed over the microspheres and in the simulations it was therefore assumed that the BSA molecules were homogeneously distributed over the lattice. The best fit was obtained using X_{model}=15 and D_{model}=1.0·10^{-3} (Fig. 7). The simulation is in good agreement with experimental data.

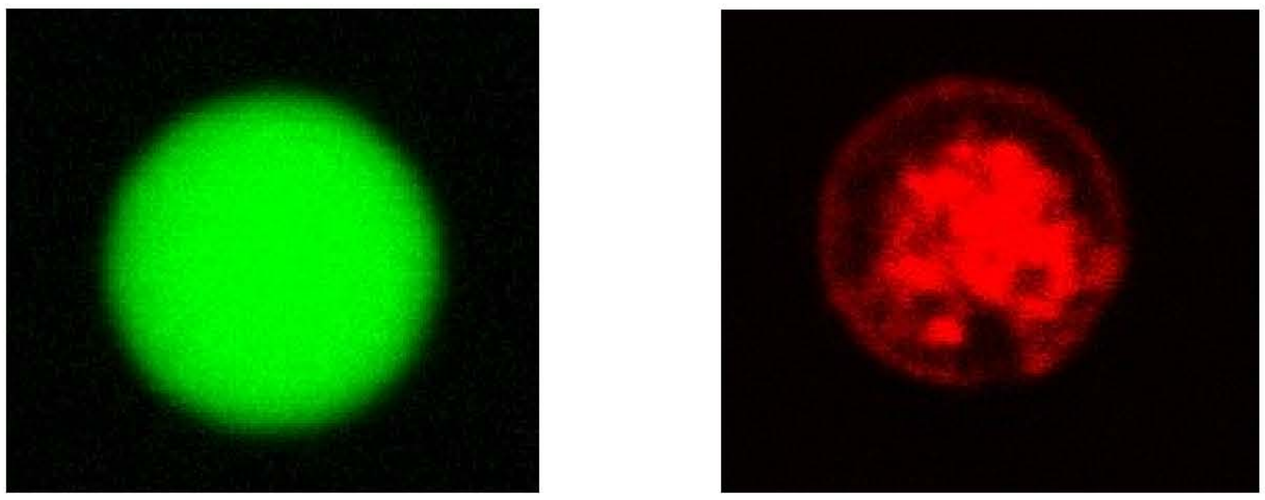


Figure 6. *(a) FITC-BSA and (b) rhodamine labeled IgG encapsulated in dex-HEMA microspheres (DS 12, water content 50%).*

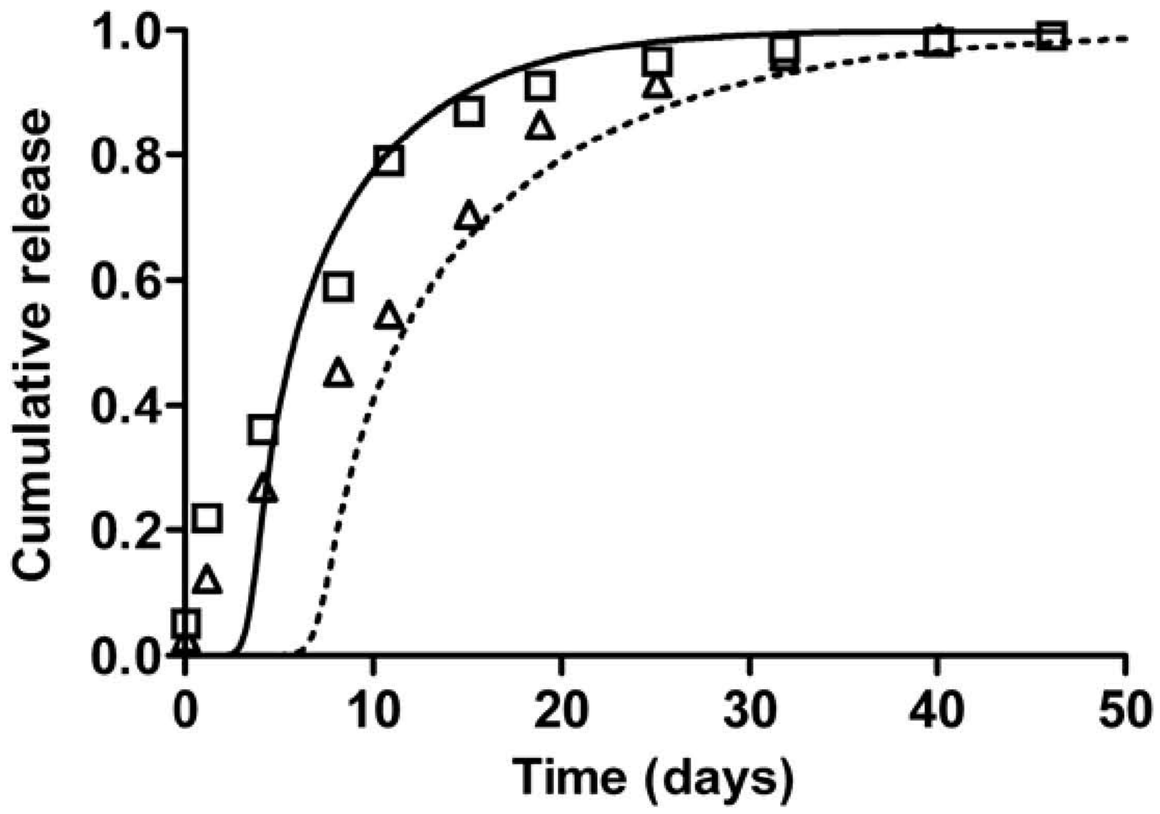


Figure 7*. Cumulative release of BSA monomer (□) and dimer (Δ) from dex-HEMA microspheres (DS 12, water content of 50%) as a function of time. The computed release curves are plotted as lines.*

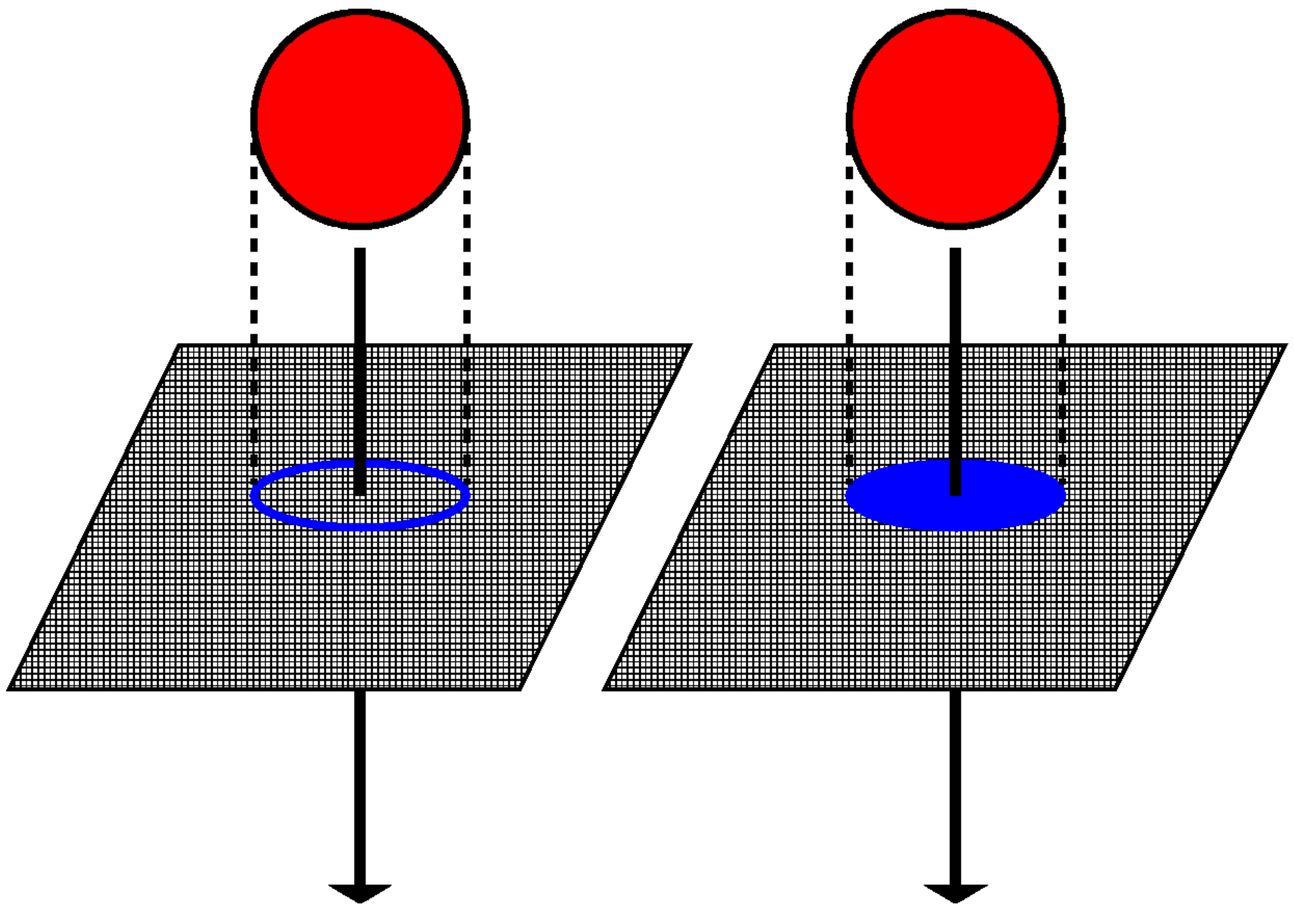


Figure 8. *Schematic representation of the movement of a protein through a plane of crosslinks. There are two extreme situations. Left: Crosslinks are hydrolyzed at the perimeter of the projection of the protein onto the plane. Right: Crosslinks at the projection of the protein are hydrolyzed.*

The obtained X_{model} and D_{model} for BSA monomer (Table 1), and the experimental release curves of BSA dimer and liposomes were used to investigate the scaling of X_{model} and D_{model} with the hydrodynamic diameter (d_h). To compute the release curve of liposomes from the microspheres, it was assumed that D_{model} is proportional to $1/d_h$ (Stokes-Einstein equation), in which d_h is the hydrodynamic diameter of the liposome. Furthermore, with increasing protein (or liposome) size X_{model} increases since for a larger protein (or liposome) more crosslinks need to be hydrolyzed to open a lattice site.

However, the precise relation between X_{model} and d_h is not a priori clear. There are two extreme situations that result in opening of the lattice site and release of the protein (or liposome) (Fig. 8). In the first situation, a lattice site is opened when all crosslinks at the perimeter of the projection of the protein on the plane are hydrolyzed. This means that X_{model} is proportional to d_h. In the second situation, a lattice site is open when all crosslinks at the surface of the projection of the protein are hydrolyzed. In that particular case, X_{model} scales with d_h^2. The real situation is expected to be an intermediate between these two extreme situations, as crosslinks are hydrolyzed randomly and not exclusively on the perimeter of the projected protein. Moreover, not all crosslinks on the projected area of the protein need to be hydrolyzed to open a lattice site. Therefore, it is expected that X_{model} scales with d_h^{α}, where $1<\alpha<2$.

In Fig. 9, the experimental and computed release curves of liposomes from dex-HEMA microspheres are shown. Best agreement between experimental data and simulations was obtained by using X_{model}=2000 and D_{model}=$2.0 \cdot 10^{-5}$ and α=1.64. Note that for liposomes X_{model} equals N_{model},

which means that liposomes can only jump to neighboring lattice sites when all model crosslinks are hydrolyzed (Fig. 2a). The Stokes-Einstein relation predicts a somewhat higher diffusivity (D_{model}=4.0·10^{-5}, Table 1). This may indicate that in the degrading hydrogel matrix the mobility of liposomes is more restricted than that of BSA.

Based on the values for X_{model} and D_{model} obtained for BSA monomer as well as α (obtained from the release of the liposomes), X_{model} and D_{model} were calculated for BSA dimer, IgG and hGH released from dex-HEMA based microspheres using the scaling rules for crosslink density (X_{model}~$d_h^{1.64}$) and diffusivity (D_{model} ~1/d_h) as discussed in the previous paragraph. In Figs. 7, 10 and 11 the experimental data are compared with the simulations.

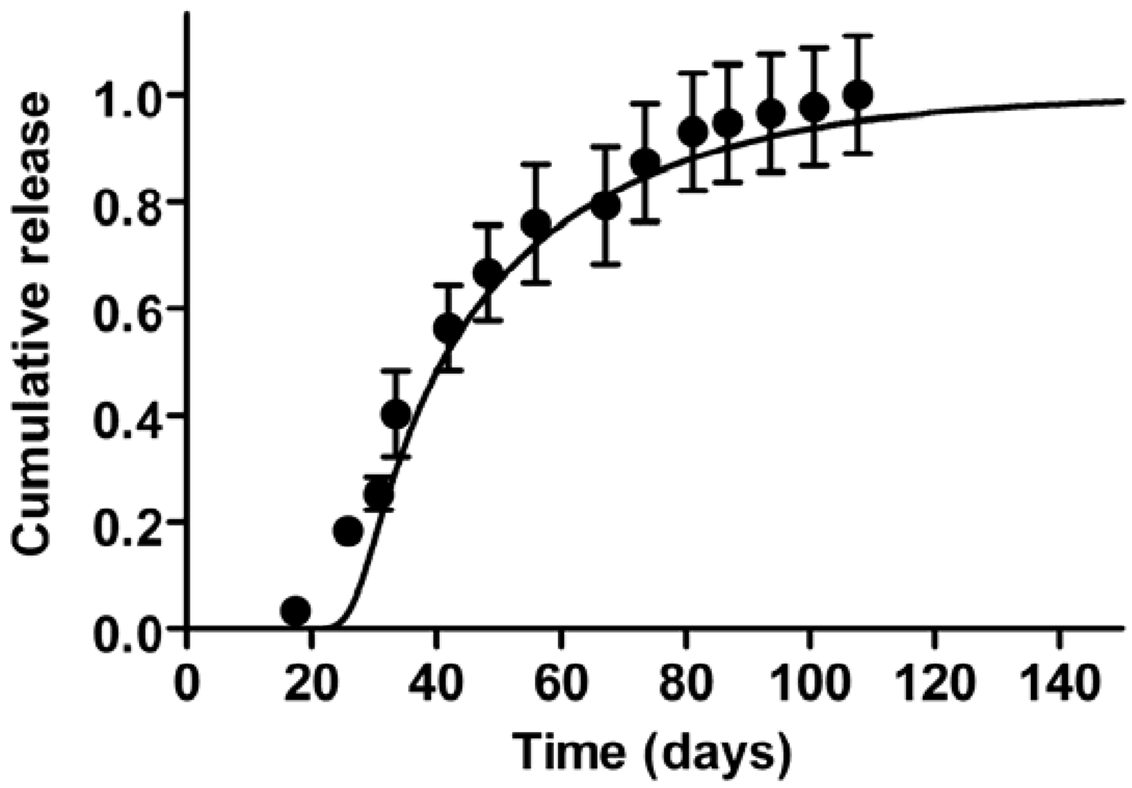


Figure 9. *Cumulative release of liposomes from dex-HEMA microspheres (DS 8 (●), water content 50%) as a function of time obtained previously by Stenekes et al.[17]. The computed release curve is plotted as a solid line.*

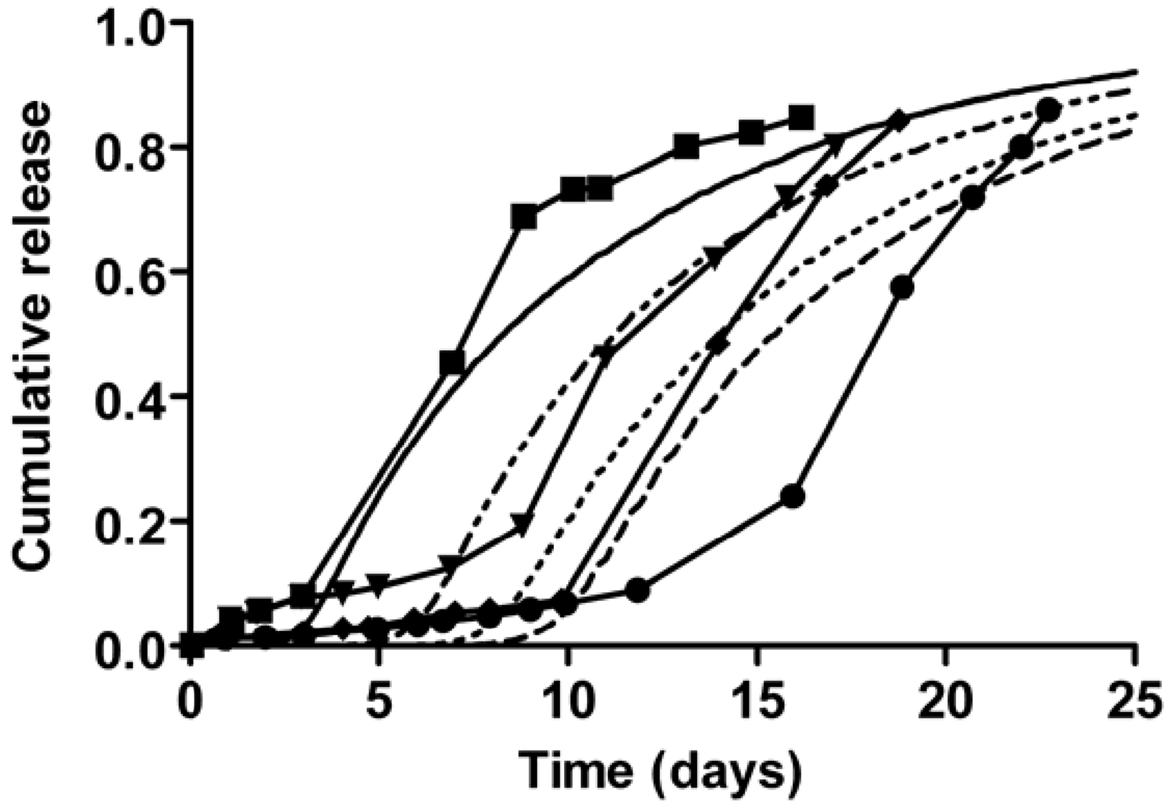


Figure 10. *Cumulative release of IgG from microspheres of DS 3(■), DS 6 (▼), DS 8(♦) and DS 11(●) (water content 50%) as a function of time. Experimental data were obtained previously by Franssen et al.[14]. The computed release curves are plotted as lines. It was assumed that the proteins were present in clusters occupying 1000 lattice sites.*

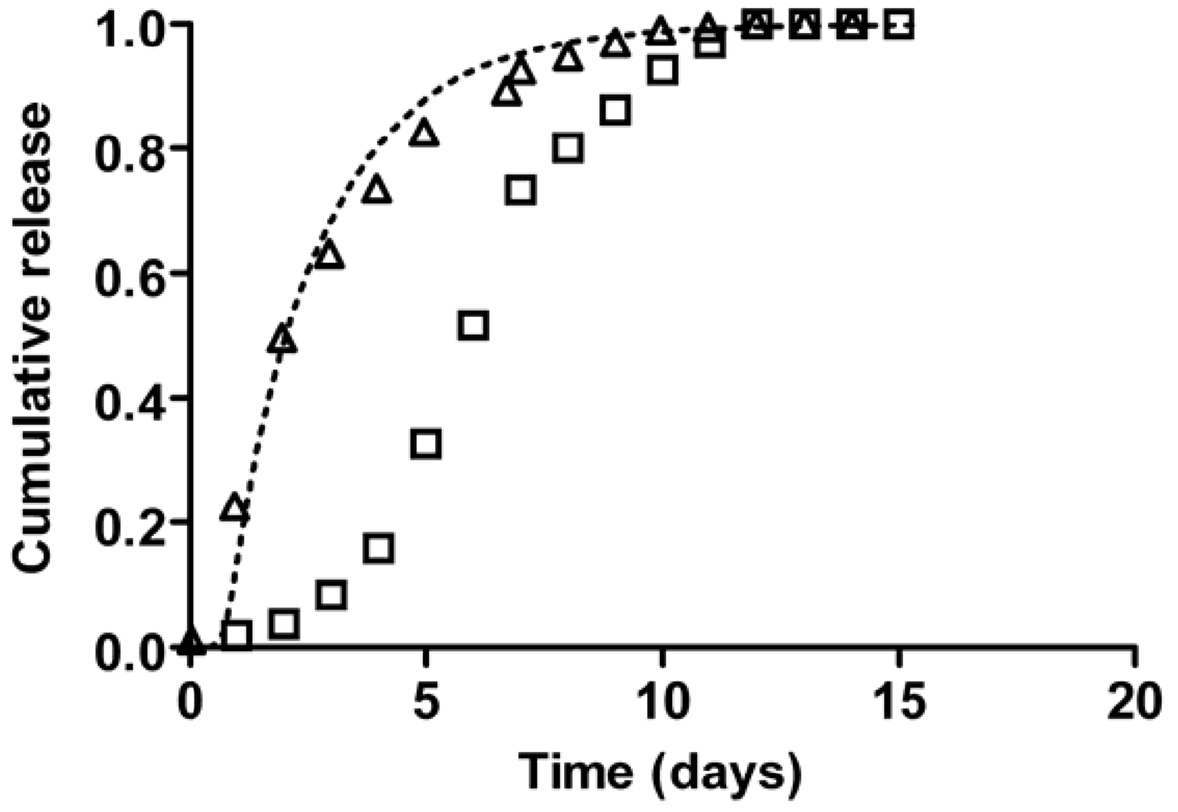


Figure 11. *Cumulative release of hGH as a function of time from microspheres of DS 16 and water content 50% prepared in the absence (□), and presence (Δ) of 0.1% tween 80. Experimental data were obtained by Vlugt-Wensink et al.*[33]*. The computed release curve is plotted as a line. In these simulations proteins were present in clusters of 1000.*

By applying the scaling relations, for BSA dimer X_{model}=43 and D_{model}=5.3·10^{-4} was predicted. Good agreement between experimental and computed release curve was obtained particularly for the last period of the release curve. Fig. 10 shows the computed and experimental release curves for IgG loaded microspheres. Confocal microscopy revealed that IgG is present in clusters of proteins inside the microsphere (Fig. 6b). Therefore, in the simulations of these systems the proteins were initially arranged in clusters. Good agreement was obtained between experimental and computed release curves, especially for the release of IgG from microspheres of DS 3 and 6 (X_{model}=9 and 15 respectively, D_{model}=6.6·10^{-4}). Differences in the lag time (but not in release rate) between experiments and simulations were found for DS 8 and DS 11(X_{model}=20 and 29 respectively, D_{model}=6.6·10^{-4}) indicating that other processes than degradation and diffusion affect the release here. A possible explanation is that IgG is present as precipitates in the microspheres. A longer lag time can be expected when dissolution of IgG from the precipitates is rate-limiting.

Fig. 11 shows the experimental release curves for hGH from microspheres prepared in the presence and absence of 0.1% tween 80. In the release curve of hGH a lag time of 2 days is observed, where in the release curve of hGH with tween 80 no lag time was present. Moreover, the release *rate* of the hGH is quite similar. Good agreement was found between the experimentally obtained release curve of hGH from microspheres prepared with 0.1% tween 80 and the computed release curve (X_{model}=16, D_{model}=1.8·10^{-3}). A possible explanation is that hGH without tween 80 is aggregated in the microspheres. Since aggregates are larger than monomers, a longer lag time and a lower diffusivity were expected. The difference in diffusivity for hGH monomer and dimer is relatively small and might not be observed in in vitro release experiments. In fact, experiments

presented in ref. 33 showed that when microspheres were prepared without tween, large precipitates of hGH were observed. Therefore, the release of hGH does not only depend on hydrolysis and diffusion (processes included in this model), but probably also on the dissolution rate of these precipitates of hGH in these microspheres.

5. Conclusions

A model was developed to investigate the release of proteins from dex-HEMA based microspheres. The effects of diffusion, crosslink density, protein loading, and clustering of proteins on the release were investigated. It was found that of the effect of X_{model} on the release is much larger than the effects of loading and clusters. It was shown that proper prediction of release curves is possible for various proteins and for microspheres of various crosslink densities (here: degree of substitution, DS) by scaling X_{model} with the hydrodynamic diameter $d_h^{1.64}$ and D_{model} with $1/d_h$ (Stokes-Einstein relation). As experimental determination of release kinetics can be time- and labor consuming, this model may play an important role in the optimization, understanding and prediction of the release of various proteins from crosslinked hydrogel based microspheres.

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Chapter 4

Effect of polymerization conditions on the network properties of dex-HEMA microspheres and macrogels

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Abstract

Dextran-hydroxy-ethyl-methacrylate (dex-HEMA) hydrogels in the form of microspheres are an attractive system for the controlled delivery of protein drugs. In this work, the microspheres were prepared by a water-in-water emulsion polymerization process. The polymerization reaction was initiated by potassium peroxodisulfate (KPS) and catalyzed by *N,N,N',N'*-tetramethylethylenediamine (TEMED). The effect of the initiator concentration, reaction temperature and pH on the mechanical and network properties of the microspheres were investigated. The size and size distribution of the microspheres, equilibrium water content, and methacrylate conversion were also determined. The mechanical properties of single microspheres were measured by a micromanipulation technique and the rheological characteristics of the same material in the form of macroscopic hydrogel gels were determined by a controlled stress rheometer. The results showed that the Young's moduli of the microspheres and of macroscopic gels measured by these two methods were in good agreement. Higher KPS initiator concentrations resulted in a more rapid polymerization with a shorter gelation and lag time, and a higher Young's modulus of the gels. An increase in temperature also resulted in a more rapid polymerization with a shorter gelation and lag time. However, the Young's modulus of the gels decreased with an increase in polymerization temperature. The pH had no significant effect on the mechanical properties of the microspheres. This study demonstrates that the network properties of dex-HEMA hydrogels can be tailored by the polymerization conditions, which opens the possibility to modulate the release rate of entrapped compounds.

1. Introduction

The progressive development in biotechnology has resulted in the availability of a great variety of protein drugs for therapeutic purposes. However, in general protein drugs have a short half-life after parenteral administration and they have a low bioavailability after oral administration. Therefore, there is an urgent need for suitable delivery systems for these relatively new therapeutic agents. Biodegradable hydrogels, hydrophilic polymeric networks that swell in water, but do not dissolve, are promising delivery systems for pharmaceutically active proteins[1-3]. Because of their high water content, hydrogels are well compatible with protein drugs and, importantly, they possess a good biocompatibility with living tissues. Dextran is a water-soluble bacterial exopolysaccharide consisting mainly of α-1,6-linked D-glucopyranose residues, and is an ideal polymer to form hydrogels due to its absent toxicity[4]. In recent years, different dextran hydrogels have been developed. Dextran hydrogels can be formed by the coupling of (metha)acrylic acid to dextran, followed by radical polymerization[5,6]. However, these hydrogels are not degradable under physiological conditions, and can only be degraded by dextranase[5,7]. Therefore, a next generation of hydrogels with hydrolytically sensitive carbonate ester groups was developed: dex-HEMA, dextran derivatized with hydroxyethyl methacrylate[8,9]. It has been shown that by varying the DS (degree of substitution, the number of methacrylates per 100 glycopyranose residues) and the initial water content of the gel, the degradation rate of the hydrogels could be tailored from days to months[10]. Moreover, the feasibility to load injectable dex-HEMA microspheres with protein drugs was demonstrated. The preparation of these protein-loaded microspheres was achieved in an all aqueous environment[11-13]. Dex-HEMA microspheres are formed by a polymerization reaction initiated by potassium peroxodisulfate (KPS) and catalyzed by *N,N,N',N'*-tetramethylethylenediamine (TEMED). It is therefore likely that the polymerization rate, which is in turn dependent on the polymerization conditions (initiator and catalyst concentration, temperature and pH)[14], may have a significant effect on network properties (crosslink density, pore size) of the microspheres. This can affect both the degradation rate of the microspheres and the release rate of an entrapped protein. Information on the network properties of these particles may be inferred from their mechanical properties. However, there is a lack of direct mechanical characterization of such small particles (several micros in diameter) due to technical difficulties. Conventionally, an indirect method is to determine the mechanical properties of macroscopic hydrogel gels by a rheometer[15,16]. The method, however, assumes that the mechanical properties of the macroscopic hydrogel gels are the same as those of microspheres and that the material is elastic. Recently, a novel micromanipulation technique has been developed, which is capable of directly measuring the mechanical properties of

single particles ranging from 1 micrometer up to 1 millimeter in diameter[17-19]. The feasibility of using this novel method to evaluate the mechanical properties of single dextran microspheres was demonstrated[12]. The objective of this work is to get insight into the network properties of dex-HEMA microspheres prepared under different conditions. The network properties (crosslink density, pore size) were derived from the mechanical properties as obtained by the micromanipulation technique. For comparison and verification, the rheological characteristics of dex-HEMA macroscopic hydrogel gels were also determined.

2. Materials and methods

2.1. Materials

PEG 10,000, dextran T40, TEMED and KPS were obtained from Fluka (Buchs, Switzerland). Hydroxyethyl methacrylate derivatized dextrans (dex-HEMA) were synthesized and characterized according to Van Dijk-Wolthuis et al.[8]. The DS (degree of substitution; the number of HEMA group per 100 glucopyranose units) was determined by proton nuclear magnetic resonance.

2.2. Preparation of microspheres and macroscopic gels

Dex-HEMA microspheres were prepared by a water-in-water emulsion method with a PEG 10,000/ dextran phase volume ratio of 40/1[20,21] using varying KPS and TEMED concentrations, polymerization temperature and pH (Table 1). The dex-HEMA used had a degree of substitution (DS) of 7. Different concentrations of KPS and TEMED in the dextran-enriched phase were prepared in 0.01M phosphate buffer. Formulation 1 represents microspheres prepared under standard conditions[21]. The concentration of KPS was varied in Formulations 2–4 and 11. Both the KPS and TEMED concentrations were varied in Formulations 5 and 6. The temperature (T) was varied in Formulations 7 and 8, and the pH in Formulations 9 and 10. For polymerization at 37 and 50 ^{o}C, a water and an oil bath were used, respectively. The polymerization at 21 ^{o}C was done at room temperature. Formulations 9 and 10 were prepared at pH 6 and 5, respectively (0.01M acetate buffer). For each formulation, three batches of approximately 5 g (dextran and PEG phase) were independently prepared. The microspheres were freeze–dried in order to maintain their stability during storage and were rehydrated in 0.1M acetate buffer pH 5 for 1 h before their mechanical properties were measured. Dex-HEMA macroscopic hydrogel gels were prepared by radical polymerization of aqueous solutions of dex-HEMA[9].

Table 1. *Preparation conditions and the equilibrium water content, volume mean diameter and methacrylate conversion of dex-HEMA microspheres*

Formulation	T (°C)	pH	$[KPS]_{dex}$ (mmol/l)	$[TEMED]_{dex}$ (mmol/l)	Equilibrium water content (%)	Volume mean diameter (μm)	Methacrylate conversion (%)
1	37	7	4.9	25.4	74.1	6.2	91
2	37	7	2.5	25.4	70.4	6.4	89
3	37	7	1.0	25.4	75.4	5.4	90
4	37	7	0.2	25.4	80.2	5.3	31
5	37	7	2.5	12.7	69.8	7.0	94
6	37	7	1.0	6.3	73.3	11.0	88
7	21	7	4.9	25.4	73.6	5.0	92
8	50	7	4.9	25.4	78.4	6.2	95
9	37	6	4.9	25.4	75.0	4.5	93
10	37	5	4.9	25.4	74.0	4.8	92
11	37	7	0.5	25.4	75.0	5.2	90

The values are the mean of three independent measurements that deviated less than 10 %.

2.3. Characterization of microspheres and macroscopic gels

Each measurement was carried out in triplicate unless otherwise stated. The size and size distribution of dex-HEMA microspheres were measured by a laser light blocking technique (AccusizerTM , model 770, Particle Sizing Systems, Santa Barbara, CA, USA). The microspheres were dispersed in reversed osmosis water. The equilibrium water content of the dextran microspheres was quantified by the blue dextran assay14. The methacrylate conversion was determined by HPLC analysis[22]. In brief, 100μl of the suspension of freshly prepared microspheres was added to 4.9 ml of 0.02M NaOH. Next, 1ml of 2M acetic acid solution was added to convert the methacrylate anion into methacrylic acid. Hundred microlitres of this sample was injected onto a RP-18 column (Lichrosphere, Merck, Darmstadt, Germany). A degassed 90/10 reverse osmosis water/acetonitrile mixture that was adjusted to pH 2 with perchloric acid was used as the mobile phase with a flow rate of 1 ml/min. The chromatograms obtained were analyzed to quantify methacrylic acid in the sample.

The polymerization kinetics and rheological characteristics of macroscopic hydrogel gels were quantified from a time-based polymerization or gelation profile obtained using an AR 1000-N controlled stress rheometer (TA Instruments, Inc., Ghent, Belgium), as described by Meyvis et al.[16].

The mechanical properties of single microspheres were measured by a micromanipulation technique. The principle of this technique is to compress a single microsphere between two parallel surfaces to a given deformation. Simultaneously the force being imposed on the particle is measured by a force transducer. The details of this technique are described elsewhere[17-19]. The size of each microsphere was measured from its microscope image. A probe with a flat end of 20μm in diameter was connected to the force transducer (0.5 g, 403 Aurora Scientific Inc, Ont., Canada), which was set to travel at 2 μm/s. Thirty microspheres from each formulation were measured. The experiments were conducted at room temperature (21°C).

3. Results and discussion

3.1. Size and equilibrium water content of microspheres

A typical volume-weighted size distribution of microspheres is presented in Fig. 1; the mean diameter is 6.2 μm (standard deviation 3.2 μm). The size distribution is Gaussian (chi-square test, 95% confidence). Table 1 shows that the volume mean diameter (D[3,0]) of the microspheres prepared under different conditions varied from 4.5 to 7 μm. Microspheres with such an average size can be administered to patients by injection. The microspheres made from Formulation 6 (mean diameter 11 μm) were significantly bigger than the others. This might result from coalescence of particles during the post-emulsion stabilization stage[21].

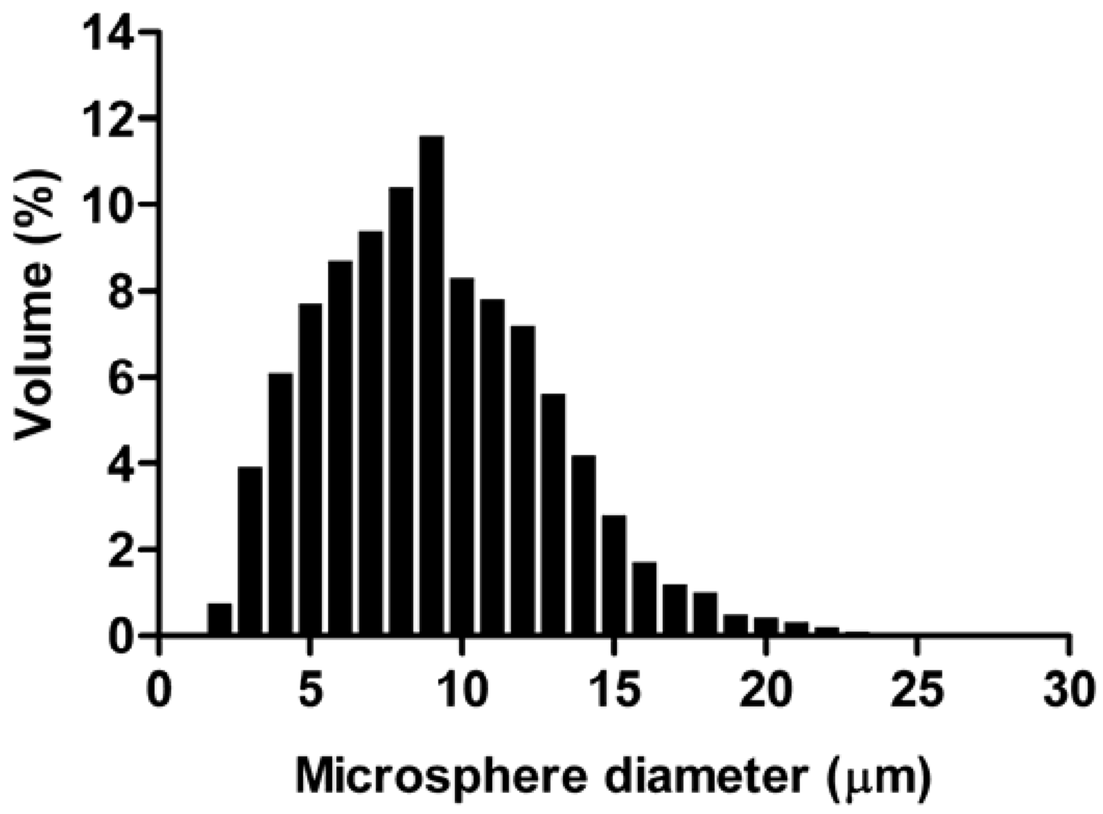


Figure 1. *A typical size distribution of dex-HEMA microspheres (Formulation 1).*

The equilibrium water content of the microspheres prepared under the different conditions varied between 70 and 75% (Table 1). However, the microspheres prepared at relatively low KPS initiator concentration (Formulation 4) had a higher equilibrium water content (~80%) than the others. This is likely due to the low methacrylate conversion, which results in a relatively low hydrogel crosslink density and thus in a high swelling capacity.

3.2. Methacrylate conversion

It has been reported that an excessive amount of the initiator (KPS) may cause undesired oxidation of an entrapped protein[23]. Therefore, it is worth to establish the minimum amount of initiator that can result in a high degree of methacrylate conversion.

The methacrylate conversion after 30 min polymerization was approximately 90%, except for Formulation 4 (conversion 31%) which was prepared with a relatively low KPS concentration (Table 1). Obviously, at this low concentration, KPS is the limiting factor of the reaction. This is expected and coincides with the previous work of Stenekes and Hennink[14].

3.3. Polymerization kinetics and rheological properties of hydrogels

Three parameters can be extracted from the timebased gelation profile (typical examples are shown in Fig. 2), namely the final shear storage modulus (*G'*), the gelation time and lag time. *G'*, taken from the plateau value of the kinetic curve, gives information on the stiffness of the hydrogel. The gelation time is arbitrarily defined as the time required for *G'* to increase from 10 to 90% of its plateau value, and the lag time is defined as the time required for *G'* to reach 10% of its plateau value. Conventionally, the gelation time is taken as time when *G'* equals the loss modulus *G''*[24] that reflects the viscous properties of the gels. However, this was not applicable for the dex-HEMA gels as it was observed that *G'* is much greater than *G''* (data not shown), and consequently there is no obvious crossover point. As the purpose of the rheological study was to verify the elastic property of macro-gel and microspheres, only *G'* results are thus presented. Both the gelation and the lag time reflect the rate of the polymerization reaction. The shorter the gelation and lag time, the faster the polymerization reaction is.

For most of the formulations listed in Table 1, the outcomes from the gelation profile, *G'*, gelation time and lag time were found to be similar to those of Formulation 1, prepared under standard conditions. However, there are several formulations that showed distinctive variations, which are summarized in Table 2.

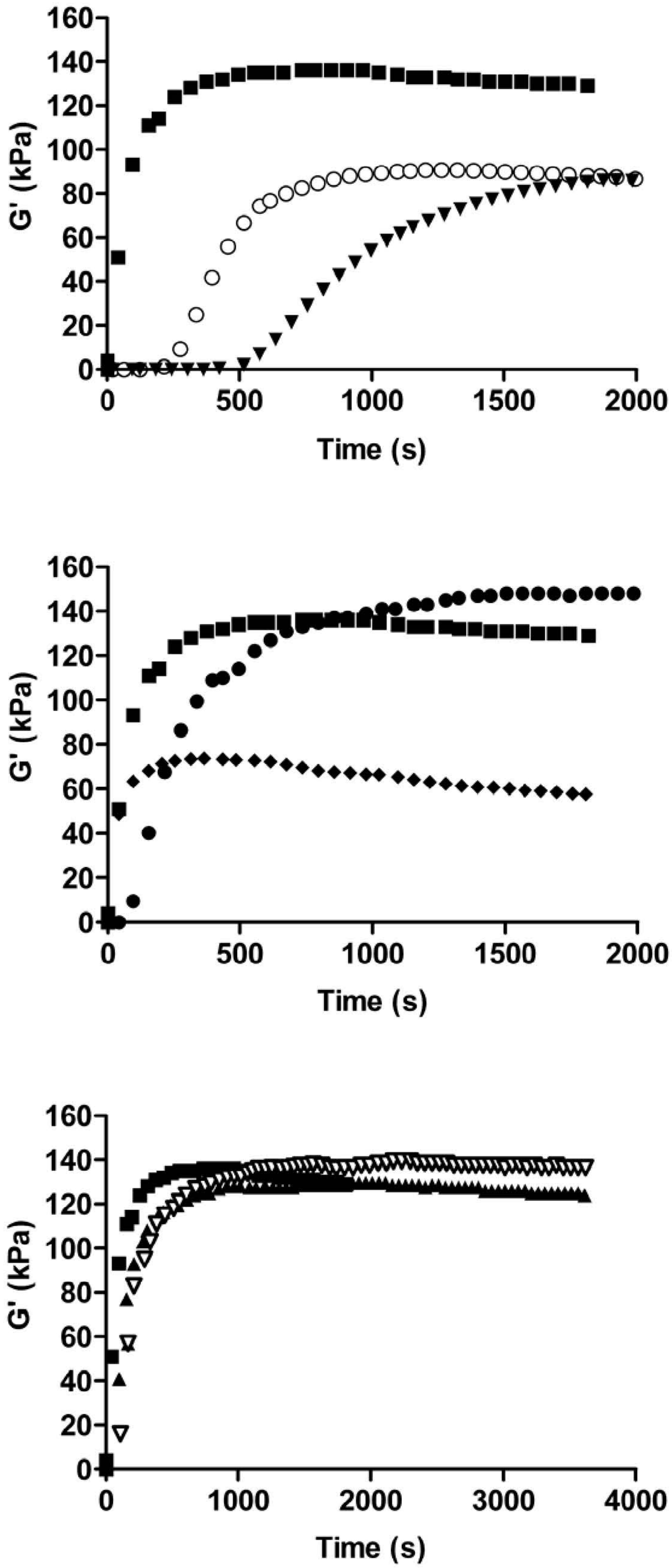


Figure 2. *Polymerization (gelation) profiles of dex-HEMA hydrogels prepared with different (a) KPS concentrations: 4.9 mmol/l (■), 0.5 mmol/l (○), 0.2 mmol/l (▼), (b) polymerization temperatures: 21 °C (◆), 37 °C (■), 50 °C (●)., (b) temperatures and (c) pH 5, (▲), 6 (▽), 7(■).*

The gelation profiles of Formulations 1, 4, and 11, which were prepared at different KPS concentrations, are presented in Fig. 2a. The results show that, as expected, with decreasing KPS concentration, the gelation proceeds more slowly. Fig. 2a also shows that the final *G'* for the gels prepared with 0.2 and 0.5 KPS mmol/l is significantly smaller than of the gels prepared at KPS 4.9 mmol/l. The low *G'* of the gel prepared at 0.2 KPS mmol/l compared with the gel prepared at 4.9 mmol/l was likely caused by the low methacrylate conversion which in turn resulted in a low crosslink density. Interestingly, the gel prepared at 0.5 KPS mmol/l had the same final *G'* as the gel prepared at 0.2 mmol/l, despite its higher methacrylate conversion. Likely, the molecular weight of the polymerized methacrylate groups for the gel prepared at higher KPS (0.5 mmol/l) is lower than that of the polymerized methacrylate in the gel prepared at 0.2 mmol/l. A lower molecular weight decreases the chain functionality of the crosslinks. It has been reported that a lower chain functionality results in a lower Young's modulus of the gels[25]. In the gel prepared at 0.2 mmol KPS, the lower methacrylate conversion might be compensated by a higher methacrylate molecular weight, resulting in a gel with nearly the same final *G'* as the 0.5 mmol KPS gel. However, the higher final *G'* of the gels prepared at 4.9 mmol/l KPS than 0.5 mmol is unexpected since both gels have the same methacrylate conversion (Table 1) and the higher KPS concentration should result in a lower molecular weight of the polymerized methacrylates corresponding to lower final *G'* [25].This discrepancy requires further investigation.

The effect of polymerization temperature on the gelation kinetics is shown in Fig. 2b. It can be seen that when the polymerization temperature was elevated from 21 to 50 °C, the time for *G'* to reach the plateau became shorter. This is logical since at higher temperature more radicals were formed which in turn caused a faster methacrylate conversion. It is very interesting to note that the hydrogel formed at 50 °C had a lower *G'* than observed for the gels formed at lower temperatures. An increase in reaction temperature increases the polymerization rate and decreases the polymer molecular weight26. A lower molecular weight of the polymerized methacrylate groups reduces the junction functionality of the hydrogel network[25], which in turn results in a lower mechanical rigidity of the network.

It has been proposed that unprotonated TEMED catalyzes the decomposition of the peroxodisulfate anion into radicals[26]. Hence the degree of protonation of TEMED (pK_as are 8.8 and 10.3 at 37 °C (Sigma, UK)), and thus the pH, may affect the polymerization rate. The polymerization was done at pH 5, 6 and 7.

Table 2. *Gelation and rheological data of different dex-HEMA hydrogels*

Formulation	T (°C)	$[KPS]_{dex}$ (mmol/l)	G' (MPa)	Gelation time (s)	lag time (s)
1	37	4.9	0.12	208	11.7
11	37	0.5	0.09	408	275
4	37	0.2	0.09	700	468
7	21	4.9	0.13	545	100
8	50	4.9	0.07	126	4.2

The values are the mean of eight independent measurements that deviated less than 15 %.

Since the concentration of the unprotonated TEMED (which is suggested to catalyze the decomposition of the peroxodisulfate anion) decreases by a factor 100 from pH 7 to 5, a substantial effect on the polymerization kinetics was expected. However, Fig. 2c shows that there was no significant effect of the pH on the gelation profile of the formulations, which strongly suggests that not the unprotonated TEMED, as proposed in literature[27], but the $TEMEDH2^{++}$ cation catalyzes the decomposition of the peroxodisulfate anion.

3.4. Mechanical characterization of microspheres by the micromanipulation technique

Fig. 3 shows a typical force versus displacement curve, obtained by the micromanipulation technique, for compressing a single hydrated dex-HEMA microsphere. The force transducer probe started to move downward at point A and touched the microsphere at B. The compression commenced immediately and the force started to increase until the microsphere underwent large deformation (point C).

It appears from the force–displacement curve in the region BC that the hydrated microsphere was only deformed under compression and did not show any rupture behavior as the force profile does not show any sudden decrease with the displacement[12,18].

In order to identify whether the microspheres are mainly elastic or elastic–plastic, single microspheres were compressed repeatedly and the results in pseudo-stress (the force divided by the cross-section area of each microsphere before it was deformed) versus deformation (the ratio between the displacement of the microsphere and its diameter) are presented in Fig. 4. As can be seen, all the curves overlap, which indicates that for the deformations investigated the dex-HEMA hydrogel microspheres are mainly elastic.

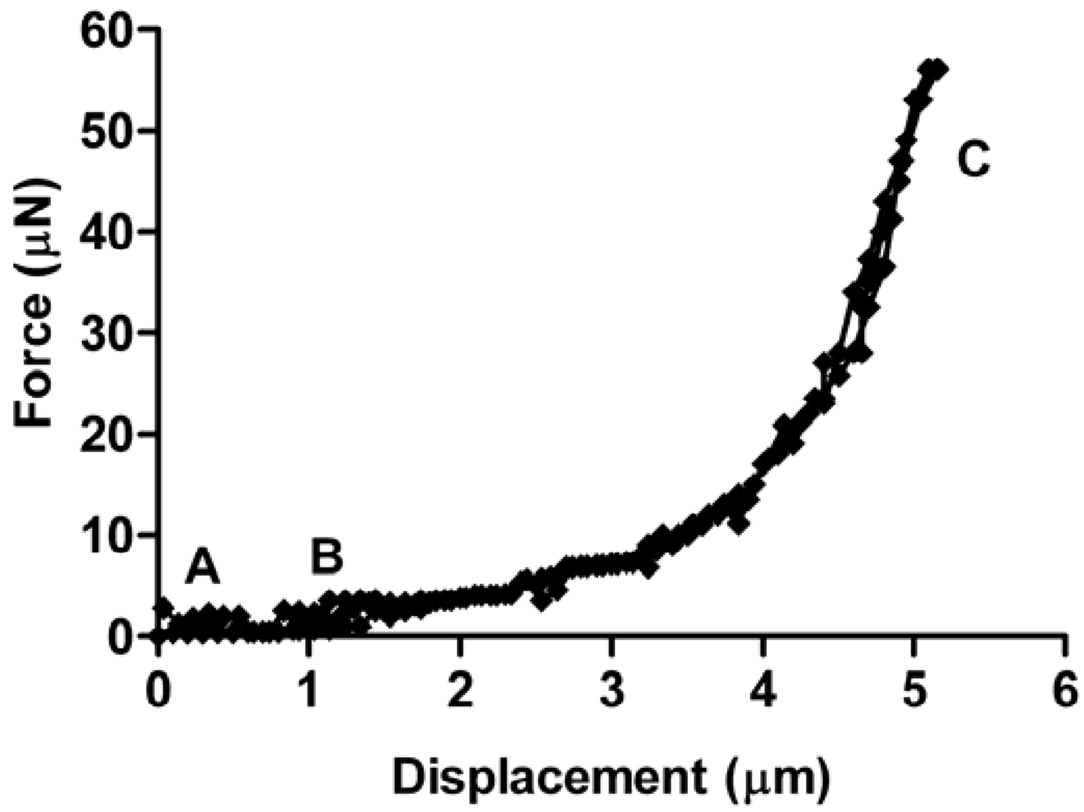


Figure 3. *A Typical force versus displacement graph obtained from compression of a hydrated microsphere by micromanipulation. The diameter of the microsphere was 5.8 μm.*

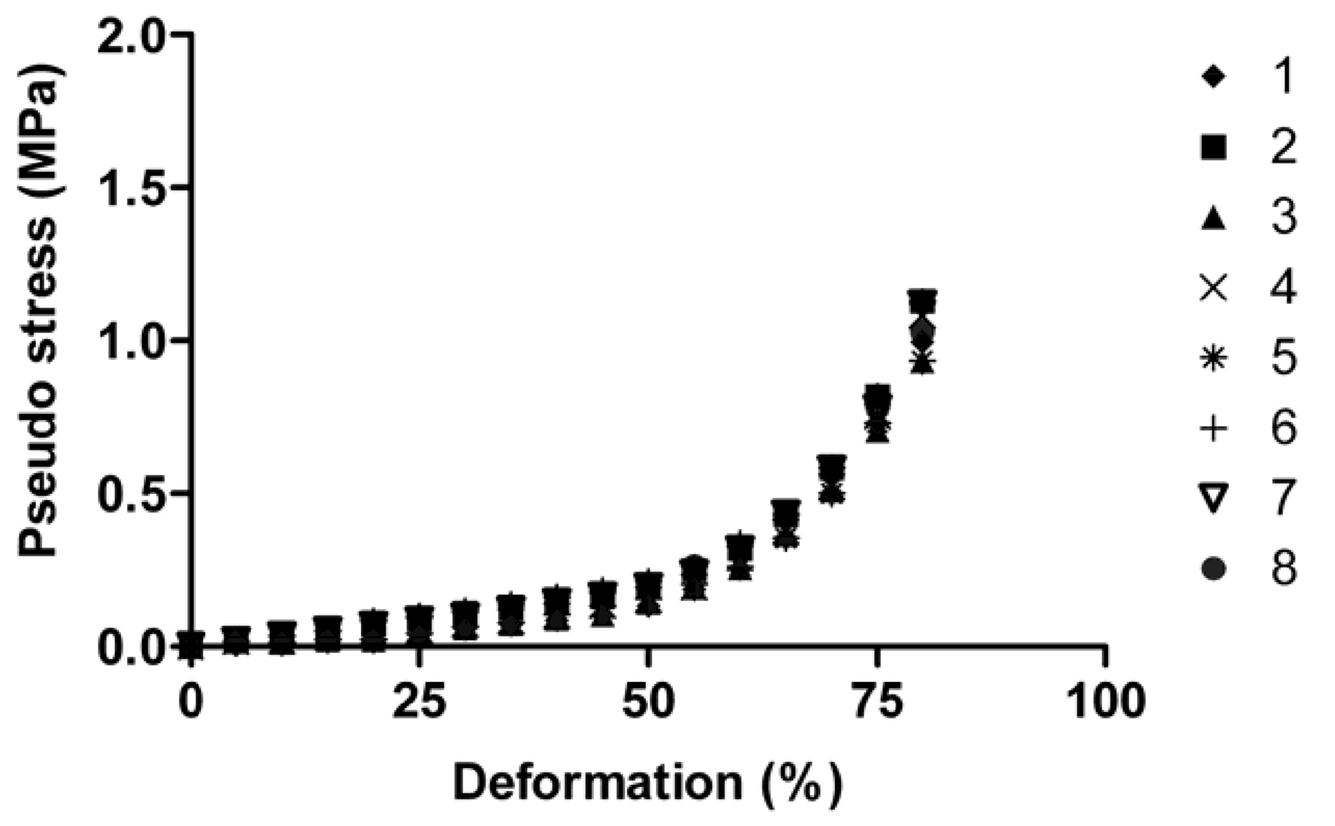
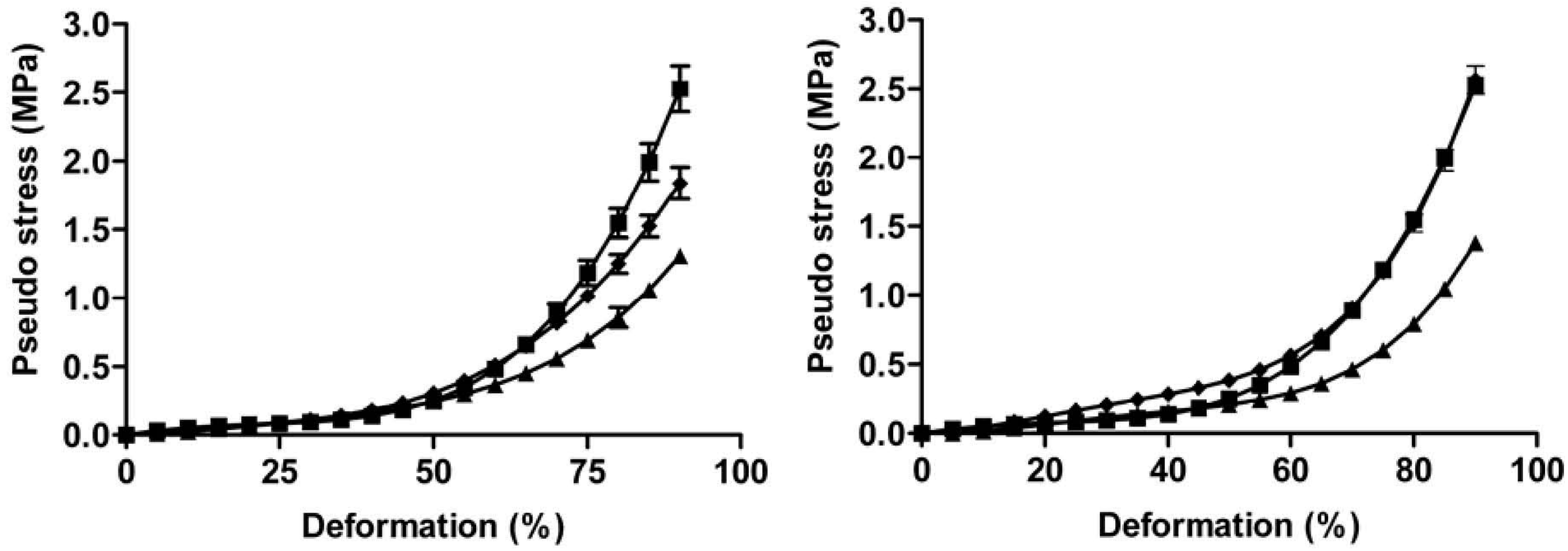


Figure 4. *Pseudo-stress versus deformation for repeatedly compressing eight times a typical dex-HEMA microsphere (Formulation 1).*

Micromanipulation was carried out on five microsphere samples which were prepared under different conditions, the corresponding hydrogel gels of which showed significant variation in their *G'* values. The relationships between the pseudo-stress and deformation of microspheres are plotted in Fig. 5a and b. It can be clearly seen that the pseudo-stress for a given deformation of the microspheres increased with the KPS concentration, particularly at higher deformation (Fig. 5a). Fig. 5b shows that there is no significant difference in the relationship of the pseudo-stress versus deformation between the microspheres polymerized at 21 and 37 °C. However, when the temperature was increased to 50 °C, the microspheres formed were weaker.



(a) *(b)*

Figure 5. *Pseudo-stress versus deformation for compressing single microspheres made with different (a) KPS concentrations: 4.9 mmol/l (■), 0.5 mmol/l (◆), 0.2 mmol/l (▲) and (b) polymerization temperatures: 21 °C (◆), 37 °C (■), 50 °C (▲). The number of microspheres measured for each formulation is 30. The error bars represent two times standard error of the mean.*

3.5. Young's modulus (E) of dextran hydrogel gels and microspheres

From the rheological study, the Young's modulus (E) of macroscopic hydrogel gels was calculated by $E=3G'$. [28] From the pseudo-stress versus deformation profile obtained from micromanipulation measurements of single microspheres, theYoung's modulus (E) of the microspheres was estimated using the initial slope of the curve[12], as demonstrated in Fig. 6.

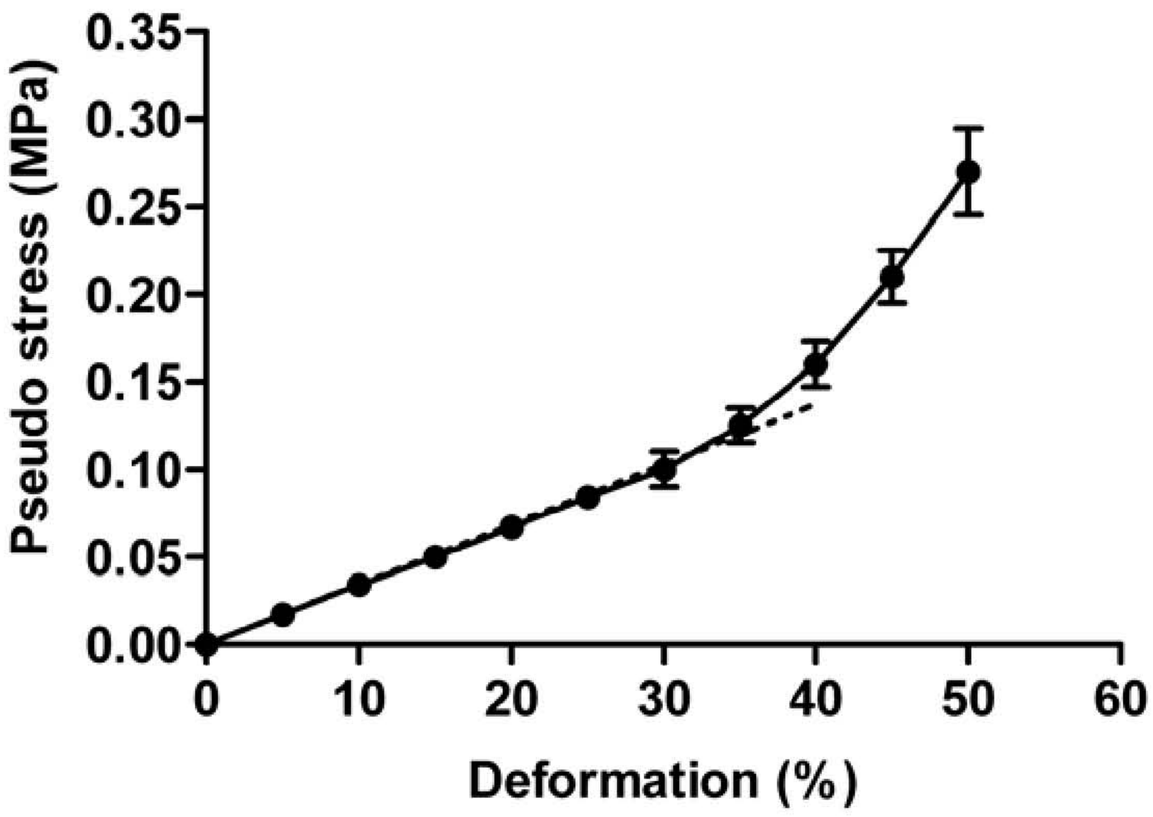


Figure 6. *Initial section of mean pseudo-stress versus deformation for compressing 30 microspheres of Formulation 1. Error bars indicate two times the standard error of the mean. Dotted line indicates the initial slope of the profile.*

The Young's moduli from both approaches are presented in Table 3. The table shows that the Young's moduli (E) of dex-HEMA hydrogels of the same composition in the form of gels and microspheres are in good agreement, which indicates that the rheological measurements on macroscopic hydrogels have validated the micromanipulation method (in agreement with Stenekes et al.[12]).

3.6. Average molecular weight between crosslinks, crosslink density and pore size

The Young's modulus of the dex-HEMA hydrogels was used to estimate the average molecular weight between the crosslinks (M_c) and the effective crosslink density (v_e). By assuming that the end-to-end distances of the chains are Gaussian, that the network deformation is isothermal and affine, and that dangling ends are absent, M_c was determined using Eq. (1) derived from the rubber elasticity theory[2,28,29]:

$$M_c = \frac{3\rho RT}{E} \tag{1}$$

where ρ is the specific density (kg/m^3), R is gas constant, T is absolute temperature, and E is Young's modulus. The specific density of the dextran microspheres was calculated from the partial specific volume of dextran and their equilibrium water content[12].

The effective crosslink density (v_e) was calculated using the following equation[28.31]:

$$v_e = \frac{\rho}{M_c} \tag{2}$$

The mean pore size of the hydrogel network of the microspheres (ξ) was estimated using the following equation [32]:

$$\xi = 0.071 \cdot \upsilon^{-1/3} \cdot M_c^{1/2} \tag{3}$$

where υ is the polymer volume fraction that can be derived from their water content[29]. The results of M_c and v_e estimated for the microspheres made of the formulations described in Table 3 are shown in Table 4. From this table it appears that the lower the KPS concentration, the greater the M_c and thus the lower the crosslink density is.

Further, with increasing polymerization temperature, M_c increases and the crosslink density decreases. The mean pore sizes of the different microspheres are also summarized in Table 4. In agreement with the data for M_c, the calculated mean pore size decreased with increasing KPS concentration but increased with the polymerization temperature.

Table 3. *Young's Moduli (E) obtained from micromanipulation measurements of hydrogels microspheres and rheological data of hydrogel gels.*

Formulation	T (°C)	$[KPS]_{dex}$ (mmol/l)	$E_{microspheres}$ (MPa)	$E_{hydrogels}$ (MPa)
1	37	4.9	0.45	0.37
11	37	0.5	0.28	0.27
4	37	0.2	0.24	0.26
7	21	4.9	0.53	0.43
8	50	4.9	0.24	0.22

The values are the mean of eight (microspheres) and three (macrogels) independent measurements that deviated less than 15 %.

Table 4. *Calculated value of the density of dex-HEMA gels (ρ), average molecular weight between crosslinks (M_c), polymer volume fraction (υ), effective crosslink density (ν_e), and pore size (ξ), for microspheres at varying KPS concentrations and polymerization temperatures.*

Formulation	T (°C)	$[KPS]_{dex}$ (mmol/l)	ρ (g/cm^3)	M_c (kg/mol)	ν_e (x10^5 mol/cm^3)	υ	ξ (nm)
1	37	4.9	0.45	18.9	6.1	0.18	17.3
11	37	0.5	0.28	30.1	3.8	0.17	22.2
4	37	0.2	0.24	33.5	3.3	0.13	25.7
7	21	4.9	0.53	15.9	7.3	0.18	15.9
8	50	4.9	0.24	34.3	3.3	0.15	24.7

The values are the mean of eight independent measurements that deviated less than 15 %.

Overall, the average pore size of the dex-HEMA hydrogel is slightly greater than that of the dex-MA hydrogels[12]. However, it should be pointed out that the calculations were done for an ideal network. The dextran networks are certainly not ideal. Therefore, the M_c/pore size data presented can only be used in a relative sense.

4. Conclusions

This study shows that single dex-HEMA microspheres are mainly elastic up to a deformation of 80%. The Young's moduli of the different microspheres derived from the micromanipulation data, were consistent with those from the rheological data. Increased KPS initiator concentrations caused a faster polymerization rate, and the formed hydrogel was stronger

(greater *G'* or *E* value), had a smaller molecular weight between crosslinks (M_c) and a smaller pore size. However, although an increase in polymerization temperature also led to a faster reaction rate, the formed hydrogel was weaker, had a larger molecular weight between crosslinks (M_c) and a bigger pore size. The pH of the polymerizing solution did not have a significant effect on both the polymerization rate and the hydrogel network properties.

This study shows that the network characteristics are dependent on the polymerization conditions. These altered network properties likely results in different release profiles of entrapped proteins, which is the subject of present investigations.

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Chapter 5

Effect of excipients on the encapsulation efficiency and release of human growth hormone from dextran microspheres

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Submitted

Abstract

In this study, the possibility was investigated to modulate the encapsulation efficiency and release of human growth hormone (hGH) from hydroxyl ethyl methacrylated dextran (dex-HEMA) hydrogel microspheres by using excipients. Microspheres were prepared by polymerization of dex-HEMA (DS, degree of methacrylate substitution (number HEMA groups per 100 glucose units) of 16) in an aqueous two-phase system of this polymer and PEG. Excipients (tween 80, pluronic F68, sucrose, NaCl, urea or methionine) were dissolved in the aqueous phases prior to the polymerization of dex-HEMA. High hGH encapsulation efficiencies (50-70 %) were obtained for microspheres prepared without excipients and with tween 80, NaCl or methionine. Substantially lower encapsulation efficiencies (27 and 19% respectively) were obtained for microspheres prepared in the presence of sucrose and urea. A biphasic release profile was observed for microspheres prepared without excipients from which hGH was released with almost zero-order release kinetics for 6 days, after a delay time of 2 days. In contrast, for microspheres prepared with tween 80, pluronic F68, sucrose, NaCl and urea the delay time was absent, and hGH was released with zero-order kinetics for 6-8 days. Microspheres prepared with methionine showed a concentration-dependent delay time varying from 0 to 2 days followed by zero order release for 6 days. Rheological measurements showed that the excipients (except methionine) had no effect on the hydrogel matrix. It was demonstrated that hGH precipitates in dex-HEMA, and likely these hGH precipitates are entrapped in the hydrogel matrices. Furthermore, it was demonstrated that in the presence of excipients smaller hGH precipitates were formed, which explains the difference in release profile. A higher hydrophobicity of the (partly) denatured hGH favors partitioning over the PEG-phase and may be the reason for the lower encapsulation efficiency for microspheres prepared with urea and sucrose. In conclusion, this study demonstrates that the release characteristics of hGH from dex-HEMA microspheres can be modulated with excipients. Especially, tween 80 and methionine are attractive excipients since hGH was encapsulated in high yield (60-70%) and the protein was released from the microspheres mainly in its monomeric form without a delay time and with an almost zero order kinetics for 6-8 days.

1. Introduction

Due to the enormous advances in biotechnology many therapeutic proteins have become available. However, generally speaking (potential) therapeutic proteins are rapidly eliminated *in vivo* and have a poor physical and chemical stability. Therefore, improved methods are needed to ensure that proteins access their target at the right concentration and for a prolonged period of time. Consequently, considerable effort has been put in the development of polymer based biodegradable systems for the controlled release of proteins[1,2]. It is one of the primary considerations in the development process of protein formulations to maintain the concentration of the protein within the therapeutic window for a time period sufficient to achieve the desired therapeutic effect. Therefore, it is important to control the release kinetics of delivery system with a wide applicability.

Microspheres based on crosslinked dextran (dex-HEMA= hydroxylethyl methacrylate derivatized dextran) are attractive systems for the controlled release of proteins[3,4]. The formation of these microspheres is based on phase-separation between aqueous solutions of poly(ethylene glycol) (as the continuous phase) and dex-HEMA (as the discontinuous phase). Upon addition of an initiator system (KPS = potassium peroxodisulfate and TEMED = *N,N,N',N'*-tetramethylethylenediamine) to the water-in-water emulsion, the dextran-bound methacrylate groups are polymerized which ultimately results in the formation of microspheres consisting of crosslinked dextran. It was shown that dex-HEMA microspheres are biodegradable and have a good biocompatibility[5]. The release is controlled by degradation of the matrix and diffusion of the protein through the matrix[4,6]. Under physiological conditions (pH 7 and 37 °C), hydrolysis of the carbonate ester linking the dextran to pHEMA results in a reduction of crosslink density and an increasing mesh size of the network over time. When the pore size in the matrix is larger than the hydrodynamic diameter of the protein, the protein will be released. Previous studies have shown that the release can be tailored by varying parameters such as water content and degree of methacrylate substitution (DS=number of HEMA groups per 100 glucose units) of the microspheres and the physical state of the protein[4,6].

In this study, the possibility was investigated to modulate the encapsulation efficiency and the release kinetics of a model protein (human growth hormone, hGH) from dex-HEMA microspheres using excipients (tween 80, pluronic F68, sucrose, NaCl, urea and methionine). These excipients were dissolved in the aqueous two phase system prior to initiation of the polymerization. Sucrose and NaCl are known precipitating agents[7-10] and likely induce the precipitation or aggregation of the protein in the PEG and dex-HEMA phase, which in turn will likely affect both the encapsulation efficiency and the release kinetics. Tween 80 and pluronic F68 are known

solubilizers of hGH and prevent its aggregation[12-15]. Urea is also often used to dissolve protein aggregates or proteins from inclusion bodies[15-17]. A previous study has shown that methionine residues in proteins can be oxidized by potassium peroxodisulfate (KPS)[18]. Therefore, methionine is added as anti-oxidant. However, as a result of this reaction, the KPS concentration available for initiation of the polymerization of dex-HEMA is reduced. It was shown by Chung et al. that a reduced KPS concentration results in an increased mesh size of the hydrogel network[19]. Therefore, it was anticipated that the release of hGH could be affected by methionine. Finally, the effect of the KPS concentration on the encapsulation efficiency and release of hGH was also established.

2. Materials and methods

2.1 Materials

Poly(ethylene glycol) (PEG) 10 kg/mol and potassium peroxodisulfate (KPS) were obtained from Merck, Darmstadt, Germany. Dextran 40 kg/mol, *N,N,N',N'*-tetramethylethylenediamine (TEMED), pluronic F68, urea, sucrose, tween 80, methionine and NaCl were purchased from Fluka (Buchs, Switzerland). Hydroxyethyl methacrylate derivatized dextran (dex-HEMA) with a degree of HEMA substitution 16 (DS 16; 16 HEMA groups per 100 dextran monomer units) was obtained from Polymer Service Centre Groningen (Groningen, the Netherlands) and synthesized and characterized according to Van Dijk-Wolthuis et al.[20]. Modified recombinant human growth hormone 190 (rhGH-190, Somategen®) as a aqueous formulation (concentration 3.2 mg/ml in a 7 mM phosphate buffer pH 7 containing 10 mM glycine/mannitol) was kindly donated by Biotechna Sicor Inc. (Irvine, CA, USA).

2.2. Methods

2.2.1. Microsphere preparation

The different batches of dextran microspheres were prepared by the water-in-water emulsion technique as described elsewhere[3]. Briefly, 3.25 g of a 40 % w/w PEG-solution in 25 mM phosphate buffer pH 7.0 was transferred into a 15 ml tube. Then, 1.69 g of a 3.6 % w/w dex-HEMA solution in 25 mM phosphate buffer pH 7.0 containing 0.3 % w/w hGH was added. The mixture was vortexed for 1 minute and subsequently, 100 µl of a TEMED solution (20% (v/v), pH neutralized with 4 M hydrochloric acid) was added, followed by vortexing for 20 s. Then, 180 µl KPS (concentration 50 mg/ml) was added and the emulsion was shaken gently. The resulting emulsion was allowed to polymerize for 1 hour at room temperature. The polymerized

microspheres were washed 5 times with 5 ml 25 mM phosphate buffer pH 7.0. Microspheres were also prepared in the presence of excipients by dissolving appropriate amounts in the buffer or protein solution used for the preparation of the dex-HEMA and PEG solutions (Table 1).

The microspheres were investigated by optical microscopy (Microscope with D12 digital camera, Olympus Optical Co. Shibuya, Tokyo, Japan) and their average volumetric particle diameter and size distributions were measured with a particle size analyzer (Malvern Mastersizer 2000). The water content and methacrylate conversion of the microspheres was determined according to the methods as previously described by Stenekes et al.[21-22].

2.2.2. In vitro release of hGH

Directly after washing, the microspheres were resuspended in 5 ml 25 mM phosphate buffer pH 7.4 and incubated in a water bath at 37 °C. Periodically, the microsphere suspensions were centrifuged for 7 min at 3200 x g, 3 ml supernatant was removed and replaced by 3 ml 25 mM phosphate buffer pH 7.4. The hGH concentration and the percentage monomer in the samples were determined with size exclusion chromatography (SEC) as described in section 2.2.5. The amount of protein, which was left in the microspheres after 12 days, was determined by nitrogen analysis (see section 2.2.6.). The amount of protein entrapped in the microspheres was calculated by adding up the amount of protein released after 12 days and the amount of protein that remained in the microspheres after 12 days. The encapsulation efficiency was obtained from the amount of protein entrapped in the microspheres divided by the amount of protein added for preparation of the microspheres. For all release curves reported, the amount of protein released after 12 days has been normalized to the released amount of protein to allow comparison of release profiles.

2.2.3. Size exclusion chromatography

The HPLC system consisted of a HPLC pump series 200, a vacuum degasser series 200, an autosampler series 200, a column LC 101 oven, and a UV detector series 200 (all Perkin Elmer Instruments, Norwalk, USA), a thermostated (35 °C) Thosohaas TSKgel G300SWXL column and a Thosohaas TSKgel SWXL guardcolumn (Montgomeryville, PA, USA) and a UV detector series 200 (Perkin Elmer Instruments, Norwalk, USA). The flow rate was 0.7 ml/min and an aqueous solution containing phosphate buffer (64 mM, pH 7.0) and 3 % (v/v) isopropanol was used as the mobile phase. The chromatograms were recorded and analyzed with the data acquisition system Totalchrom (Perkin Elmer Instruments, Norwalk, USA).

2.2.4. Nitrogen analysis

To determine the amount of protein that was left in the microspheres after a release period of 12 days, nitrogen analyses were done by pyro-chemiluminescent nitrogen assay using a nitrogen analyzer. Placebo microspheres were used to substract background signal. The nitrogen analyzer consisted of an autosampler (ELS 2100) and the samples were injected into a nickel boat, which was transported into the furnace tube TN 30000, and a cold trap TX/TS module and a UV detector (all thermo Euroglass corp. Delft, Holland). The injected volume was 500 µl. The argon flow was set at 340 ml/min, the oxygen flow was set at 15 ml/min and the ozonator at 110 ml/min. The temperature of furnace 1 and 2 was set at 1000 °C, while the temperature of the NOx scrubber was set at 320 °C. A calibration curve was obtained by injecting was 500 µl 0.1-10 µg/ml glycine. The hGH-190 concentration was calculated by assuming that this protein contains 16.7 % w/w nitrogen[23]. The emission of the NO_2 -radical produced by reaction of NO (formed after pyrolysis of the samples) with ozone was measured and analyzed with the data acquisition system of Thermo Euroglass DIN 38409 (Thermo Euroglass corp. Delft, Holland).

2.2.5. Rheological properties of macroscopic hydrogels prepared in the presence of excipients

Dex-HEMA macrogels were prepared in the presence of the different excipients and their rheological properties were measured with dynamic mechanical analysis (DMA). Hydrogels were prepared by dissolving 450 mg dex-HEMA (DS 16) in 840 µl of the selected solution, resulting in a concentration of 0.1 % tween 80, 0.5 % pluronic F68 or 25 % sucrose, 5.7 M urea or 2 M NaCl respectively, in the macrogels. Macrogels were also prepared with different concentrations of KPS (4.4 mmol/l, 1.6 mmol/l). After dissolving dex-HEMA the solutions were vigorously vortexed and centrifuged for 5 min at 13,000 g to remove air bubbles from the solution. Then, 75µl of a 7.6% (v/v) TEMED (adjusted to pH 7.4 with 4N HCl) solution was added. The solutions were cooled to 4 °C to slow down the polymerization and to increase the handling time during hydrogel preparation. A KPS solution (135 µl, 14.4 mg/ml) was mixed with the dex-HEMA solution, which was subsequently quickly transferred into a mould and allowed to polymerize for 1 hour at room temperature to obtain hydrogel cylinders with a diameter of about 5 mm and a height of 6 mm. The mould was precoated with a PEG solution to facilitate removal of the gels.

The rheological properties of the obtained hydrogels were determined by dynamic mechanical analysis (DMA) as described previously[24]. Briefly, the DMA measurements were carried out with a DMA 2920 Dynamic Mechanical Analyzer (TA Instruments, New Castle, England) equipped with a compression clamp (upper plate 6 mm, lower plate 45 mm) installed with sandpaper glued on both plates. The experiments were performed by imposing various static forces between 0.1 and 8.0

N, depending on the gel, with amplitude of 10 mm and a frequency of 1 Hz. The modulus was determined for each static force. The compression modulus was obtained by plotting the modulus versus the static force and extrapolation to a static force of zero. For each formulation, at least three gels were measured. The experiments were performed at room temperature.

2.2.6. Precipitation of hGH in dex-HEMA solution

Seven ml of the stock solution of hGH was diluted with 14 ml 25 mM phosphate buffer pH 7.0. This protein solution was concentrated over a Centricon® filter YM-10 with cut-off 10 kDa (Milipore, Bedford, USA) by centrifugation for 30 min at 4600 x g . The concentration of the hGH solution was determined with SEC (section 2.2.5). The concentrated hGH solution was split into two samples of 500 µl. To one of these samples 0.2 g dex-HEMA was added to obtain a 30 % w/w dex-HEMA (w/w) solution. To the other sample, first NaCl (0.06 g) was added to obtain a concentration of 2M. Hereafter, dex-HEMA (0.2 g) was added to obtain a 30 % w/w dex-HEMA (w/w) solution. After incubation of 1 hour at room temperature, the samples were centrifuged (3200x g for 30 min). The supernatant was removed, diluted 50 times with 25 mM phosphate buffer pH 7.0 and the concentration of protein was determined with SEC (see section 2.2.5). The loss of protein by precipitation was calculated by dividing the amount of protein in the supernatant by the added amount of protein. The precipitate collected in the pellet was investigated with a light microscope.

3. Results and Discussion

3.1. Microsphere formation

Table 1 summarizes the characteristics of the hGH loaded microspheres prepared with the various excipients. The volume mean diameter of microspheres ranged between 9 to 14 µm. microspheres. The excipients had only marginal effects on the water content and methacrylate conversion of the microspheres (Table 1). The water content of the microspheres ranged from 53 to 63 %, whereas the methacrylate conversion ranged from 86 to 96 %, which is in good agreement with data reported previously[22]. For microspheres prepared with 1 % methionine or 1.6 mmol/l KPS (note that for the standard formulation 4.4 mmol/l KPS was used), slightly lower methacrylate conversions were found (respectively 86% and 88 %, respectively).

Table 1. *Characteristics of hGH loaded microspheres.*

Formulations	Particle size (μm)	Equilibrium water content (%)	Methacrylate conversion (%)	Encapsulation efficiency (%)	Recovery (%)	Fraction protein released as monomer (%)
KPS*						
- 4.4 mmol/l (=standard)	10	62	93	58	88	79
- 1.6 mmol/l	ND	57	88	64	89	83
Methionine						
- 0.1 %	9	63	94	59	89	81
- 0.5 %	ND	59	91	68	91	81
- 1 %	9	61	86	63	94	84
Tween 80 0.1 %	12	61	95	58	91	84
Pluronic F68 0.5 %	14	54	91	47	91	73
Sucrose 25 %	14	53	94	27	88	60
Urea 8 M	10	60	96	19	91	45
NaCl 2M	10	63	95	56	92	74

Note[1]: , ND=not determined
Note[2]:For particle size, water content, MA conversion the values are the average of 3 independent measurements that deviated less than 5 %. For encapsulation efficiency, total release, percentage monomer, the values are the average of 2 independent measurements that deviated less than 5 %.
** The KPS concentrations correspond to the KPS concentrations in the dex-HEMA phase prior to emulsification[22].*

The compression moduli of the macroscopic hydrogels prepared with the different excipients are presented in Fig.1. This figure shows that the moduli of the hydrogels prepared with NaCl, sucrose, pluronic F68, tween 80 and urea, were the same as the compression modulus of hydrogels of the standard formulation. This indicates that the presence of these excipients during polymerization did not affect the network structure. For hydrogels prepared with methionine, added as a protein anti-oxidant[18], a concentration-dependent decrease in elastic modulus was found ($P<0.05$). Since KPS oxidizes methionine, its concentration for initiation of the polymerization of the HEMA groups decreases. Fig. 1 also shows that the compression modulus of hydrogels prepared with 1.6 mmol/ml is lower than the compression modulus of the standard formulation prepared with 4.4 mmol/l KPS. Thus, the lower modulus found for gels prepared in the presence of

methionine can be very well explained by a lower KPS concentration available for initiation of the polymerization of the HEMA groups and in line with the drop in methacrylate conversion (1 % methionine; Table 1). In a previous study, it was demonstrated that the compression moduli of hydrogels and microspheres correspond very well[25]. Therefore, the effects of excipients on the macrogels are supposed to be representative for the effects on the microspheres.

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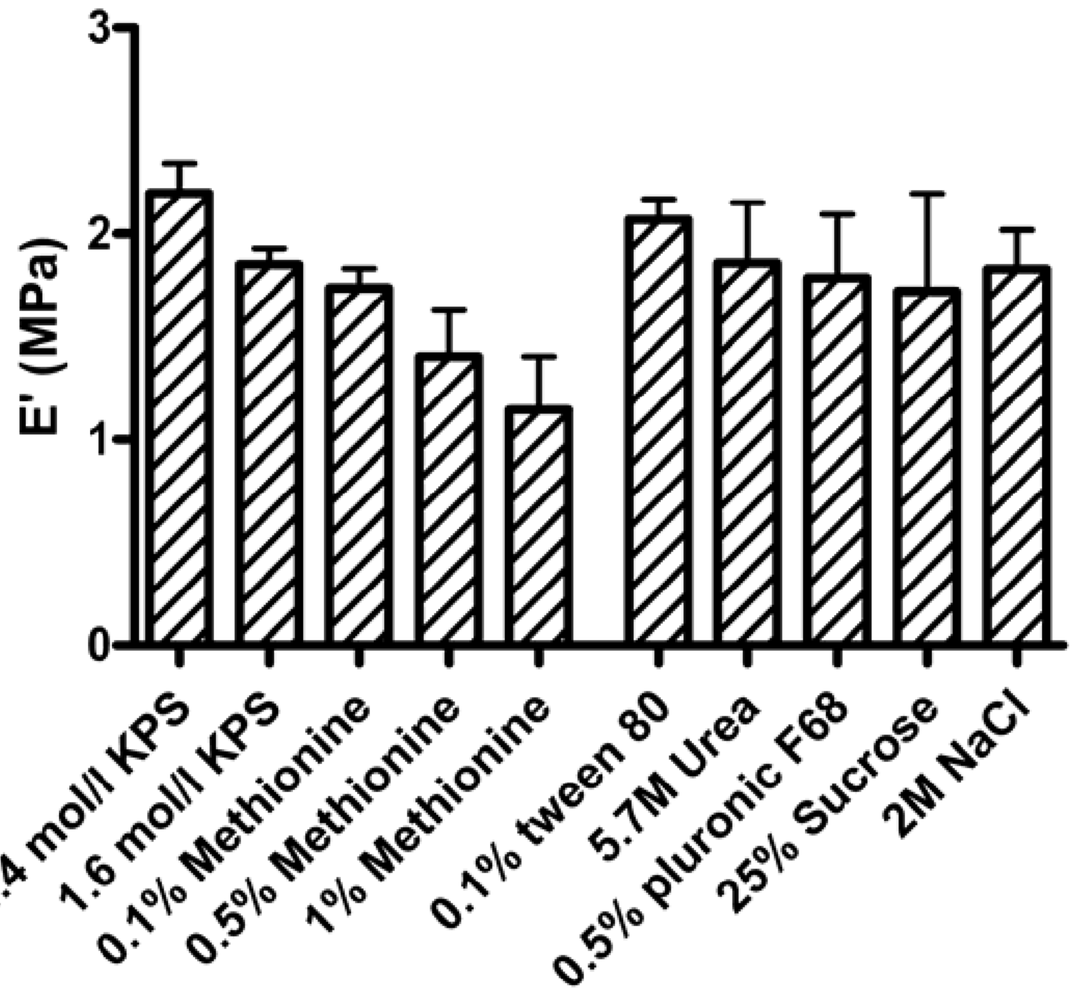
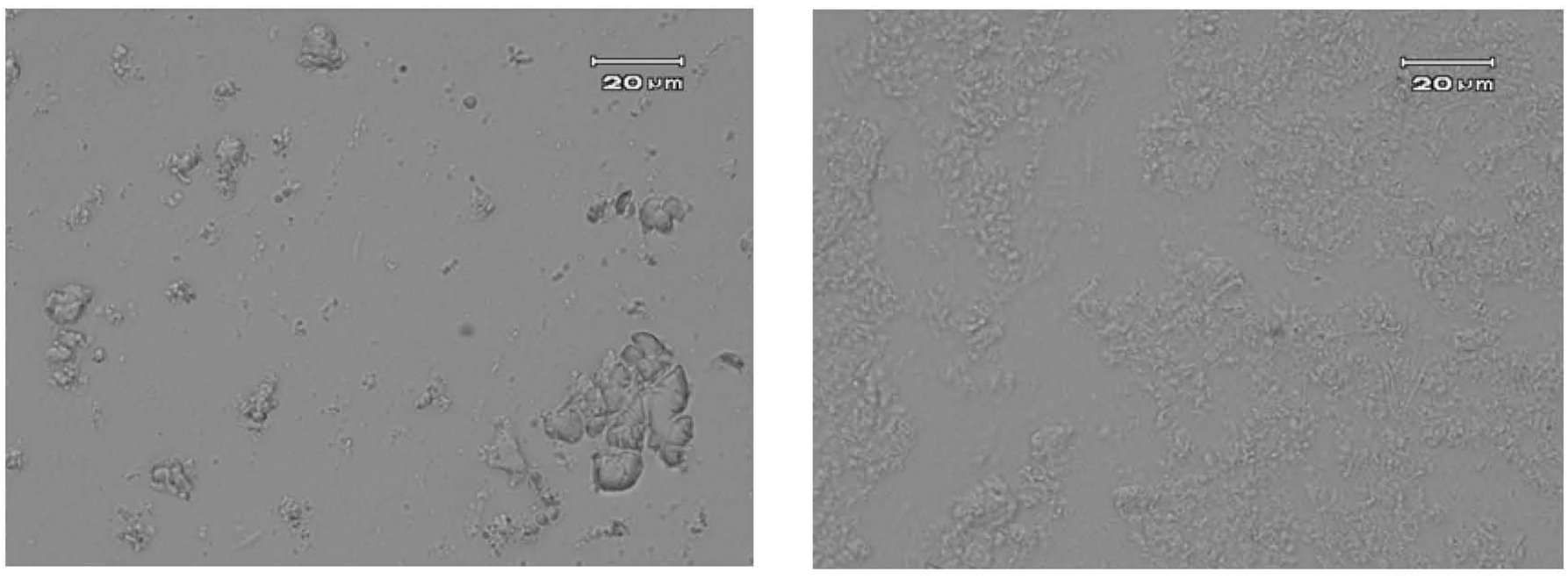


Figure 1. *Compression moduli (E') of macroscopic hydrogels prepared with different excipients, measured by DMA (average ±s.d., n=5).*

3.2. Precipitation of hGH

HGH readily precipitated when 30 % (w/w) dex-HEMA was added to a solution of 21 mg/ml hGH in 25 mM pH 7.4 phosphate buffer. Centrifugation of the samples and determination of the amount of hGH in the supernatant revealed that around 50 % of the hGH was precipitated. From the encapsulation efficiency of hGH and the water content of the microspheres (Table 1) it is calculated that the dex-HEMA and protein concentration in the dex-HEMA phase during microspheres preparation were much higher (40 % w/w and 52 mg/ml, respectively) than the protein and dex-HEMA concentration tested in this precipitation experiment. It is known that protein precipitation increases with an increasing protein concentration or an increasing concentration of precipitant[11,26]. Therefore, it is very likely that in the emulsified dex-HEMA droplets hGH precipitates are formed and that after polymerization, these precipitates are encapsulated in the microspheres.

The effect of NaCl on the precipitation of hGH in a 30 % w/w dex-HEMA solution was also investigated. First a solution of hGH (21 mg/ml) in 2M NaCl was prepared. In this solution no visible precipitation of hGH was observed. However, hGH readily precipitated when dex-HEMA (30 % w/w) was dissolved in this solution; only 30 % hGH of the initial amount of hGH was recovered in the supernatant, indicating that 70 % was precipitated. Light microscopy analysis shows that the precipitates formed in the presence of NaCl (~1μm) were about 10 times smaller than those formed in absence of NaCl (~10 μm) (Fig. 2). In general, the presence of NaCl stimulates preferential dehydration of proteins. Na^+ and Cl^--ions are excluded from the protein surface and therefore encourages the reduction of solvent exposed area[7-10]. Dextran is able to reversibly precipitate proteins in aqueous solution[26]. Its mechanism is explained by the excluded volume theory[11,27,28].



(a) *(b)*

Figure 2. *Microscopic image of (a) hGH precipitate obtained in a 30 % (w/w) dex-HEMA solution, (b) hGH precipitate obtained in a 30 % (w/w) dex-HEMA solution also containing 2M NaCl (magnification: 40x).*

In principle, at high concentrations of dex-HEMA or NaCl, a further reduction of the exposed area is favored by increasing the precipitate size. However, in situations of supersaturation (such as in the presence of both dex-HEMA and NaCl) precipitation by nucleation is favored over precipitate growth processes, which will result in smaller particles[29]. This implies that after polymerization, the microspheres prepared with NaCl contain smaller precipitates than the microspheres prepared without NaCl.

3.3. Encapsulation efficiency

Table 1 shows that the encapsulation efficiency of hGH in the microspheres of the standard formulation, and in the microspheres prepared with tween 80, pluronic F68, NaCl or methionine ranged from 47 to 68 %; the encapsulation efficiency of hGH in microspheres prepared with sucrose and urea was substantially lower (27 % and 19 %, respectively). The encapsulation efficiency of hGH depends on the amount of hGH in the dex-HEMA phase prior to polymerization which in turn is determined by the hGH partitioning over the dex-HEMA and the PEG phase. Partitioning of a dissolved protein in an aqueous PEG/dextran two-phase system depends on its net charge, its hydrophobicity, its solubility in both phases as well as on the type and concentration of ions present in the system[30-34]. In section 3.1.2 it was demonstrated that hGH precipitates in the dex-HEMA phase and consequently, the protein encapsulation efficiency is also dependent on the amount of protein that precipitates in the dex-HEMA phase. As the encapsulation efficiency of hGH in microspheres prepared with tween 80 (58 %), pluronic F68 (47 %), NaCl (56 %) or methionine (59-68 %) is similar to the encapsulation efficiency of hGH in the microspheres from the standard formulation (58%), apparently, these excipients do not affect the protein solubility and therefore its partitioning.

Denaturation of the protein results in exposure of the relatively hydrophobic core of the protein to the solvent. In general, an increased hydrophobicity of proteins results in an increased partitioning over the PEG phase[30-34], and consequently in a decreased concentration in the dex-HEMA phase, which explains the observed lower encapsulation efficiency of hGH in dex-HEMA microspheres prepared with urea as excipient. The reason for the low encapsulation efficiency of hGH in dex-HEMA microspheres prepared in the presence of sucrose is unclear. One might speculate that sucrose induces conformational changes of hGH and as a consequence, the hydrophobicity of hGH changes resulting in a lower encapsulation efficiency.

3.4. Release of hGH from dex-HEMA microspheres

In Fig. 3, the normalized release curves of hGH from dex-HEMA microspheres prepared with and without excipients are shown. Basically, two different types of release curves are observed. In the first type of release profiles, hGH was released over a period of 6 days (for microspheres prepared with NaCl, sucrose, pluronic F68, tween 80) or 8 days (microspheres prepared with urea). There was no burst release observed, except for microspheres prepared with pluronic F68, which showed a burst of around 15 % of the loaded dose. Microspheres prepared with the standard method showed a different release pattern. During the first two days ('delay time') hardly any hGH was released and thereafter, hGH was released with almost zero-order release kinetics during the following 6 days. For all formulations tested, the protein recovery (total amount of protein released after 12 days) was 90 % (Table 1).

Previous studies have shown that delay time in the release of proteins from dex-HEMA microspheres increases with a decreasing mesh size of the microspheres matrix, lower protein loading or by the presence of clusters of proteins in the microspheres[4-6]. Table 1 shows that microspheres prepared with tween 80, pluronic F68, NaCl, sucrose and urea have the same characteristics in terms of equilibrium water content and methacrylate conversion as the microspheres of the standard formulation. Moreover, Fig. 1 demonstrates that the compression modulus of the hydrogels is not affected by the presence of these excipients during polymerization.

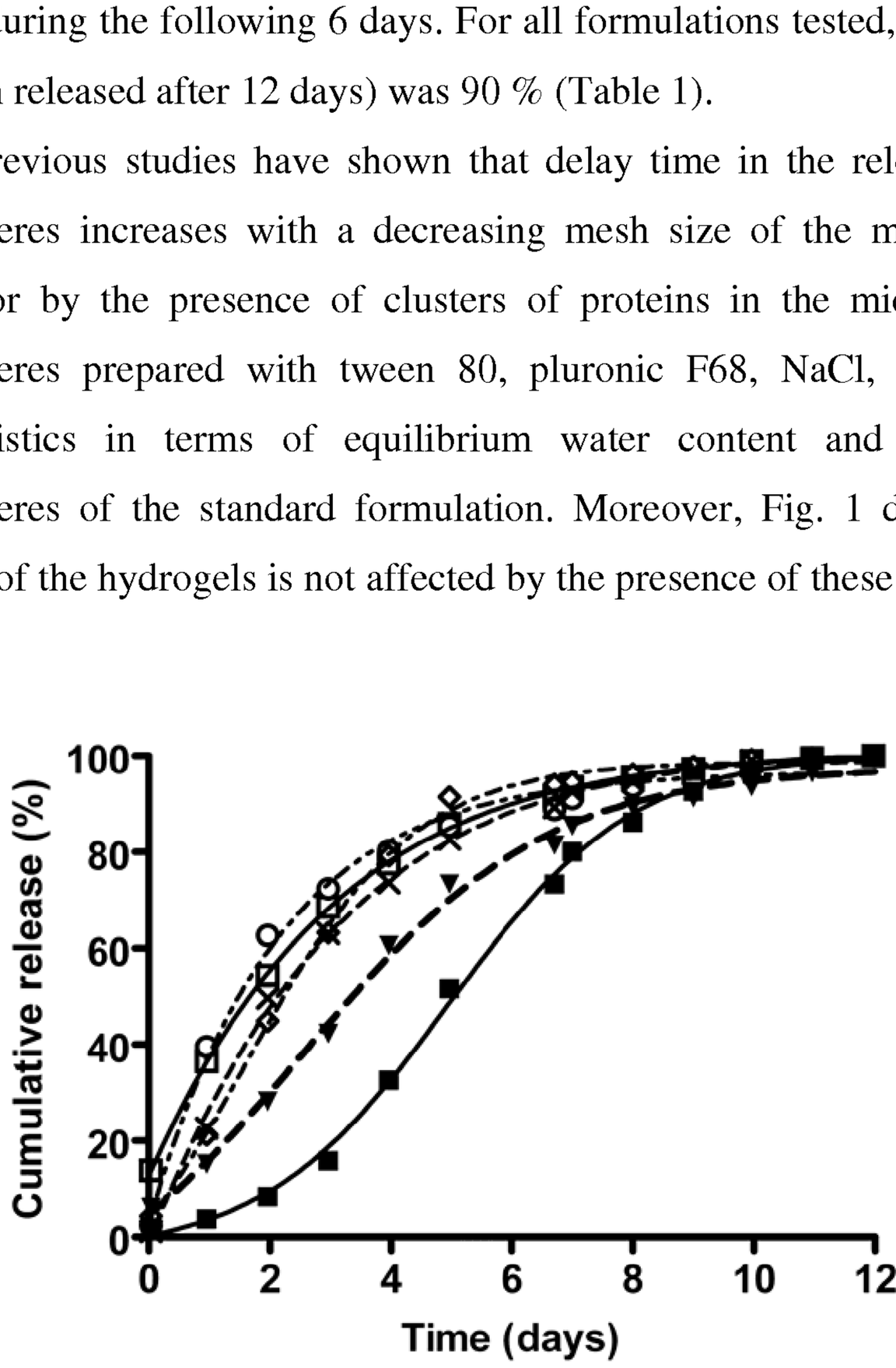


Figure 3. *Cumulative release of hGH from microspheres of the standard formulation (prepared with KPS 4.4 mmol/l)(■), urea 8M (▼), tween 80 0.1% (×), NaCl 2M (◊), pluronic F68 0.5 % (□), sucrose 25% (o). The values are the average of two independent measurements and deviated less than 5 %.*

This indicates that the different microspheres have the same mesh sizes. Moreover, the microspheres prepared with and without excipients have around the same encapsulation efficiency (50-70%) and recovery (90%), except for microspheres prepared with urea or sucrose, and hGH is mainly released as monomer (70-80%, Table 1). Consequently, the observed differences in release curves might be attributed to the presence of protein clusters in the microspheres. It is indeed shown that hGH precipitates in dex-HEMA (section 3.1.2) and that likely these aggregates are entrapped in the microspheres after polymerization. The results in section 3.1.2 also suggest that in microspheres prepared in the presence of NaCl smaller precipitates of hGH were encapsulated than in microspheres prepared in absence of excipients. In a previous study, it was indeed found that with increasing protein-cluster size, the release delay time was prolonged[6] which was explained by the restricted mobility of proteins inside a cluster. Nevertheless, the effects of cluster size on the release delay time were small, since in this model it was assumed that clusters of proteins consist of a group of monomeric proteins that were neither chemically nor physically linked and of which the protein molecules can move independently from each other once a sufficient number of crosslinks was hydrolyzed. In the present study it was shown that precipitates were encapsulated in the microspheres. This implies that once sufficient crosslinks are hydrolyzed to release a protein molecule, first the precipitate needs to dissolve before the dissolved protein molecules can be released. Likely, protein dissolution is a slow process in a matrix with a high concentration of dextran, and consequently, the delay time is increased due to the slow dissolution of the protein precipitate. To further explain this, a schematic presentation of the release of proteins from dex-HEMA microspheres containing large and small precipitates is given in Fig. 4. In Fig. 4a the release of protein from microspheres in which large precipitates are entrapped is indicated. At a certain time *t*, a certain number of crosslinks is hydrolyzed and there is an open network connection between the protein precipitate and the surface of the microsphere. However, to release all protein molecules from the precipitate, first the protein at position 1 needs to be dissolved and released, then protein 2, etc. In Fig. 4b the release of protein from microspheres in which small precipitates are entrapped is shown. At time t, the same number of crosslinks is hydrolyzed as in Fig. 4a, however, there are more open network connections between protein precipitates and the surface of the microsphere. Moreover, as the surface-to-volume ratio of a small precipitate is higher than that of a large precipitate and as a result more protein molecules are released in a certain time frame from microspheres with a small precipitate than from microspheres containing large precipitates.

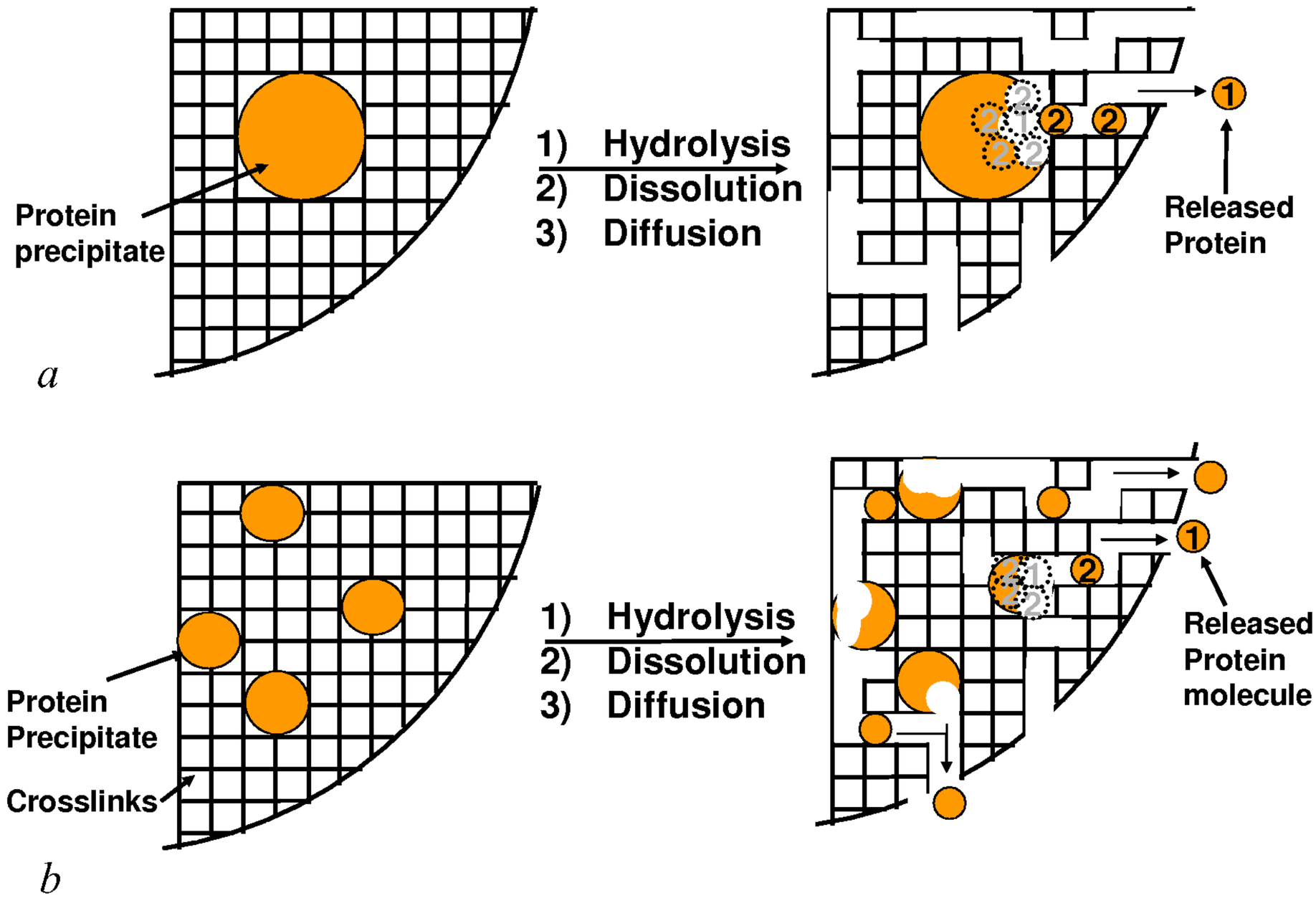
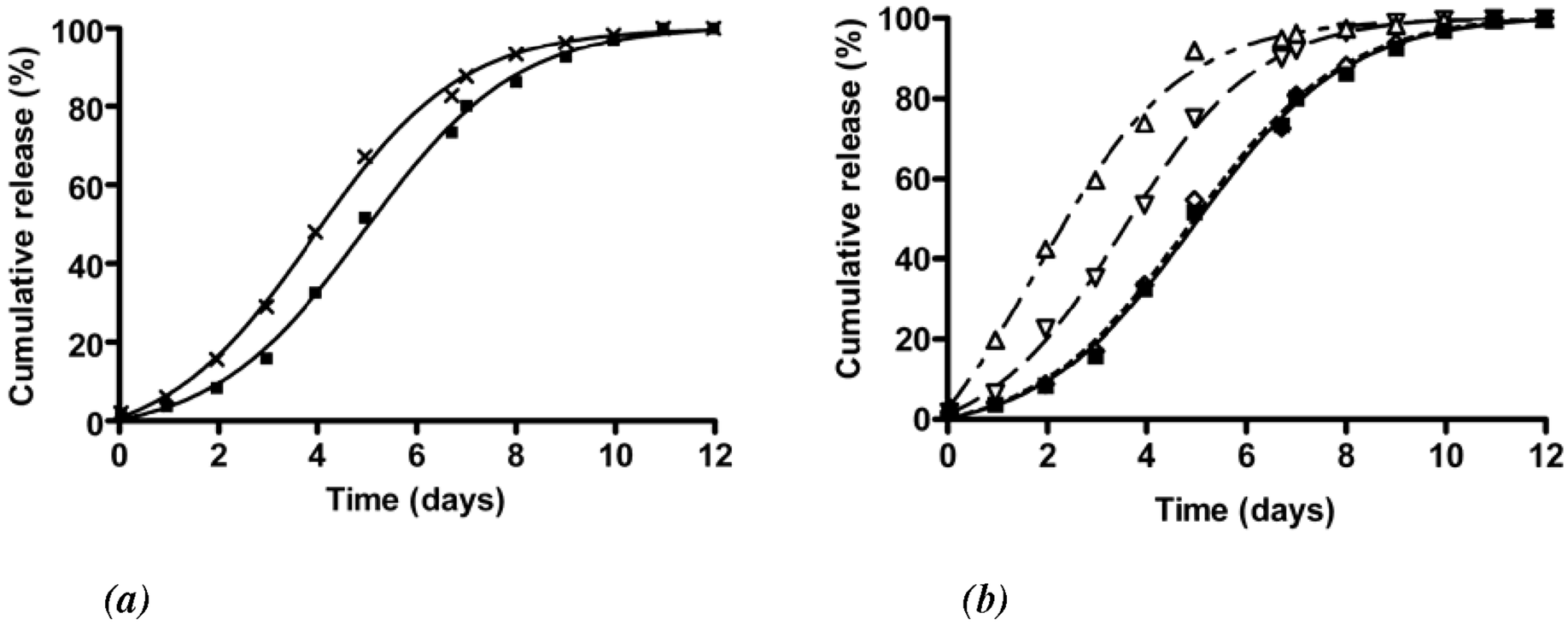


Figure 4. *Schematic representation of the release of protein molecules from a microsphere containing large protein precipitates (a), and small protein precipitates (b). (Note that each compartment (□) represents a number of crosslinks that needs to be hydrolyzed before a protein can enter this compartment[6]. The numbers indicate the (original) position of the protein molecule inside the precipitate).*

The slightly longer release time observed for microspheres prepared in the presence of urea as compared to the other microspheres (Fig. 3) is explained by the higher fraction dimer and multimers in the released amount of protein (73 % as dimer or multimer; Table 1), of which the released was delayed in time (data not shown). Removal of urea (due to washing of the microspheres) can result in incomplete refolding hGH or in the formation of dimers and multimers. Due to their larger size and lower diffusivity protein dimers (and multimers) release slower from the dex-HEMA matrices than monomeric protein molecules and explains the longer release time of hGH from the microspheres prepared with urea as excipient. The reason for larger fraction of dimers obtained in the release samples of microspheres prepared with sucrose is unclear. As indicated in section 3.1.4, one might speculate that sucrose induces conformational changes of hGH and as a consequence, after release more dimers are formed in the release buffer, after release from the microspheres.



(a) *(b)*

Figure 5 *Cumulative release of hGH from microspheres prepared with (a) 0% (■), 0.1 % (◊), 0.5 % (▽) and 1% (Δ) methionine and (b) 4.4 mol/l (■) and 1.6 mol/l (×) KPS. The reported values are the average of two independent measurements that deviated less than 5 %.*

In Figs. 5a and b the release profiles of hGH from dex-HEMA microspheres prepared with different methionine and KPS concentrations are shown. All microspheres except the ones prepared with 1 % methionine showed a biphasic release profile of hGH of which the delay time depended on the KPS or the methionine concentration. The release profiles of microspheres prepared under standard conditions or with 0.1 % methionine showed a delay time of 2 days. A delay time of one day was found for microspheres prepared with 1.6 mol/l KPS and 0.5 % methionine (Figs. 5a,b). For microspheres prepared with 1% methionine, no delay time was observed. Table 1 indicates that the methacrylate conversion and water content of microspheres prepared with and without methionine were similar whereas Fig. 1 shows that the compression modulus of dex-HEMA gels decreases with an increasing methionine concentration. A previous study has shown that a lower compression modulus indicates a lower crosslink density and therefore a larger mesh size[25]. This explains the shorter delay time for microspheres prepared with increasing methionine concentrations; a lower number of crosslinks needs to be hydrolyzed to release a protein from the microspheres.

Table 1 shows that with increasing methionine concentration, the methacrylate conversion remained high (>90 %, 0.5 % methionine) whereas Fig. 1 and 5a shows that both the compression modulus of the hydrogel network and the release delay time decreased. In contrast, a decreasing KPS concentration immediately results in a decreased methacrylate conversion (Table 1). Previous studies have shown that residual monomers are generally toxic[36,37] and it is therefore preferred that the methacrylate conversion is high. Taken together, it can be concluded that tailoring the delay time of the release of hGH without affecting the methacrylate conversion can be established by increasing the methionine concentration.

4. Conclusions

This study demonstrates that the encapsulation efficiency and the release of hGH from dex-HEMA microspheres can be modulated by the nature and concentration of the excipients. Especially, tween 80 and methionine are potential candidates to tailor/optimize the release of hGH as their encapsulation efficiency is high, the methacrylate conversion is almost quantitative and the protein is essentially released in its monomeric form.

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Chapter 6

The *in vitro* degradation behaviour of microspheres based on crosslinked dextran

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Abstract

The aim of this study was to investigate the *in vitro* degradation of hydroxyl ethyl methacrylated dextran (dex-HEMA) microspheres. Dextran microspheres were incubated in phosphate buffer pH 7.4 at 37 ^{o}C, and the dry mass, mechanical strength and chemical composition of the microspheres were monitored in time. The amount and nature of the formed degradation products were established for microspheres with different crosslink densities by FT-IR (Fourier transformed infrared spectroscopy), NMR, mass spectrometry, SEC analysis and XPS (X-ray photoelectron microscopy). Dex-HEMA microspheres DS 12 (degree of HEMA substitution; the number of HEMA groups per 100 glucose units) incubated at pH 7.4 and 37 ^{o}C showed a continuous mass loss, leaving after 6 months a residue of about 10 % (w/w) of water-insoluble products. NMR, mass spectrometry and SEC showed that the water-soluble degradation products consisted of dextran, low molecular weight pHEMA (M_n ~ 15 kg/mol), and small amounts of unreacted HEMA and HEMA-DMAP (intermediate reaction product of the Baylis-Hillman reaction of HEMA with DMAP (4-dimethyl aminopyridine)). Microscopy revealed that the water-insoluble residue consisted of particles with a similar shape and size as non-degraded microspheres. However, these particles had lost their mechanical strength as evidenced from micromanipulation experiments. FT-IR and XPS revealed that these particles consisted of pHEMA of which a small fraction was soluble in methanol (M_n ranging between 27-82 kg/mol). The insoluble material likely consisted of lightly crosslinked pHEMA. In conclusion, *in vitro* degradation of dex-HEMA microspheres results in the formation of water-soluble degradation products (mainly dextran), leaving a small water-insoluble residue mainly consisting of pHEMA.

1. Introduction

Biodegradable polymeric microparticles have been intensively investigated as controlled release systems for pharmaceutically active proteins[1]. A major advantage of these microparticles is the possibility to tune the release rate of the proteins by their degradation rate[1-3]. Moreover, there is no need for surgical removal of these systems after they have released their payload. In general, the suitability of devices based on biodegradable polymers as a protein releasing matrix depends not only on the possibility to tune the release rate, but also on the degradation time, and the biocompatibility of the polymers and their degradation products[2,3]. Therefore, it is important to have good insight into the nature of the products that are formed during degradation of the drug delivery system.

Systems based on poly(D, L-lactic-co-glycolic acid) (PLGA) have been widely studied as controlled release system, because of their biodegradability and good biocompatibility. Under physiological conditions, these systems ultimately degrade into lactic and glycolic acid, which may enter the Krebs Cycle or are excreted through the normal pathways. However, there are a number of problems associated with the application of these systems for protein delivery, among which the use of organic solvents for preparation of the protein loaded particles, the pH drop during degradation and subsequently incomplete release as a result of undesired protein aggregation/degradation[4-7]. Similar problems were also observed for microspheres based on other hydrophobic biodegradable polymers such as poly(orthoesters) (POE) and poly(anhydrides)[8-11]. The hydrophobic character of these polymers requires protein unfriendly formulation techniques such as solvent removal or hot melt encapsulation[12,13].

Owing to their high water content, hydrogels are generally compatible with both living tissue and proteins[14-17]. Therefore, hydrogel-based microspheres are a good alternative for the microsphere systems based on hydrophobic polymers. Franssen and Stekenes et al. described a method to prepare hydrogel-based dextran microspheres in an all aqueous system, thereby avoiding the use of organic solvents[18,19]. With this method, microspheres were obtained by polymerization of aqueous droplets containing methacrylated dextran in a continuous poly(ethylene glycol) (PEG) phase. However, these microspheres were essentially non-degradable without the addition of a matching enzyme (dextranase)[20,21]. Biodegradable microspheres, that contain an additional carbonate ester in their crosslinks, which are hydrolytically sensitive under physiological conditions, were obtained by crosslinking hydroxyethyl methacrylated dextran (dex-HEMA)[22]. Important features of these microspheres are their high encapsulation efficiency for proteins, a low or even absent burst release and the possibility to tailor the release profiles by the degree of

substitution of dex-HEMA (DS, number of HEMA groups per 100 glucose units) and the initial water content of the microspheres[22-24]. *In vivo* studies performed by Cadée et al. showed these microspheres to be biocompatible[24].

Previous studies have shown that dex-HEMA microspheres degrade under physiological conditions through hydrolysis of the carbonate esters linking dextran and pHEMA[22,24-30]. Therefore, dextran and pHEMA are the expected degradation products. It was indeed demonstrated that dextran, a water-soluble polymer, was released from dex-HEMA microspheres and macrogels when the main fraction of crosslinks was hydrolyzed[22,24-30]. However, the solubility and molecular weight of the expected pHEMA degradation products have not been studied so far. Recently, Weaver at al. reported that the solubility of pHEMA depends on the degree of polymerization (DP)[31]. Homopolymers up to a DP of 20 are water-soluble, whereas higher molecular weight pHEMA is water-swellable rather than water-soluble. Therefore, the degradation of dex-HEMA microspheres may result in the formation of water-insoluble degradation products.

The aim of this study was to investigate the *in vitro* degradation of hydroxylethyl methacrylated dextran (dex-HEMA) microspheres. Dextran microspheres with different crosslink densities were incubated in phosphate buffer pH 7.4 at 37 °C, and the mass, mechanical strength and chemical composition of the microspheres were monitored in time. The amount and nature of the formed soluble degradation products were established by FT-IR, NMR, XPS, mass spectrometry, and SEC analysis.

2. Materials and methods

2.1 Materials

Poly(ethylene glycol) (PEG) 10 kg/mol, potassium peroxodisulfate (KPS), cesium iodide (CsI), sodium iodide (NaI) and potassium bromide (KBr) were obtained from Merck, Darmstadt, Germany. Dextran 20 kg/mol and 40 kg/mol, N,N,N',N'-tetramethylethylenediamine (TEMED), 4-dimethylaminopyridine (DMAP) and hydroxyethyl methacrylate (HEMA) were purchased from Fluka (Buchs, Switzerland). 2,2'-Azobis(isobutyronitrile) (AIBN) was obtained from Polysciences, Inc. (Eppelheim, Germany). Hydroxyethyl methacrylate derivatized dextrans (dex-HEMA) with a DS of 8, 12, 16 and 20 were synthesized and characterized according to Van Dijk-Wolthuis et al.[32]. The degree of HEMA substitution (DS, the number of HEMA groups per 100 glucose units) was determined by proton nuclear magnetic resonance. Disposable dialysers based on cellulose mixed

esters membrane MWCO 50 kg/mol were obtained from Sigma-Aldrich (Buchs, Switzerland). DMSO-d_6 and methanol-d_4 were obtained from Cambridge Isotope Laboratories (Andover, USA).

2.2. Preparation and degradation of the microspheres

2.2.1. Preparation of the microspheres.

To analyze the nature and the quantity of both the soluble and insoluble degradation products of dex-HEMA microspheres with different crosslink densities, different batches of microspheres (DS 8, 12, 20; fixed water content of 50%) were prepared at 100 g scale (yielding 1.2 g dry dex-HEMA microspheres). To collect enough sample material to accurately determine the mass loss and to follow the evolution of the chemical composition, the mechanical strength and the morphology of the microspheres in time, one batch of microspheres (DS 12, initial water content 50% (w/w)) was prepared at 1 kg scale (yielding 12 g dry dex-HEMA microspheres). The preparation of the microspheres at 100 g and 1 kg scale was essentially done as described elsewhere[22]. In detail, for a 100 g batch, 12 g of a 10 % solution of dex-HEMA (DS 8, 12 or 20) in 10 mM phosphate buffered saline pH 7.0 (PBS) was added to a solution of PEG in PBS (22.4 g, 40 % (w/w)) and PBS was added to a final mass of 100 g, resulting in a PEG/dex-HEMA volume ratio of 40:1, based on PEG/dex-HEMA phase diagrams.[14] The PEG/dex-HEMA/water mixture was emulsified for 55 minutes at 1500 rpm using a three bladed impeller (2 cm in diameter). Next, 4 ml TEMED solution (20 % (v/v), pH adjusted to 7.0 with 4 M hydrochloric acid), was added and stirring was continued for 5 minutes. The stirring rate was set to 500 rpm, 2.7 ml of 50 mg/ml KPS solution was added and stirring was continued for 1 more minute. Then, the stirring was stopped and the emulsion was incubated at room temperature for 60 minutes to polymerize the dextran grafted HEMA groups. The microspheres were purified with water by 5 centrifugation (3200 x g, 20 min) and washing steps (15 ml water). Finally, the microspheres were resuspended in a minimal volume of water and freeze-dried. For the preparation at 1 kg scale 10 fold larger amounts were used. The emulsification was performed in a 2 L reaction vessel with a three bladed impeller (4.5 cm in diameter) and a stirring speed of 1500 rpm. Average particle diameter and size distributions of the microspheres were measured with a Malvern Mastersizer 2000 (Malvern instruments Ltd, Worcestershire, United Kingdom) with water as eluent. After preparation, the methacrylate conversion was determined as described previously[33].

2.2.2. Degradation of dex-HEMA microspheres

Freeze-dried microspheres (DS 12, 2 g) were rehydrated in 5 ml PBS containing 0.02 % NaN_3 (pH 7.4) and transferred into a disposable dialyser. Six dialysers, each filled with 5 ml

microsphere suspension, were transferred into a vial containing 500 ml buffer (10 mM PB, 100 mM NaCl, 0.02% NaN_3, pH 7.4) and subsequently incubated at 37ºC. At regular time intervals, one of the dialysers was removed and the entire incubation buffer was refreshed. The concentration of dextran in the removed incubation buffer was determined with SEC. Cumulative release curves were obtained by summation of the amount of dextran released at each time point. The microspheres from the removed dialyser were analysed using a light microscope (microscope with D12 digital camera, Olympus Optical Co. Shibuya, Tokyo, Japan). Hereafter, the microspheres were extensively washed with water (15 ml, 5 times centrifugation, 3200 x g, 20 min) and freeze-dried. The mass of the freeze-dried microspheres was determined. The freeze-dried microspheres were analysed with Fourier transformed infrared spectroscopy (FT-IR) and X-ray photoelectron microscopy (XPS). The mechanical strength of the microspheres after rehydration was analysed with a micromanipulation technique as described by Stenekes et al.[34].

2.2.3. Quantification and analysis of degradation products

To collect, quantify and identify the water-soluble and water-insoluble degradation products, dex-HEMA microspheres (DS 8, 12 and 20) were hydrolyzed under accelerated conditions. In detail, 240 mg freeze dried microspheres were suspended in 4 ml 0.1 M NaOH and incubated for 5 days at 70 °C. Next, the suspension (pH adjusted 7.0 with 0.1 M HCl) was centrifuged (3200 x g, 5 min) and the supernatant (fraction A) and the insoluble residue (fraction B) were separated. Fraction A was freeze-dried until about 2 ml remained and subsequently 8 ml methanol was added. The sample was centrifuged to separate the methanol-soluble part (A2) from the methanol-insoluble part (A1). Sample A1 was washed several times with methanol to remove residual methanol-soluble degradation products, dried and weighed. Sample A2 was dried in air and weighed. The molecular weight and molecular weight distribution of the polymers in sample A1 dissolved in water and A2 dissolved in DMF were determined using SEC (see section 2.3.1.) and their chemical composition was established with NMR (see section 2.3.4).

Fraction B was washed several times with water to remove residual water-soluble compounds. Then, 16 ml methanol was added to the water-insoluble part (B) to obtain a methanol-soluble (B1) and a methanol-insoluble fraction (B2). The methanol-insoluble fraction (B2) was washed several times with methanol, dried, weighed and analyzed by FT-IR. The methanol in fraction B1 was almost completely removed (room temperature) until about 1 ml of this solution remained. The molecular weight and the molecular weight distribution of the polymer in this fraction were determined with SEC as described in section 2.3.1. Then, the samples were further dried in air and the chemical composition was established with FT-IR (see section 2.3.6).

2.2.4. Analysis and release of HEMA-DMAP

Ten mg microspheres (DS 8, 12 or 20) were incubated at room temperature in 5 ml 10 mM NH_3 solution (pH 9) at room temperature for 3 days. At regular time intervals, the suspension of degrading microspheres was centrifuged (3200 x g, 20 min) and the supernatant was analyzed by HPLC (see section 2.3.2) and by ESI-MS (see section 2.3.3). As a control, 10 mg dex-HEMA was incubated under the same conditions and the concentration HEMA-DMAP determined with HPLC (see section 2.3.2).

2.3. Analyses

2.3.1. Size exclusion chromatography

SEC analyses of the water-soluble degradation products were performed with a system consisting of an HPLC pump series 200, a vacuum degasser series 200, an autosampler series 200, a column LC 101 oven, a differential refractometer PL-RI 800 (all Perkin Elmer Instruments, Norwalk, USA) and 2 thermostated (35°C) Shodex KB-800 series columns (OHpak KB-800P, 6 mm x 50 mm, guard column; OHpak KB-806M 8mm x 300mm, exclusion limit $2 \cdot 10^7$; Showa Denko, Tokyo, Japan). The flow rate was 0.7 ml/min and the mobile phase was an aqueous solution of 20 mM phosphate buffer pH 7.0 also containing 200 mM NaCl. The columns were calibrated with dextran standards of known molecular weight and narrow molecular weight distribution (Fluka Chemie AG). The chromatograms were recorded and analyzed with data acquisition system Perkin Elmer Totalchrom (Perkin Elmer Instruments, Norwalk, USA).

The methanol-soluble degradation products were analyzed using the same HPLC system. The PLgel 5µm MIXED-C columns (Polymer Laboratories Inc, Amherst, USA) were thermostated at 50 °C. The flow rate was 0.7 ml/min and the mobile phase consisted of DMF with 0.02 M LiCl. The columns were calibrated with PEG standards of known molecular weight and narrow molecular weight distribution (Fluka Chemie AG).

2.3.2. Reversed-phase high performance liquid chromatography

Reversed-phase HPLC was used to determine the methacrylate conversion of the microspheres and to determine the concentration HEMA and HEMA-DMAP in the release samples (section 2.2.4). The HPLC system consisted of a pump series 200, a vacuum degasser series 200, an autosampler series 200, a column LC 101 oven, and a UV detector series 200 (all Perkin Elmer Instruments, Norwalk, USA) and a thermostated (35 °C) RP-18 column (Symmetry C18, 4.6x150 mm) with an 8 x 3 mm precolumn (all Waters, Ireland). The flow rate was 1.0 ml/min and a mobile phase of water/acetonitrile 10/90 v/v (adjusted to pH 2.0 with perchloric acid) was used. A

calibration curve was obtained by injecting 50µl of 0.5-25 µg/ml MA or HEMA. The concentration of HEMA-DMAP was determined using a calibration curve of DMAP obtained by injecting 50 µl in the concentration range 0.5-25 µg/ml. The chromatograms were recorded and analysed with data acquisition system Totalchrom (Perkin Elmer Instruments, Norwalk, USA).

2.3.3. Electrospray mass spectrometry

The samples obtained by degradation of microspheres in an aqueous NH_3 solution (see section 2.2.4.) were injected on a tandem quadrupole mass analyzer Micromass Quattro LC (Micromass, Cheshire, United Kingdom). The cone voltage was 30 V, the capillary voltage was 2.8 kV, the scan range was m/z 0-1500, the flow rate was 4 µl/min, the source temperature was 60 ºC and the desolvation temperature was 80 ºC. Positively charged ions were produced using an electrospray probe. Calibration was done with a mixture of 96 mg NaI and 2.5 mg CsI in 50 ml 2-propanol. For MS/MS analysis, the collision energy was 25 eV and argon was used as collision gas. The mass spectra were analysed using Masslynx Software (Micromass, Cheshire, United Kingdom). The exact mass measurements were carried out with a Micromass LC ToF (Micromass, Cheshire, United Kingdom) using nano-electrospray ionisation. Cone voltage was 30 V and capillary voltage 1.2 kV. Calibration was performed with PEG 400 in acetonitrile/water containing 20 mM ammonium acetate.

2.3.4. ^{1}H-NMR spectroscopy

NMR spectra were recorded with a Gemini 300 MHz spectrometer (Varian associates Inc. NMR instruments, Palo Alto, CA, USA). Approximately 30 mg of material was dissolved in 0.8 ml of a suitable solvent. For measurements in methanol, the signal at 3.31 ppm was used as the reference line, whereas in DMSO-d_6/D_2O (7:1), the central DMSO line was set at 2.5 ppm.

2.3.5. Micromanipulation

The mechanical properties of the microspheres were measured by a micromanipulation technique. Briefly, a single microsphere was compressed between two parallel surfaces to a certain deformation. Simultaneously, a force transducer measured the force being imposed on the microspheres as function of the applied deformation. The details of this technique are described elsewhere.[34-38]

2.3.6. Infrared Spectroscopy

The Fourier transformed infrared spectra (FT-IR) of the freeze-dried microspheres and the water-insoluble degradation products (fraction B1 and B2 after drying; section 2.2.3) were recorded in transmission mode on a Bio-Rad FTS6000 FT-IR spectrometer with Win-IR Pro software (Cambridge, MA, USA). Spectra were obtained from a KBr tablet compressed at 10 tons with a hydraulic press. Each tablet contained 2 % (w/w) of microspheres or reference polymer (dextran, pHEMA or a mixture of both). Scans (128) were co-added at 2 cm^{-1} resolutions at a scan speed of 0.16 cm/s (5 kHz laser modulation). The spectra were corrected for water vapor at 3700-3800 cm^{-1}. PHEMA was obtained by polymerization of HEMA (4 g, 30.1 mmol) with AIBN (0.52 g, 3.2 mmol) in 40 ml of freshly distilled dioxane at 80 ºC for 2 hours. After this, pHEMA was precipitated in hexane and dried under vacuum at 40 ºC. The spectra obtained from the pHEMA and dextran (different mass ratios) were used to calculate the composition of the different microsphere samples. The composition of degraded microspheres was determined by comparing the peak heights to a reference sample of a mixture of pHEMA and dextran of a known composition.

2.3.7. X-ray photoelectron spectroscopy

To analyze the surface composition of non-degraded and degraded microspheres, X-ray photoelectron spectroscopy (XPS) analyses were performed. XPS is a surface sensitive technique, with a probing depth in the range of 1-5 nm. The XPS C*1s* spectra were obtained using a Vacuum Generators CLAM-2 hemispherical analyzer operating at 100 eV pass energy. A Mg Kα source (Vacuum Generators twin-anode XR2E2) was used at 120W. The angle between the surface and the analyzer axis was 15° and the angle between the analyzer and the X-ray source axis was 33.5°. For XPS analysis, the freeze dried dex-HEMA microspheres were hydrated in a small amount of water and the resulting paste was spread onto the XPS sample plate and dried in air. Samples of pHEMA, dextran and degraded microspheres were obtained by powder compression with a pressure of 10.4 kg/cm^2 using an infrared tablet press. The C*1s* spectra were subsequently recorded 3 times to check for the absence of sample degradation due to radiation and charging stability during analysis. The spectra obtained from the pHEMA and dextran (different mass ratios) were used to calculate the surface composition of the different microsphere samples.

Assuming that the outer layer of a microsphere is a mixture of pHEMA and dextran, the resulting signal intensity $I(E_{tot})$ can be considered as a superposition of the pHEMA and dextran signals according to:

$$I(E)_{tot} = cI(E)_{dexran} + (1-c)I(E)_{pHEMA} \quad (1)$$

In this equation, $I(E)_{dextran}$ and $I(E)_{pHEMA}$ are the signal intensities of dextran and pHEMA respectively, E is the binding energy of the electrons and c is the fraction of dextran in the mixture.

3. Results and Discussion

3.1. Characterization of the prepared microspheres and release of HEMA and HEMA-DMAP

In table 1, the water content, particle size and methacrylate conversion of the different microsphere formulations are given. The volume mean diameter ranged between 19 and 32 μm. The dex-HEMA microspheres were larger than the microspheres previously prepared by Franssen et al. (10 μm),[22] which might be attributed to the difference in manufacturing scale. The microspheres in Ref. 22 were prepared at a 5 g scale and emulsification was performed with a vortex (at 1500 rpm). In the present study, the microspheres were prepared at 100 g scale in a reactor equipped with an impeller stirring at 1500 rpm. The use of a larger amount of reaction mixture (100g), and different dimensions of the reaction vessel can results in a in a lower shear stress per volume emulsion, which in turn results in larger spheres.[19] In agreement with previous findings, the methacrylate conversion exceeded 90% for all formulations.[33]

Table 1. *Particle size and methacrylate conversion of microspheres.*

DS	process scale	volume mean diameter (μm)	methacrylate conversion (%)
8	100 g	19	98
12	100 g	20	92
12	1 kg	23	94
20	100 g	32	90

The values are the mean of 3 independent measurements that deviated less than 10 %.

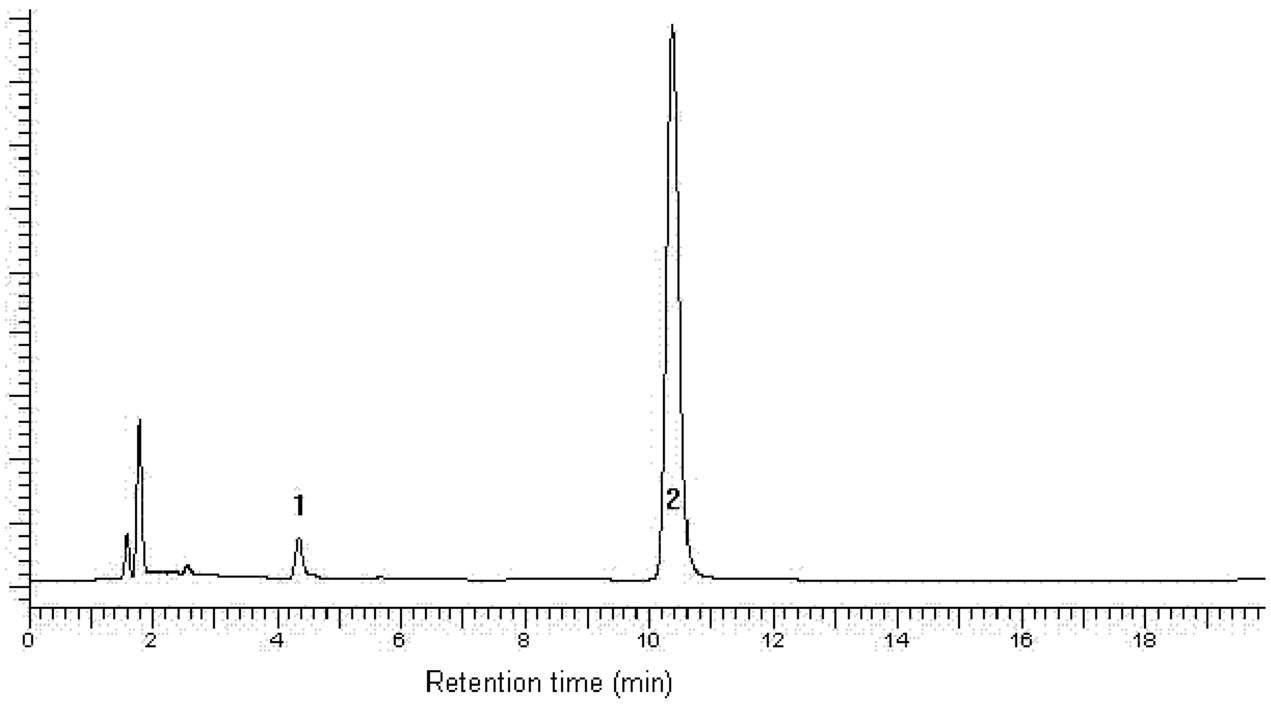


Figure 1. *RP-HPLC chromatogram of an accelerated degradation sample (t = 1h, pH=9) containing HEMA-DMAP (1) and HEMA (2) and buffer salts (peaks eluted before Rt=2 min).*

HPLC-analysis of the water-soluble degradation products samples revealed besides the presence of HEMA (rt = 10.5 min) also that of another molecule (rt = 4.4 min, λ_{max}=289 nm) (Fig. 1). It was found that both compounds were released from the microspheres at the same rate during 2 days of incubation in a 10 mM NH_3 pH 9 at room temperature, which suggests that this compound, like HEMA, was coupled to dextran via a carbonate ester. The exact mass of this compound as established with LC/MS/MS was 252.1552, which is in agreement with the bruto formula $C_{13}H_{20}N_2O_3$. This suggests that this molecule may be the protonated reaction product formed by the addition reaction of HEMA ($C_6H_{10}O_3$, Mw 130 g/mol) to DMAP ($C_7H_{10}N_2$, Mw122 g/mol), which is used as a catalyst for the coupling of HEMA-imidazole carbamate (HEMA-CI) to dextran[32]. Fig. 2 shows the MS/MS spectrum and the possible structure of the HEMA-DMAP molecule, as derived from this spectrum. The ions in this MS/MS spectrum at *m/z* 209 and 123 in the MS/MS spectrum are either formed by cleavage of respectively ethanal from the HEMA-DMAP adduct ($[M+H-44]^+$) and further cleavage of methacrylic acid from the ion *m/z* 209 or formed by immediate cleavage of HEMA from HEMA-DMAP ($[M+H-130]^+$). The ion at *m/z* 136 is formed by cleavage of acrylic acid from the ion at *m/z* 209.

Most probably, the reaction of HEMA with DMAP to form HEMA-DMAP is based on the Baylis-Hillman reaction of an activated alkene or alkyne with an aldehyde or ketone (scheme 1)[39]. Baylis-Hillman reactions usually require Lewis bases, such as tertiary amines, e.g. 1,4-diazabicyclo[2,2,2]octane (DABCO) and DMAP, as catalysts[39,40]. Likely, the HEMA-DMAP adduct (Scheme 2) is an intermediate of the Baylis-Hillman reaction, which is stabilized by the intramolecular salt-bridge that is formed between the positively charged quaternary N-atom and the negatively charged enolate anion.

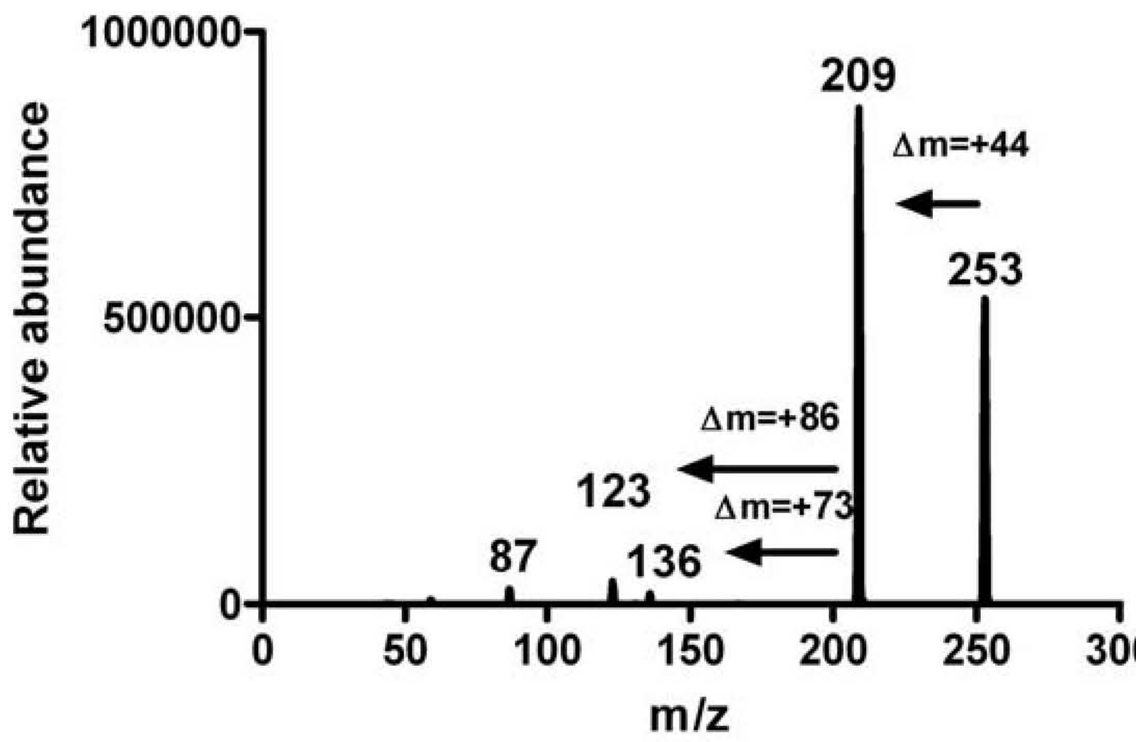
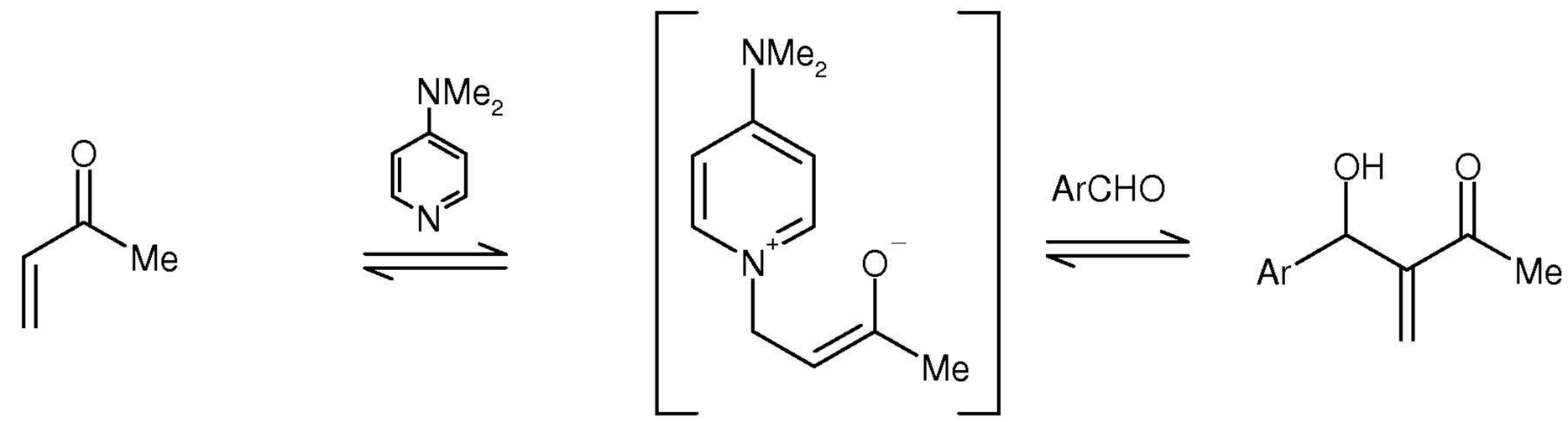
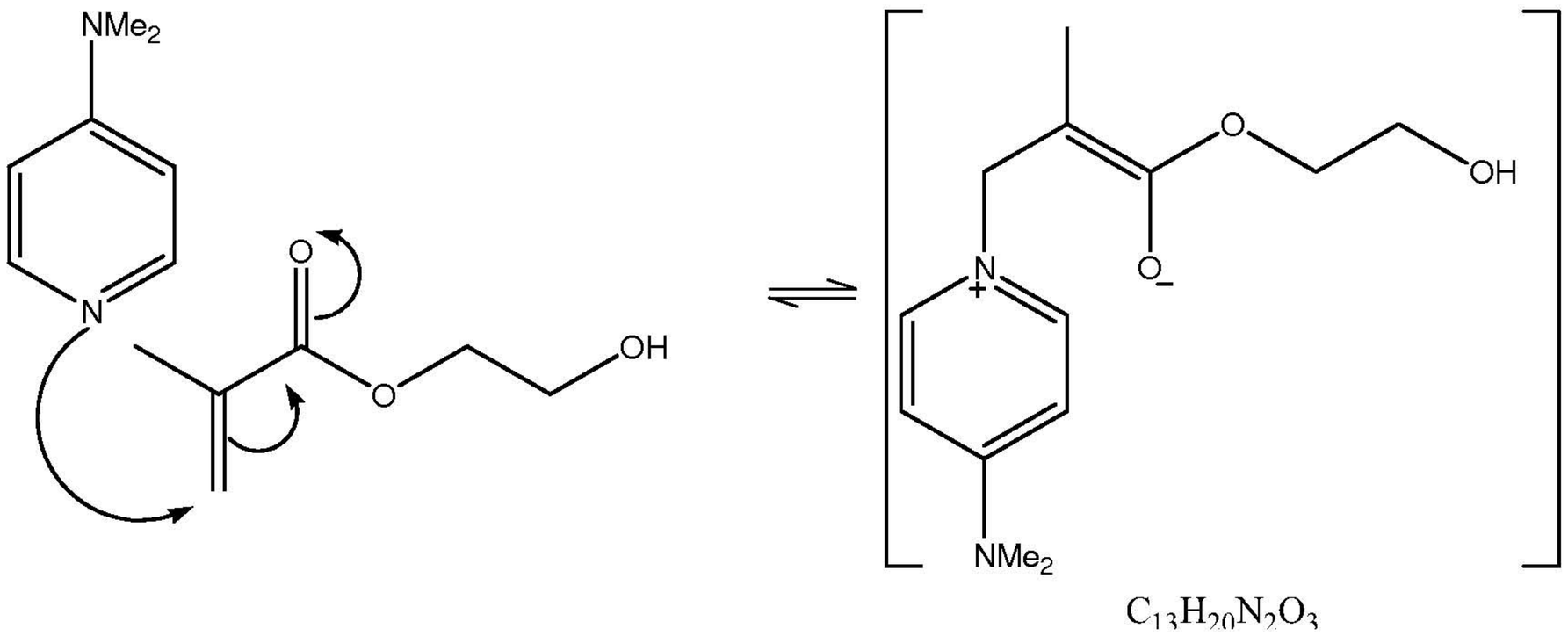


Figure 2. *The MS/MS spectrum of the Baylis-Hillman intermediate of HEMA and DMAP (right).*



Scheme 1. *The Baylis-Hillman reaction of a methyl vinyl ketone with an aryl aldehyde catalyzed by DMAP.*



Scheme 2. *The Baylis-Hillman reaction of DMAP with HEMA.*

3.2. Degradation of dex-HEMA microspheres and analysis of degradation products.

To study the *in vitro* degradation of dex-HEMA microspheres, the mass loss and dextran release were determined for microspheres of dex-HEMA DS 12 (water content 50%) at pH 7.4 and 37 °C (Fig. 3). This figure shows that the microspheres lost 94 % of their mass in 6 months. After this period, no further degradation was observed (results not shown) leaving a solid residue of 6 % of the initial mass of the microspheres. As mentioned in the introduction, the expected degradation products of the microspheres are dextran and pHEMA. It is also shown in Fig. 3 that dextran was released quantitatively within 6 months, which suggests that the solid residue consists of pHEMA. In addition, it can be calculated that the expected mass loss for microspheres of dex-HEMA DS 12 after complete release of dextran is 91 %. This might indicate that during the degradation besides dextran, a small amount of water-soluble pHEMA is released.

The chemical composition of the solid residue present at 2.8 months and 5.7 months was analyzed with FT-IR (Fig. 4a) and compared to that of non-degraded microspheres. The most significant bands resolved in the spectrum of the non-degraded microspheres are: 1726 cm^{-1} [ν(C=O)], 1150 cm^{-1} [ν(C-O)], 1162 cm^{-1} [$\gamma(CH_3)$, τ(OH)], 1050 cm^{-1} [δ(C-OH)] and 980 cm^{-1} [ν(C-O) and δ(C-OH)] in which ν is bond stretching, τ is torsion, δ is bending, γ rocking.[41-43]. It is clearly visible that in the spectra of the solid residue which remained at 2.8 months and 5.7 months of degradation the intensity of the band at 1726 cm^{-1} had increased and the peaks at 1074 cm^{-1} [ν(C-O), alcohol] and 1021 cm^{-1} [ν(C-O) ester] were more resolved as compared to those in the spectra of non-degraded microspheres. To enable the interpretation of these data properly, FT-IR spectra of pHEMA, dextran and different mixtures of dextran and pHEMA were taken (Fig. 4b).

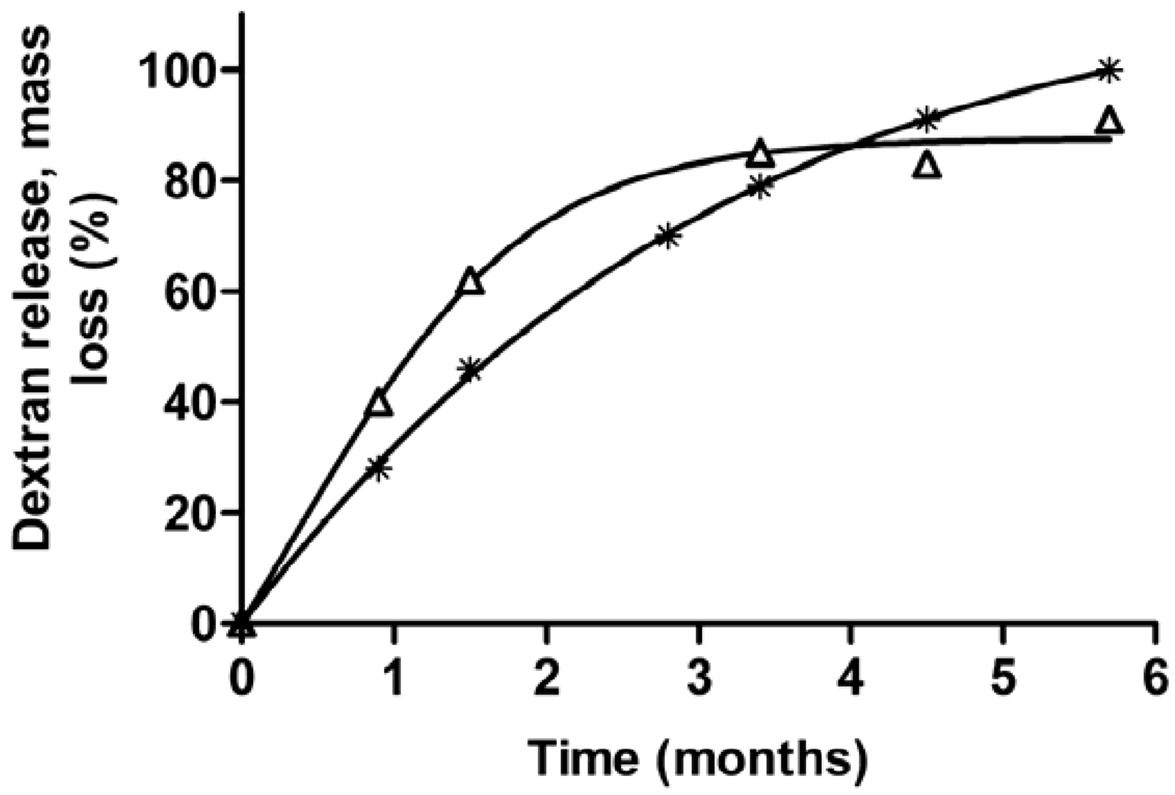
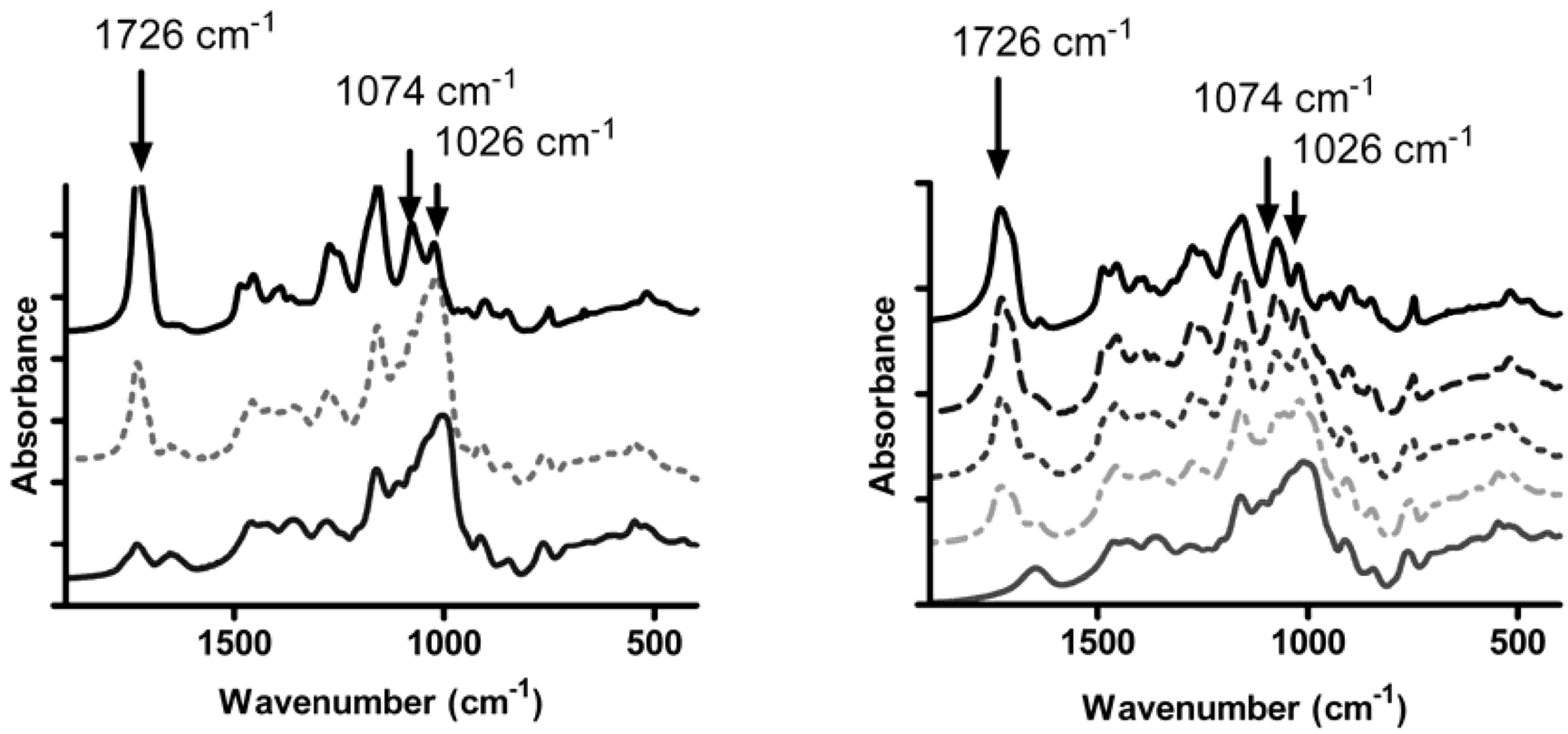


Figure 3. *Dextran release (∗) and mass loss (Δ) of dex-HEMA microspheres (DS 12, initial water content 50 %) incubated at 37 °C and pH 7.4 as a function of time.*

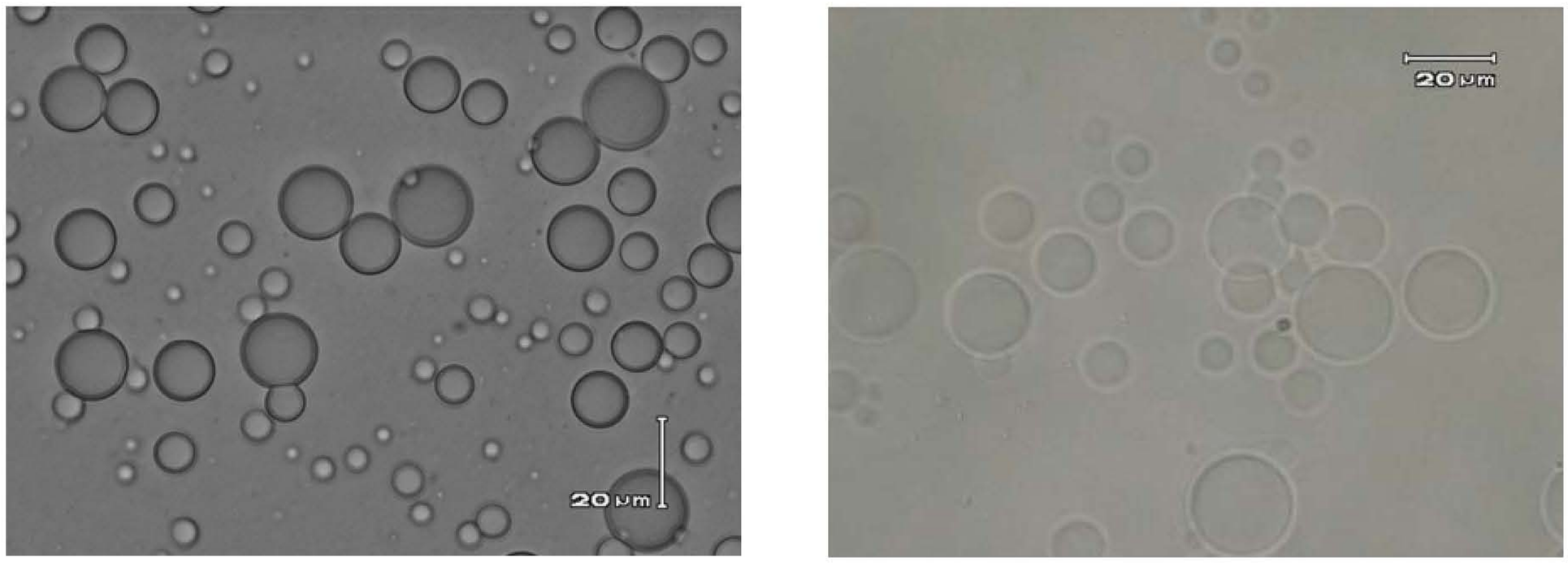


(a) *(b)*

Figure 4. *FT-IR spectra in the region 1900-400 cm^{-1} of non-degraded microspheres DS 12 (—, top), and degraded microspheres after 2.8 months (- - -) and 5.7 months (—, bottom) of incubation (pH 7.4, 37 ^{0}C) (a). FT-IR spectra of pHEMA and dextran, as well as their physical mixtures in the region between 1900 cm^{-1} and 400 cm^{-1}. From top to bottom: pHEMA/dextran 100/0 (—), 80/20 (– –), 50/50 (- - -), 40/60 (– - –), 0/100 (—) ((w/w) (b).*

With increasing mass ratio of pHEMA the intensity of the ester band at 1726 cm^{-1} [ν (C=O)] increases, whereas the peak intensity of the cyclic alcohols in dextran (1050 cm^{-1} [δ(C-OH)]) decreases. Therefore, by combining the results of Fig. 4a and 4b it can be concluded that the insoluble degradation products are enriched in pHEMA. Quantitative analysis of the FT-IR spectrum of the water-insoluble material that was present after 6 months indicated that this material consisted essentially of pHEMA (89±6%). These FT-IR analyses were not sensitive enough to detect trace amounts of dextran in the remaining insoluble degradation products.

Light microscopy revealed that the water-insoluble residue consisted of spherical particles. The size and shape of these particles was not significantly different from the non-degraded microspheres (Fig. 5). However, during degradation the particles became more transparent than the non-degraded microspheres, which implies that the difference in refractive index between these hydrated microspheres and surrounding medium (water) had decreased. This can be attributed to a higher extent of hydration of the degraded microspheres as a result of the release of dextran (Fig. 3).



(a) *(b)*

Figure 5. *Microscopy image of microspheres (DS 12) in phosphate buffer (10 mM, 100mM NaCl, 0.02 % NaN_3, pH=7.4). Non-degraded microspheres (a). Degraded microspheres (DS 12, 5.7 months, pH=7.4, 37^0C) (b).*

The mechanical strength of the microspheres as a function of the degradation time was investigated by the micromanipulation technique. Fig. 6 shows that the mechanical rigidity of the microspheres represented by the pseudo elasticity modulus (calculated from the initial slope of the pseudo stress strain curves[34]) drastically decreased in time, from 1.4 ±0.1 MPa (t = 0) to 0.0070±0.0005 MPa (t = 2.8 months). This can be ascribed to hydrolysis of the crosslinks in the dex-HEMA network, resulting in a decrease of the crosslink density and thereby of the mechanical strength[34]. After 3 months, the compression force acting on the microspheres was below detection level of the micromanipulation force transducer. This indicates that at this time point when 70 % of the total amount of dextran has been released from the microspheres, they essentially had lost their mechanical strength.

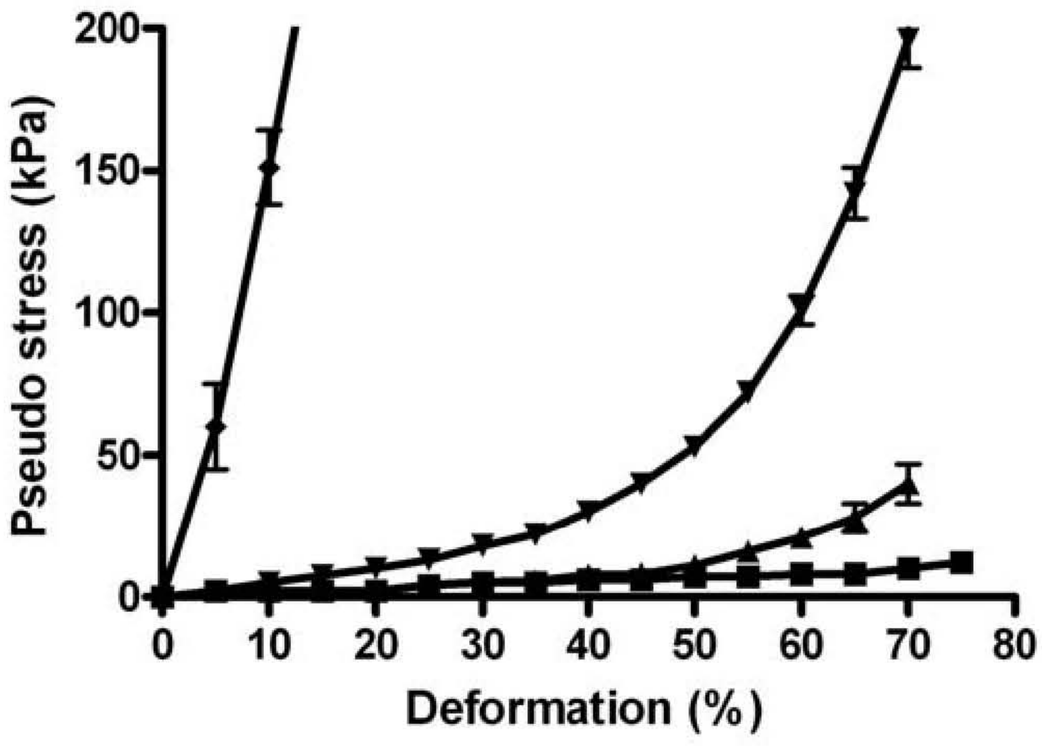
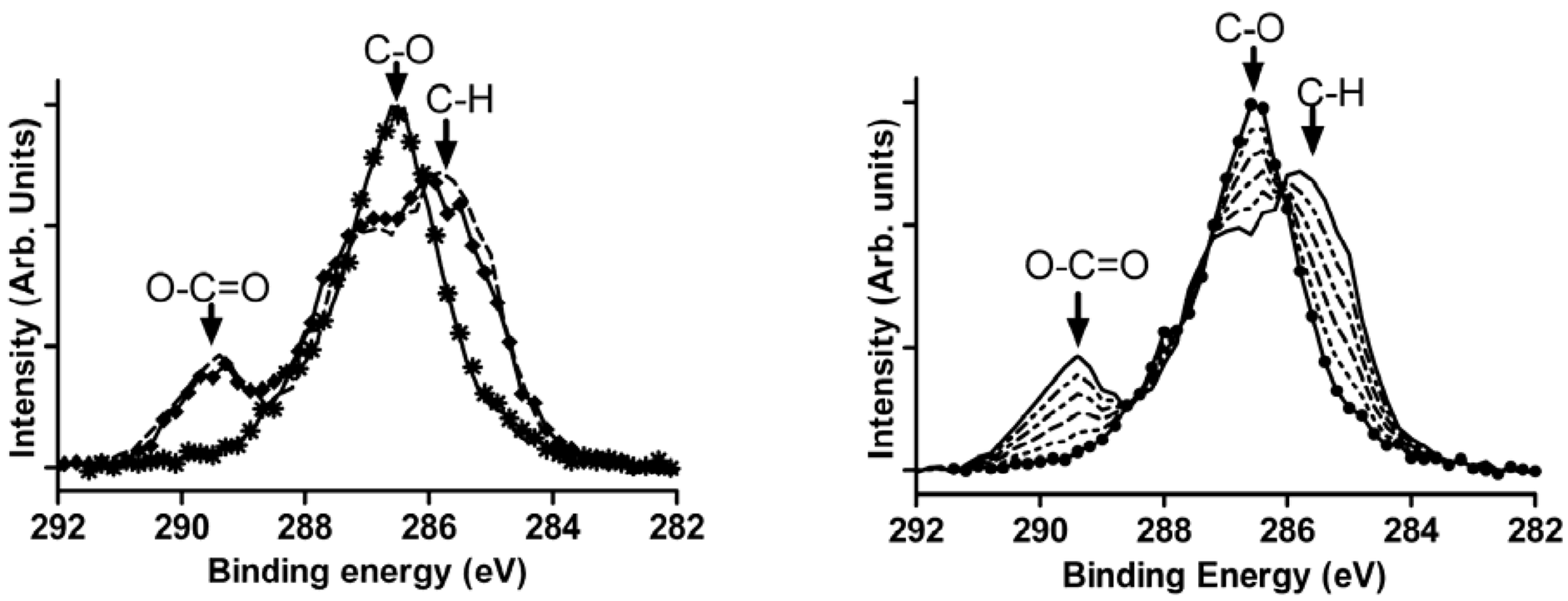


Figure 6. *Micromanipulation compression profiles of dex-HEMA microspheres at t= 0 (♦), 1 (▼), 1.5 (▲), 2.8 (■) months.*

The surface composition of the microspheres was investigated with XPS. Fig. 7 shows the C*1s* spectra of non-degraded dex-HEMA microspheres, the insoluble residue isolated at 5.7 months, pHEMA and dextran (Fig. 7a) and different mixtures of pHEMA and dextran (Fig. 7b). In the spectrum of dextran and the non-degraded microspheres, the peaks originating from C-O of the alcohols and glycosidic bonds in dextran are clearly visible (Figs 7a/b). In the spectrum of pHEMA and the insoluble degradation products, O-C=O (of the ester group present in pHEMA), C-O (present in pHEMA (partly overlapped by C-H signal of pHEMA)), C-H groups of pHEMA. (Fig 7 A/B) are observed[44,45]. In Fig. 7b the calculated spectra of different mixtures of dextran and pHEMA are shown. As expected, with increasing ratios pHEMA/dextran, the peaks of O-C=O and C-H become more apparent.

When the peak intensities in the spectrum of the non-degraded microspheres (Fig. 7a) were fitted with equation 1 (section 2.3.7), the calculated mass percentage of pHEMA in the outer layer of non-degraded microspheres was 5 ± 4%. Provided that pHEMA and dextran were homogeneously distributed in the outer layer of the microspheres, for non-degraded microspheres prepared from dex-HEMA DS 12 a mass percentage of 9% pHEMA was expected. The calculated composition is in good agreement with the expected composition and with the bulk composition as calculated from the FT-IR spectra.



(a) *(b)*

Figure 7. *XPS C1s spectrum of pHEMA (- - -), dextran (—), non-degraded dex-HEMA microspheres of DS 12 (*), and degraded dex-HEMA microspheres of DS 12 (5.7 months, pH 7.4, 37°C) (♦) (a), XPS C1s spectrum of pHEMA (—), dextran(—•—), and mixtures of pHEMA and dextran in ratios 20/80 (- - -), 40/60 (– –), 60/40 (– - –), 80/20 (– - - –) (b).*

Fitting the peak intensities of the insoluble residue that was present at 5.7 months, revealed a pHEMA content of more than 91 ±6 %. FT-IR analysis showed that the insoluble residue consisted for 89±5 % of pHEMA. This suggests that also for these insoluble residues their surface composition is similar to their bulk composition and that they essentially consist of pHEMA.

3.3. Analysis of mass fraction and composition of water-soluble and water-insoluble degradation products

The composition and mass fraction of the water-soluble products and the water-insoluble residue of microspheres with different crosslink densities (obtained by polymerization of dex-HEMA of DS 8, 12, 20), degraded under accelerated conditions (0.1 M NaOH, 70°C, 3 days) were established (Table 2).

It was shown by NMR that the water-soluble fraction (A) mainly consisted of dextran and a small fraction of water-soluble pHEMA. As indicated in Table 2, the mass percentage of dextran (A1) and water soluble pHEMA (A2) was respectively, 86-99 % and <1%. (The mass percentage of dextran recovered in the water-soluble degradation products is consistent with the expected amount of dextran in the microspheres (87-94 % w/w). The water-insoluble degradation products (B) consisted of a small fraction of methanol-soluble pHEMA (B1;<1% w/w, independent of the crosslink density of the microspheres) and methanol-insoluble pHEMA (B2), which increased with DS from 4 to 14 % w/w (Table 2). This indicates that pHEMA is mainly recovered as methanol-insoluble product.

Table 2. *Solubility, identity and mass percentage of different degradation products of dex-HEMA microspheres (DS 8, 12 or 20).*

Fraction (see section 2.2.3)	water-soluble	methanol- soluble	chemical composition	mass percentage (%)		
				DS 8	DS 12	DS 20
A1	+	-	Dextran	99	96	87
A2	+	+	LMW pHEMA	1	1	<1
B1	-	+	HMW pHEMA	<1	<1	<1
B2	-	-	Insoluble pHEMA*	4	7	14

+ = soluble, - = insoluble, LMW = low molecular weight; HMW = high molecular weight.
** Solvent removal was incomplete due to inclusion of solvent during drying*

The number average molecular weight of the water-soluble pHEMA (A2) was between 14 and 16 kg/mol with a polydispersity between 1.2 and 2.0 (Table 3), which is consistent with previous observations by Weaver et al.[31]. For dextran in fraction A1 it was found that, independent of the formulation, M_n=20 kg/mol, M_w = 37 kg/mol. Note that for dextran 40 kg/mol, M_n= 21 kg/mol; M_w = 40 kg/mol.

The molecular weight of the insoluble pHEMA could not be determined, as it dissolved neither in methanol nor in other organic solvents (DMSO, DMF). The insoluble fraction showed significant swelling in DMSO. Since high molecular weight pHEMA is soluble in DMSO[46], this result suggests that the insoluble residual material consists of a loosely cross-linked pHEMA. The mechanism behind the crosslinking pHEMA is not clear yet and requires further investigation.

Table 3. *Molecular weights of degradation products from various microspheres.*

DS	Chemical composition	M_n (kg/mol)	M_w (kg/mol)	PDI
8	LMW pHEMA	16	20	1.2
	HMW pHEMA	65	103	1.6
12	LMW pHEMA	16	28	2.7
	HMW pHEMA	82	162	2.0
20	LMW pHEMA	14	18	1.3
	HMW pHEMA	26	48	1.8

M_n=Number average molecular weight, M_w = Weight average molecular weight, PDI= poly dispersity index

4. Conclusions

This study demonstrates that the degradation of dex-HEMA microspheres in phosphate buffer pH 7.4, 37 °C results in the formation of the water-soluble degradation products (~86-99%) and a small fraction of water-insoluble degradation products (~4-14%). Under physiological conditions, water-soluble degradation products are gradually released as a result of the hydrolysis of crosslinks and consist of dextran, and small amounts of low molecular weight pHEMA (M_n ranging between 14-16 kg/mol), unreacted HEMA and HEMA-DMAP (intermediate reaction product of the Baylis-Hillman reaction of HEMA with DMAP). Water-insoluble degradation products consist of

slightly crosslinked pHEMA and a small fraction of methanol-soluble, high molecular weight pHEMA (M_n ranging between 27-82 kg/mol).

Dex-HEMA microspheres are promising biocompatible controlled delivery devices. For these systems, a low level of impurities and complete removal of the degradation products at the site of administration is preferred. Therefore, the *in vivo* degradation and the processing of the insoluble residue by macrophages[47] and the mechanism of formation of crosslinked pHEMA are currently under investigation. Finally, the synthesis of dex-HEMA is optimized to avoid the formation of HEMA-DMAP.

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Chapter 7

Synthesis and characterization of dextrans substituted with hydrophilic polymerizable side groups and their application in protein-releasing microspheres – an introductory study

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Abstract

The aim of this study was to synthesize a series of new biodegradable polymerizable dextrans that can be used to prepare hydrogels that degrade at physiological conditions into fully water-soluble degradation products and that are suitable to prepare protein-releasing microspheres. These polymerizable dextrans were obtained by derivatization of dextran with HPMAm (N-(2-hydroxypropyl)methacrylamide), lac-HPMAm (N-(2-hydroxypropyl)methacrylamide-monolactate), lac_2-HPMAm (N-(2-hydroxypropyl)methacrylamide-dilactate), or HEMAm (N-(hydroxyethyl)methacrylamide) and were synthesized by activation of their terminal hydroxyl group with 1,1'-carbonyl diimidazole (CDI) followed by coupling of the activated compounds to dextran in dimethyl sulfoxide (DMSO). Dextran derivatized with hydroxyethyl methacrylate (dex-HEMA) was used as a contol. It was demonstrated that the degradation of hydrogels based on dex-HEMAm and dex-HEMA resulted in the formation of both water-soluble and water-insoluble degradation products. In contrast, the degradation of both macrogels and microspheres based on dex-(lac1,2-)HPMA resulted in the formation of only water-soluble degradation products. Therefore, swelling and degradation kinetics were only investigated using these polymerizable dextrans. The degradation time depended on the degree of substitution (DS, number of polymerizable side groups units per 100 glucose units) and nature of the polymerizable side group and increased according to the following order: dex-lac_2-HPMAm< dex-lac-HPMAm< dex-HEMA< dex-HPMAm The release characteristics of microspheres based on the HPMAm-dextrans and loaded with bovine serum albumin (BSA) were investigated. The release profiles were dependent on the number of lactic acid units in the crosslinks and the DS. Microspheres based on dex-lac_2-HPMA are most attractive, because BSA loaded microspheres based on this polymerizable dextran showed high encapsulation efficiency (86%), low burst release (2%) and almost linear release for 3 days. Importantly, quantitative release of the encapsulated protein from these microspheres in a period of 5 days was observed. Moreover, as observed for macroscopic hydrogels, the microspheres degraded into fully water-soluble degradation products within 12 days after the release period.

1. Introduction

Biodegradable microspheres obtained by radical polymerization of dextran derivatized with hydroxyethyl methacrylate (HEMA) (dex-HEMA) are very attractive systems for the controlled release of proteins[1,2]. Advantages of this system are the high encapsulation efficiency of proteins, an all-in-water preparation procedure and the possibility to tailor the release of proteins by changing the crosslink density of the microspheres[2,3]. Moreover, it was demonstrated that dex-HEMA based microspheres degraded *in vivo* and have a good biocompatibility[4].

Microspheres are formed by polymerization of the HEMA groups linked to dextran in a water-in-water emulsion of dex-HEMA in poly(ethylene glycol) (PEG)[5,6]. Under physiological conditions, degradation of dex-HEMA microspheres proceeds via hydrolysis of the carbonate ester links between dextran and polyHEMA (pHEMA), first releasing the encapsulated protein and in later stage degradation products like dextran and pHEMA[2,7-9]. It was demonstrated in Chapter 6 of this thesis that the degradation of dex-HEMA microspheres results in the formation of water-soluble degradation products (mainly dextran and low molecular weight pHEMA) and a small fraction water-insoluble degradation products (mainly high molecular weight pHEMA)[9]. Therefore, dextran derivatized with more hydrophilic polymerizable groups, and thus improved solubility of the resulting degradation products are preferred. Furthermore, the molecular weights of these degradation products should be below the renal excretion threshold (~Mw< 50kDa).

In this study results are presented on the development of new polymerizable dextrans that can be used for the preparation of microspheres for the controlled release of proteins and that degrade under physiological conditions (pH 7.4, 37°C) into fully water-soluble degradation products. The investigated candidates were dextrans derivatized with hydroxypropyl methacrylate (HPMAm), and hydroxyethyl methacrylamide (HEMAm) (chemical structures in Fig. 1a). Due to the amide instead of the ester function, these monomers are more hydrophilic than HEMA; log P (=logarithm of n-octanol/water partition coefficient) is -0.21 and 0.11 for HEMAm and HPMAm, respectively, whereas log P=0.47 for HEMA[10]. Consequently, it is expected that polymers of HEMAm and HPMAm have a better water-solubility than pHEMA. HPMAm was selected as polymerizable group since pHPMAm, one of the degradation products of dex-HPMAm hydrogels is widely investigated and its safety is well established[11-13]. Higher ester stability of secondary alcohols (HPMAm) compared to esters of primary alcohols (HEMAm and HEMA) might decrease the hydrolysis rate of the carbonate ester[14, 15] and consequently the degradation rate of the hydrogels.

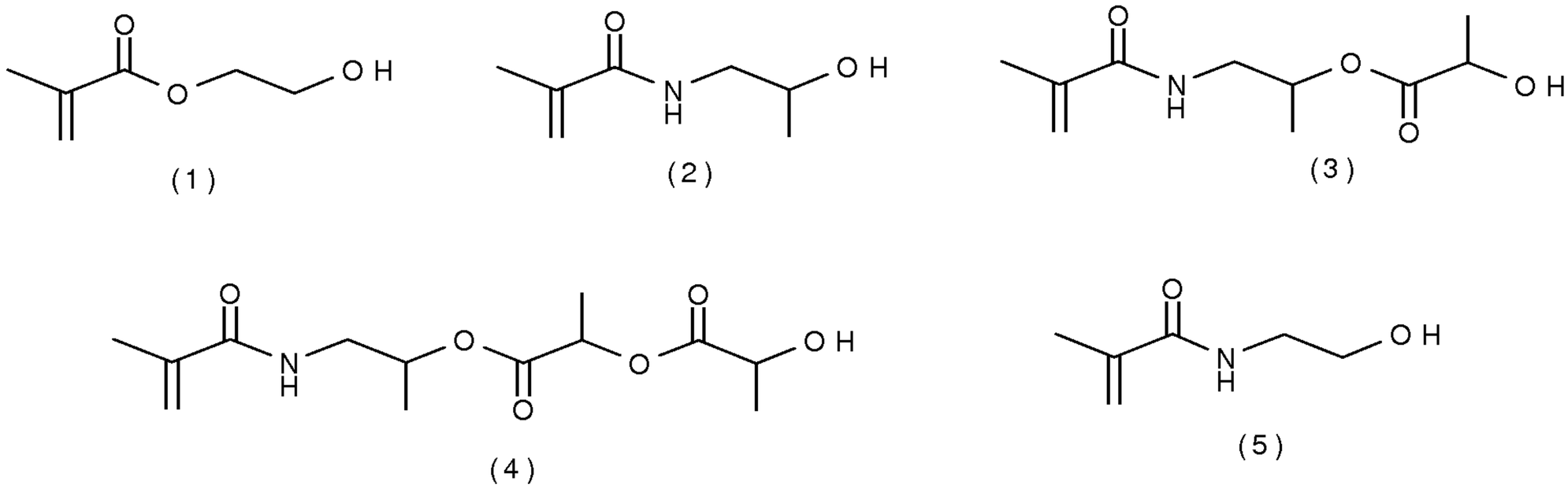

Figure 1. *Structure of hydroxyethyl methacrylate (HEMA, 1), hydroxypropyl methacrylamide (HPMAm, 2), HPMAm-lactate (3), HPMAm-dilactate (4) and hydroxyethyl methacrylamide (HEMAm, 5), which were used for the substitution of dextran chains.*

Previous studies have demonstrated that due to the presence of a lactate spacer in dex-lactate-HEMA, these hydrogels have higher degradation rates than the corresponding dex-HEMA hydrogels[1,16]. Therefore, dextran was also derivatized with HPMAm-monolactate and HPMA-dilactate (Fig. 1).

In summary, in this introductory study, results are presented on the derivatization and polymerization of dextran with HPMAm, HPMAm-monolactate, HPMAm dilactate and HEMAm. The formation of water-soluble degradation products was investigated in an accelerated degradation study. Furthermore, the degradation and swelling kinetics were investigated using macrogels based on these derivatized dextrans and the release of BSA, a model protein, from microspheres formed of those dextrans that resulted in the formation of only water-soluble degradation products (dex-($lac_{1,2}$-)HPMAm) was studied.

2. Materials and methods

2.1. Materials

Hydroquinone monomethylether, dextran (from *Leuconostoc ssp.*, T40), dimethylsulfoxide (DMSO), poly(ethylene glycol) (PEG) 10,000 and potassium peroxodisulfate (KPS) were obtained from Merck, Darmstadt, Germany. *N,N,N',N'*-tetramethylethylenediamine (TEMED) was obtained from Fluka, Buchs, Switzerland. 4-(*N,N*-dimethylamino)pyridine (DMAP) was purchased from Acros Chimica, Geel, Belgium. Dichloromethane (DCM) was purchased from Biosolve LTD, Valkenswaard, The Netherlands. ^2H_2O (99.9% ^2H) was purchased from Cambridge Isotope laboratories, Inc, Andover, MA, USA. BSA (bovine serum albumin, fraction V), deuterated chloroform ($CDCl_3$, 99.8% ^2H), deuterated dimethylsulfoxide (DMSO-d_6, 99.9% ^2H) and

carbonyldiimidazole (CDI) were obtained from Sigma-Aldrich Chemie GmbH, Steinheim, Germany. Hydroxyethyl methacrylate derivatized dextrans (dex-HEMA) with degree of substitution (DS, the number of HEMA groups per 100 glucose units) of 5, 7 and 12 were obtained from Polymer Service Centre, Groningen, The Netherlands. 1,4-Dioxane 99(% (Fluka Chemie AG) was purified by distillation. 2,2-Azobis(isobutyronitrile) (AIBN) was from Fluka Chemie AG (Buchs, Switzerland). Hydroxypropyl methacrylamide (HPMAm) was synthesized as reported by Oupicky et al.[17]. HPMAm mono-lactate and di-lactate (further abbreviated as HPMAm-lac and HPMAm-lac_2, respectively) were synthesized as described by Neradovic et al.[18]. Hydroxyethyl methacrylamide was synthesized as reported by Kosvintsev et al.[19].

2.2. Methods

2.2.1. Synthesis of dex-HEMAm, dex-HPMAm, dex-HPMAm-lac and dex-HPMAm-lac2

For the preparation of dex-HEMAm, dex-HPMAm, dex-HPMAm-lac and dex-HPMAm-dilactate the same procedure was followed as previously described for the synthesis of dex-HEMA[1]. Briefly, the hydroxyl groups of HPMAm, HPMAm-$lac_{1,2}$ and HEMAm were activated with CDI (molar ratio 1:1) yielding HPMAm-CI, HPMAm-$lac_{1,2}$ CI and HEMAm-CI, respectively. These activated compounds were subsequently coupled to dextran in DMSO in the presence of DMAP[1]. The reaction mixture was stirred for 4 days at room temperature under nitrogen flow. After this period, the degree of substitution of the formed dextran derivatives was determined with ^{1}H NMR spectroscopy. For this purpose, 0.5 ml of the reaction mixture was dropped into 10 ml methanol to precipitate the derivatized dextran. The precipitate was filtered under vacuum, rinsed with methanol and DCM, and dried before ^{1}H NMR analysis. Based on previous results with dex-HEMA, a maximum couplings efficiency of 85 % was expected within 4 days[1]. Assuming a similar coupling efficiency for this new series of polymerizable dextrans, DS 12 was expected to be obtained after 4 days. The reaction mixture was neutralized with HCl, transferred into a dialysis tube and dialyzed for 5 days against dematerialized water at 4ºC. Next, the polymer solution was frozen in liquid nitrogen and subsequently freeze-dried in a Christ, Alpha 1-2 freeze-dryer (Osterode, Germany). After lyophilization, the DS of the resulting methacrylamide dextran derivative was determined with ^{1}H NMR.

2.2.2. ^{1}H NMR spectroscopy

^{1}H NMR spectra were recorded with a Gemini 300 MHz spectrometer (Varian Associates Inc. NMR Instruments, Palo Alto, CA, USA). Approximately 30 mg of material was dissolved in

0.8 ml solvent. For measurements in $CDCl_3$ or in DMSO-d_6 containing 12% (v/v) $^2H_20$, the chemical shifts were given relative to TMS as external reference.

2.2.3. Preparation of dextran based macrogels

Macrogels were obtained by radical polymerization of aqueous solutions of derivatized dextran. In detail, 450 mg dextran derivative was dissolved in 945 µl of 10 mM sodium phosphate buffer (PB, pH 7.4) in a 2 ml Eppendorf vial. Next, 30 µl of a 20% (v/v) TEMED solution neutralized to pH 7.2 with 4 M hydrochloric acid (HCl) were added followed by the addition of 75 µl of KPS in PB (20 mg/ml). This solution was mixed and polymerized for 1 hour at room temperature resulting in the formation of a cylindrically shaped hydrogel with an initial water content of 70% (w/w).

2.2.4. Preparation of dextran based microspheres

Microspheres were obtained by the water-in-water emulsion method as described previously by Stenekes et al.[6]. Briefly, a standard formulation (without protein) was prepared by weighing 3.25 g of 40 % w/w PEG-solution in 25 mM sodium phosphate buffer pH 7.0, in a 15 ml tube. Then, 1.69 g of 3.6 % w/w dex-HEMA (or other dextran derivatives) solution in the same buffer was added. In the equilibrium state the volume ratio PEG/dex was 40/1. The mixture was vortexed for 1 minute. Subsequently, 100 µl of a TEMED solution (20% (v/v), pH neutralized o pH 7.0 with 4 M HCl was added, followed by vortexing for 20 s. Then, 180 µl KPS (concentration 50 mg/ml) was added and the emulsion was shaken gently. The resulting emulsion was allowed to polymerize for 1 hour at room temperature. The polymerized microspheres were washed 5 times with 5 ml 25 mM phosphate buffer pH 7.0.

BSA-loaded microspheres were prepared by dissolving the dex(-$lac_{1,2}$)-HPMA in a 5.5 mg/ml BSA solution in PBS. The encapsulation efficiency of BSA was obtained from the amount of protein added for the preparation of the microspheres minus the amount of protein retrieved in the wash fractions (as determined with high liquid performance chromatography (HPLC, see section 2.2.9) divided by the amount of protein added for preparation of the microspheres.

2.2.5. Accelerated degradation of macrogels and microspheres

To investigate the formation of water-soluble degradation products the hydrogels or microspheres were degraded under accelerated conditions by incubation them in 5 ml 2 M NaOH at 37 °C. After 4 days, the mixtures were neutralized with 4 M HCl and analyzed by optical microscopy for the presence of insoluble particles.

2.2.6. Degradation and swelling kinetics

After polymerization, the hydrogels were removed from the cups vials, weighed (W_0) and incubated in 15 ml 100 mM sodium phosphate buffer, pH 7.4, containing 0.03 M NaCl and 0.02 % NaN_3 (PBS) at 37°C. At regular time intervals, the weight (W_t) of the gels was determined and used to calculate the swelling ratio W_t/W_0. Simultaneously, the incubation buffer was replaced by fresh PBS. The swelling profile of a gel was determined in duplicate.

2.2.7. Water content of the microspheres

The water content of the dextran microspheres was determined according to the procedure earlier described by Stenekes et al.[20]. Briefly, the microspheres were washed with ultra pure water (5 times, 5 ml), frozen with liquid nitrogen and subsequently freeze-dried in a Christ, Alpha 1-2 freeze-dryer (Osterode, Germany). Forty mg of the freeze-dried microspheres was resuspended in 0.4 ml of an aqueous solution of dextran blue (initial concentration 6 mg/ml). After incubation at room temperature for one hour, the hydrated microspheres were separated from the solution by centrifugation (3 min, 3900 x g). The increase in dextran blue concentration of the supernatant was quantified spectrophotometrically (λ=610 nm) using a Perkin-Elmer Lambda 2 UV/VIS spectrophotometer (Überlingen, Germany).

2.2.8. In vitro release of BSA and degradation of microspheres

For the release experiments, the freeze-dried, protein-loaded microspheres were suspended in 5 ml PBS. Periodically, the microspheres were centrifuged for 7 min at 3900 x g, Next, 3 ml supernatant was removed and replaced by 3 ml fresh PBS. The BSA concentration in the release samples was determined with high performance liquid chromatography (HPLC) as described in section 2.2.9. The burst release is defined as the percentage of the encapsulated amount hGH released after 12 h of incubation. After 6 days a sample was taken and analyzed under a microscope equipped with a digital camera (Olympus Optical Co. Shibuya, Tokyo, Japan) to investigate the swelling of the microspheres.

2.2.9. High performance liquid chromatography

The HPLC system used consisted of a HPLC pump series 200, a vacuum degasser series 200, an autosampler series 200, a column LC 101 oven, and a UV detector series 200 (all Perkin Elmer Instruments, Norwalk, USA) and a thermostated (80 °C) RP-18 column (Alltima C18 EPS, 7x33 mm) with a 0.5-µm prefilter (all Alltech, Deerfield, USA). The flow rate was 1 ml/min. After equilibration for 0.5 min at 99 % A (100 % water, 0.1 v/v% TFA), a gradient was run from 99 % A

(99.9 % water, 0.1 v/v% TFA) to 40 % A (99.9 % acetonitrile, 0.1 v/v% TFA) in 1 min, followed by a equilibration for 1 min at 40 % A. Hereafter the column was equilibrated for 1 min at 100 % B, followed by 1 min at 99 % A. A calibration curve was obtained by injecting 50 µl of 0.02-1.5 mg/ml BSA. The chromatograms were recorded and analyzed with the data acquisition system Totalchrom (Perkin Elmer Instruments, Norwalk, USA).

3. Results and discussion

3.1. Synthesis of dex-HEMAm, dex-HPMAm, dex-lac-HPMAm and dex-lac2-HPMAm

HEMAm, HPMAm, HPMAm-mono and dilactate were coupled to dextran after activation of their terminal hydroxyl groups with CDI according to the method previously described by van Dijk-Wolthuis et al. for the synthesis of dex-HEMA[1]. As evidenced from ^{1}H-NMR analysis, the activation with CDI of the monomers mentioned above was 100 % and the activated monomers were obtained in almost quantitative yield[1]. After 4 days, the coupling efficiency of HEMAm-CI and HPMAm-lac$_2$-CI to dextran in DMSO using DMAP as catalyst was around 85%, which is comparable to the incorporation efficiency of HEMA-CI as previously reported[1], and yielded dextrans with a degree of substitution (DS) of 11 and 12, for dex-HEMAm and dex-lac$_2$-HPMAm respectively. It was found that the coupling of HPMAm-CI and HPMAm-lac-CI proceeded slower, yielding an incorporation efficiency of respectively 31 % (after 4 days) and 52 % (after 7 days). The DS of the obtained dex-HPMAm and dex-lac-HPMAm was consequently lower than the expected one (DS 12) and amounted to 7 and 4, respectively.

3.2. Accelerated degradation of macrogels and microspheres

After 4 days of incubation in 2M NaOH at 37 °C, microscopic analysis revealed the presence of a water-insoluble residue for both dex-HEMAm macrogels and microspheres. Prolongation of the incubation time did not result in dissolution of these water-insoluble residues of the dex-HEMAm macrogels. Light microscopy revealed that the water-insoluble residue left consisted of spherical particles. It was expected that polymers derived of HEMAm were better water-soluble than pHEMA, as the log P value of HEMAm is lower (-0.21) than that HEMA (0.47)[10]. Indeed, pHEMAm (Mw 120 kDa) synthesized in our lab is water-soluble to at least 10 mg/ml, whereas pHEMA is water-soluble when its molecular weight is below (~Mw 3 kDa)[21]. Therefore, it is suggested that insoluble residual material that remained after accelerated degradation consists of a loosely cross-linked pHEMAm. The mechanism behind the crosslinking

of pHEMAm is not clear yet and requires further investigation. In contrast to dex_HEMAm gels, the dex-($lac_{1,2}$-)HPMAm macrogels and microspheres completely dissolved after 4 days of incubation in 2M NaOH at 37 °C.

3.3. Swelling and degradation of macrogels

The swelling and degradation kinetics of the different dextran hydrogels was investigated by incubating different dextran macrogels in buffer pH 7.4 at 37°C and measuring their weight in time. Fig. 2 shows the swelling profile of the hydrogels prepared from the various polymerizable dextrans. Like previously observed for dex-HEMA hydrogels, the hydrogels showed a phase of progressive swelling in time, which is caused by the hydrolysis of crosslinks, followed by a dissolution phase[2,7,16].

Table 1 summarizes the swelling and degradation characteristics of the macrogels prepared from the new polymerizable dextrans and dex-HEMA macrogels. Also summarized in Table 1 are the pseudo first-order reaction rate constants k_{obs} derived from the swelling profile. Previously, van Dijk-Wolthuis et al. calculated the hydrolysis rate of the crosslinks in the dextran-based hydrogels from the swelling profile assuming pseudo first-order reaction kinetics[8]. They further assumed that at the time that the gels start to dissolve (i.e., the time at which the swelling ratio of the gels reaches a maximum, Fig. 2) the dextran chain bears approximately one crosslink per chain.

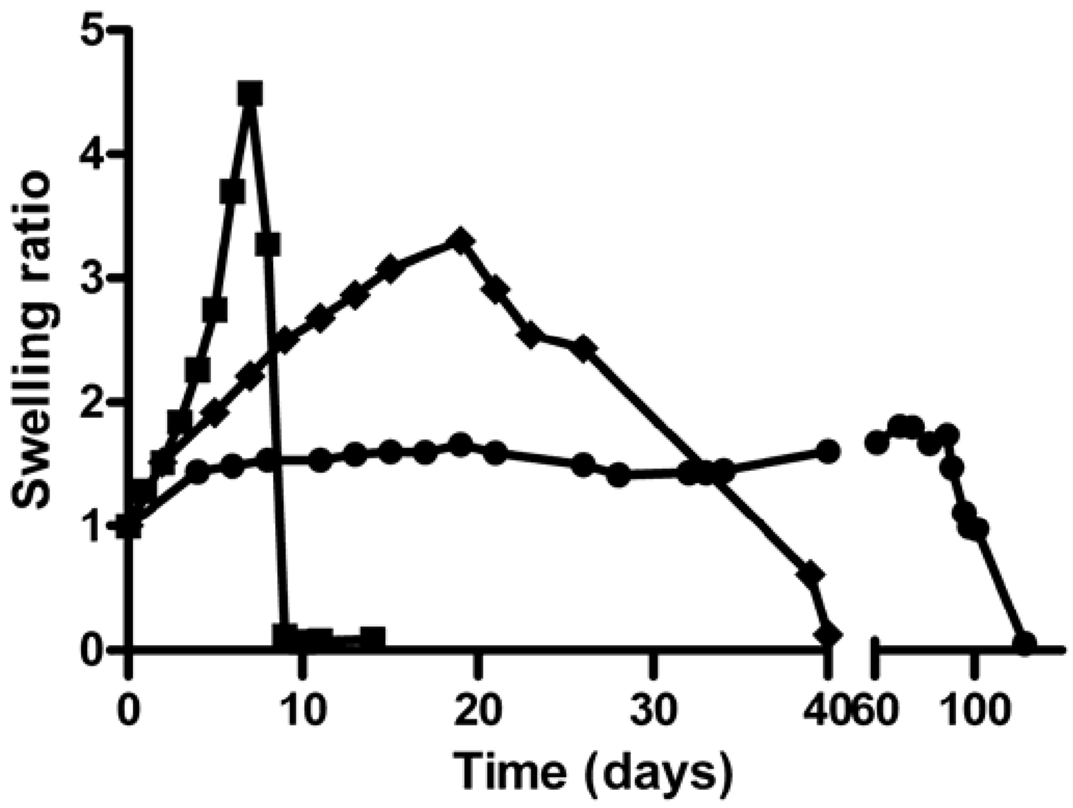

Figure 2. *Swelling ratio of degrading macroscopic dex-HPMAm DS 4 (●),dex-lac-HPMAm DS 7 (♦) and dex-lac_2-HPMAm DS 12 (■) hydrogels. The values are the mean of two independent measurements that typically deviated about 10%.*

Since the number of crosslinks at the start of the degradation can be derived from the degree of substitution of dextran, the pseudo first-order hydrolysis rate constant k_{obs} for the hydrolysis of crosslinks in the dextran based hydrogels can be calculated. It was proven that this pseudo first-order reaction rate constant k_{obs} determined from the swelling profile was in good agreement with the pseudo reaction rate constant k_{obs} determined from the degradation of dex-HEMA (before polymerization) in solution[8]. Table 1 shows that the dex-HEMA DS 5 gel reached maximum swelling within 22 days (resulting in a k_{obs} for dex-HEMA of $1.3x10^{-6}$ s^{-1}) and degraded in 32 days. The time to reach maximum swelling and the degradation time of the dex-HPMAm DS 4 hydrogel was much longer: 86 days and 126 days respectively (resulting in a k_{obs} for dex-HPMAm of $0.3x10^{-6}$ s^{-1}). It has been reported that the presence of increasingly branched aliphatic groups has a retarding effect on the alkaline catalyzed hydrolysis of esters[15]. Therefore, the lower degradation rate of dex-HPMAm hydrogels in comparison with dex-HEMA hydrogels can be ascribed to the higher stability of esters of secondary alcohols (HPMAm) compared to esters of primary alcohols (HEMA). Importantly, degradation of the dex-HPMAm gel resulted in complete dissolution of the degradation products, whereas after degradation of the dex-HEMA hydrogels still a water-insoluble residue was visible.

The dex-lac-HPMAm gel showed a higher maximum swelling (3.0) than a dex-HEMA gel of the same DS (2.3; see Table 1). Since pHPMAm is more hydrophilic than pHEMA (log P_{HEMA}= 0.47, log P_{HPMAm}= 0.11) [10], degrading dex-lac-HPMAm hydrogels have a higher water absorbing capacity than degrading dex-HEMA gels, which might explain the difference in maximum swelling. The time to reach maximum swelling and the degradation time of the dex-HEMA and dex-lac-HPMA gels is almost similar (20 and 40 days respectively) but degradation of dex-lac-HPMAm gels resulted in complete dissolution of the gel, whereas, as observed previously (Chapter 6) after the degradation of dex-HEMA gels water insoluble degradation products were left. Table 1 shows that the hydrolysis rate constant (k_{obs}) of dex-lac-HPMAm hydrogels is $1.9x10^{-6}$ s^{-1}, which is much greater than the hydrolysis rate constant of dex-HPMAm hydrogels (k_{obs}= $0.3x10^{-6}$ s^{-1}) and almost similar to k_{obs} of HPMAm-monolactate (k_{obs} = $2.2x10^{-6}$ s^{-1} at 37 °C, and pH = 7.5), as previously observed by Neradovic et al.[21]. This suggests that degradation of dex-lac-HPMAm hydrogels primarily proceeds via hydrolysis of the lactate esters between HPMAm and dextran and not via hydrolysis of the carbonate esters.

Table 1. *Swelling and degradation characteristics of the various macroscopic dextran hydrogels*

Composition	DS	Maximum Swelling	$t_{max\ swelling}$ (days)	k_{obs} (x 10^{-6} s^{-1})	$t_{degradation}$ (days)	only water-soluble degradation products
dex-HPMAm	4	1.8	86	0.3	126	Y
dex-lac-HPMAm	7	3.3	20	1.9	40	Y
dex-lac_2-HPMAm	12	4.5	7	5.6	8	Y
dex-HEMA	5	2.5	22	1.3	32	N
dex-HEMA	7	2.3	20	1.4	40	N
dex-HEMA	12	2.3	25	1.6	126	N

$t_{max\ swelling}$: time to reach maximum swelling
k_{obs} : reaction rate constant for the hydrolysis of crosslinks
$t_{degradation}$: time to reach complete degradation

Table 1 shows that dex-lac_2-HPMAm gels reached a considerably higher maximum swelling ratio (4.5) than dex-HEMA (2.3) of equal DS or dex-lac-HPMAm (3.3) and dex-HPMAm (1.8) of lower DS. Moreover, the dex-lac_2-HPMAm gels reached much faster their maximum swelling (7 days) and degraded much faster (8 days) than the dex-HEMA gels (time to reach maximum swelling: 25 days, degradation time: 126 days). Furthermore, degradation of dex-lac_2-HPMAm gels resulted in the formation of water-soluble degradation products, whereas for the dex-HEMA water insoluble degradation products were formed, which confirm the observation in section 3.2. Likely, the much stronger swelling of dex-lac_2-HPMAm hydrogels compared to dex-HEMA hydrogels and dex(-lac)-HPMAm hydrogels can not only be attributed to the higher hydrophilicity of pHEMAm compared to pHEMA. Likely, the higher swelling of degrading dex-lac_2-HPMAm hydrogels might be attributed to the change in hydrophilicity when HPMAm-dilactate polymers are degraded to pHPMAm-(monolactate). Previously Soga et al. reported that the cloud point of pHPMAm-dilactate is 13 ^{o}C[23]. During of the degradation of dex-lac_2-HPMAm hydrogels, pHPMAm-dilactate is hydrolyzed to pHPMAm-monolactate (cloud point at 65 ^{o}C) and finally to pHPMAm, which is fully water-soluble[22]. This means that during degradation besides a decrease in crosslink density, which results in an increase in swelling, the gels become also more hydrophilic because pHPMAm-dilactate is converted in time into pHPMAm-monolactate and pHPMAm. This increase in hydrophilicity will increase the water-absorbing capacity of the degrading dex-lac_2-HPMAm gels. The combined effect (decrease in crosslink density and increase in network hydrophilicity) likely explains the observed high swelling ratio of the dex-lac_2-HPMAm gels observed during degradation. The much faster degradation rate of dex-lac_2-HPMAm compared to dex-lac-HPMAm or dex-HEMA hydrogels can be attributed to the fact that a larger number of hydrolytically sensitive groups are present in a crosslink[1,16].

3.4. Microsphere characteristics and protein release

Since the degradation of dex-(lac$_{1,2}$-)HPMAm macrogels and microspheres resulted in the formation of water-soluble degradation products, encapsulation and release experiments were only performed with these microspheres. The size of the prepared dex-HPMAm and dex-lac$_{1,2}$-HPMAm microspheres ranged between 13 and 17 μm and the water content between 67-68 % (Table 2), which is comparable to the microspheres prepared of dex-HEMA[22].

The encapsulation efficiency of BSA was 28, 74 and 86 % for dex-HPMAm, dex-lac-HPMAm and dex-lac$_2$-HPMAm, respectively, whereas the respective burst release was 15, 8 and 2 %. The low encapsulation efficiency and high burst release of microspheres prepared of dex-HPMAm (DS 4) can be ascribed to the low crosslink density of the microspheres. Likely, many pores in the microspheres are larger than the hydrodynamic diameter of the protein, which will result in both extraction of the protein from the microspheres during washing and a burst release.

In Fig. 3, the release of BSA from dex-HPMAm (DS 4), dex-lac-HPMAm (DS 7) and dex-lac$_2$-HPMAm (DS 12) microspheres is shown. For dex-HPMAm and dex-lac-HPMAm microspheres, 30 and 72 %, respectively, of their total encapsulated amount of BSA was gradually released over a period of 5 days. Further prolongation of the release period to 12 days did not increase the total amount of released BSA. For dex-lac$_2$-HPMAm BSA loaded microspheres slightly S-shaped release was observed and BSA was released quantitatively over a period of 5 days.

Table 2. *Characteristics of various dextran microspheres*

Composition	DS	Particle size (μm)	Water Content (%)	Encapsulation efficiency (%)	Burst Release (%)	Total release (%)
dex-HPMAm	4	17	69	28	15	33
dex-lac-HPMAm	7	15	67	74	8	72
dex-lac$_2$-HPMAm	12	13	68	86	2	101

Measurements are average of three independent measurements that deviated less than 10 %.

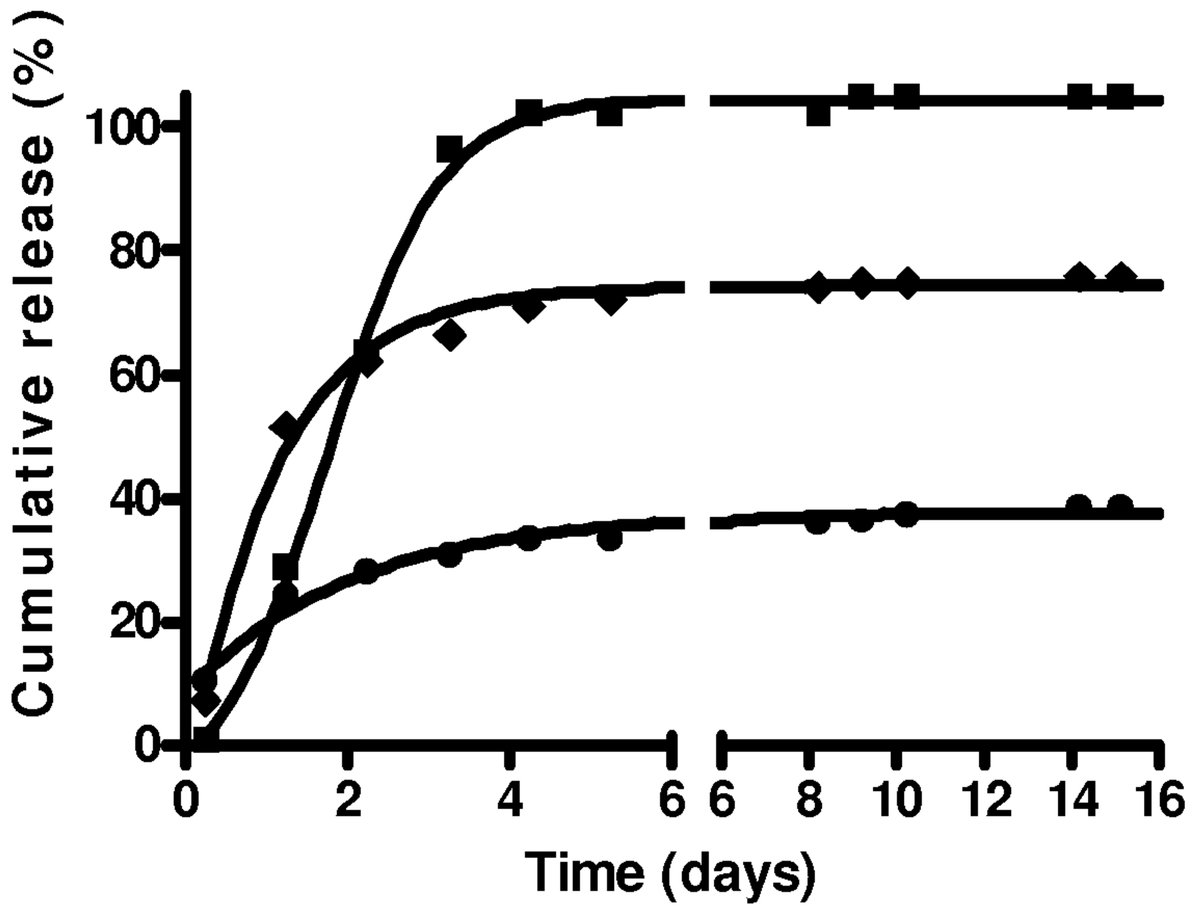

Figure 3. *Release of BSA from dex-HPMAm DS 4 (●),dex-lac-HPMAm DS 7 (♦) and dex-lac$_2$-HPMAm DS 12 (■) microspheres. The values are the mean of two independent measurements that deviated less than 5 %.*

Due to the low crosslink density of dex-HPMAm and dex-lac-HPMAm, many pores in the microspheres are larger than the hydrodynamic diameter of the protein. Therefore, the release of (not-aggregated) BSA is mainly governed by diffusion, which explains the shape of the release curves. Inspection of the samples after this release period revealed that microspheres prepared of dex-lac-HPMAm and dex-HPMAm were still present after this period. As no second pulse of BSA release was observed, even after 15 days, protein aggregates likely were entrapped in the dex-HPMAm. Such aggregates might only be released in a much later stage or when the microspheres completely disintegrate. This, however, was not investigated.

As a result of the higher crosslink density, most pores in the dex-lac$_2$-HPMAm microsphere network are smaller than the hydrodynamic diameter of the protein. Therefore, the release is slightly delayed, as first pores sizes in the hydrogel network need to increase (e.g. by hydrolysis of crosslinks or swelling) before protein molecules can be released. Hereafter, the release is governed by swelling, degradation and diffusion, resulting in an almost linear release profile. The period over which the BSA was released (5 days) from these microspheres is shorter than previously reported for BSA released from dex-HEMA microspheres of similar DS (10 days)[23]. This can be ascribed to the higher swelling (see Fig. 4) and degradation rate of dex-lac$_2$-HPMAm microspheres. Importantly, after 5 days, complete release of the loaded BSA was obtained for microspheres prepared of dex-lac$_2$-HPMAm microspheres, whereas 30 and 72 % of the encapsulated amount of BSA was released after 5-12 days for microspheres based on dex-HPMAm and dex-lac-HPMAm respectively (Table 2). Inspection of the samples, after 16 days revealed that the microspheres prepared of dex-lac$_2$-HPMAm, were fully degraded.

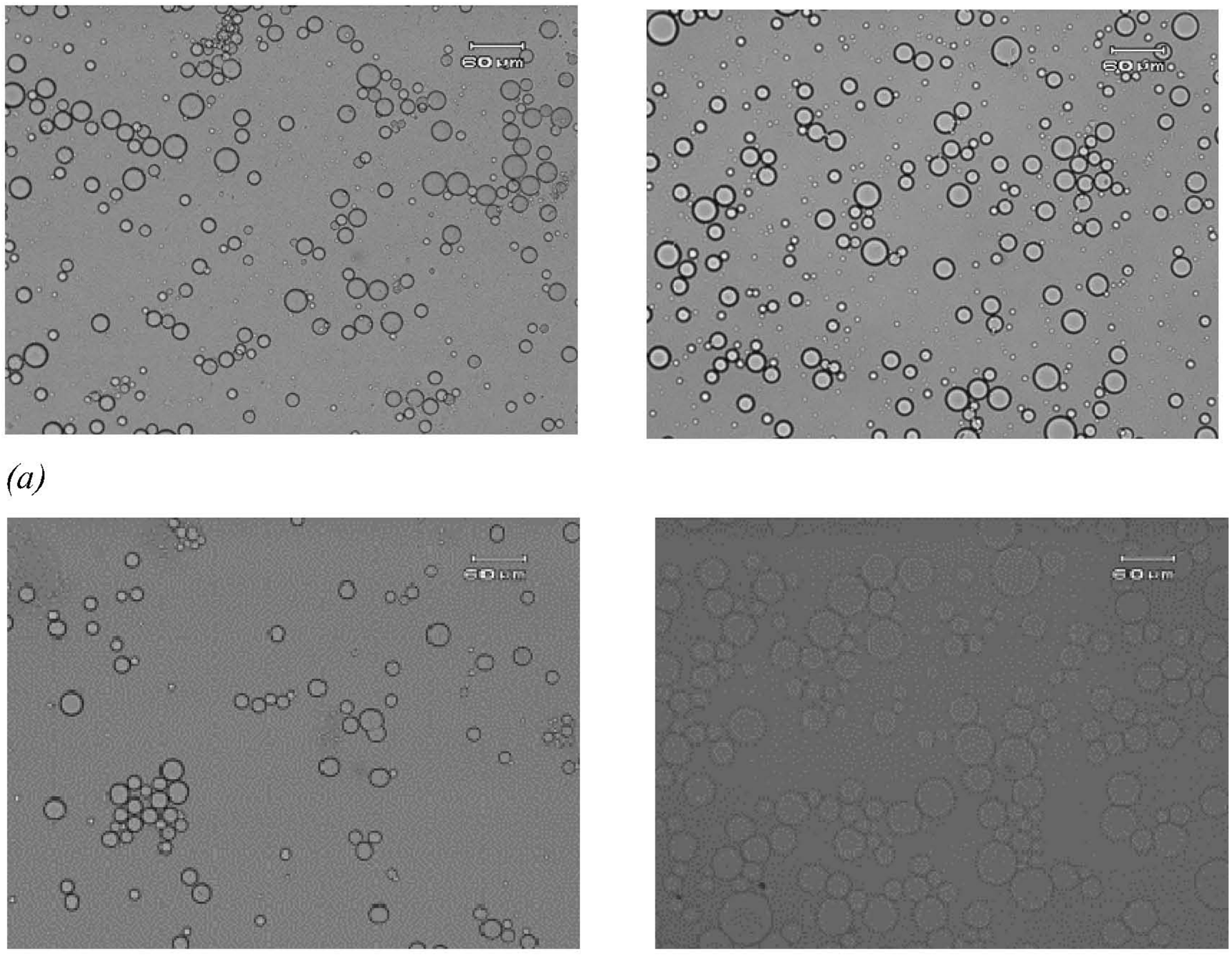

Figure 4. *Microscopic images of microspheres composed of (a) dex-HEMA DS 12 (b), dex-lac$_2$-HPMAm DS 12 at t=0 (left) and after 5 days in PBS at 37°C (right).*

4. Conclusions

Dex-HEMAm and dex(-lac$_{1,2}$)-HPMAm were synthesized macroscopic hydrogels as well as microspheres were investigated for their degradation characteristics . In contrast to microspheres and macrogels based on dex-HEMAm and dex-HEMA, degradation of systems prepared of dex-(lac$_{1,2}$-)HPMA resulted in the formation of only water-soluble degradation products. The swelling of hydrogels based on the new dextrans was generally greater than that of dex-HEMA. The degradation rate depended on the DS and the nature of the grafted side group and increased according to the following order: dex-HPMAm<dex-HEMA<dex-lac-HPMAm<dex-lac$_2$-HPMAm. It was possible to prepare protein-loaded microspheres of HPMAm variants of which the release of a model protein (BSA) was dependent on the number of hydrolyzable bonds in the crosslinks and the type of polymerizable group. Especially, microspheres prepared of dex-lac$_2$-HPMAm microspheres are attractive as controlled release system since protein-loaded microspheres based on this polymer were obtained with high encapsulation efficiency, low burst release and quantitative

release of the loaded protein. Importantly, these microspheres completely degraded into water-soluble degradation products. To further evaluate the feasibility of these new systems, future research should be focused on analysis of molecular weight of the degradation products, on the possibility to tailor the release and integrity of (pharmaceutically relevant) proteins by changing DS and water-content. Also, the biocompatibility and *in vivo* degradation of microspheres based on these polymers needs further investigation.

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Chapter 8

Summary and Perspectives

Summary

During the past three decades, recombinant DNA and hybridoma technologies have resulted in the commercial availability of a significant number of pharmaceutically active proteins. However, many of the pharmaceutical proteins that are currently on the market are only therapeutically useful following a regimen of frequent dosing through injections or intravenous infusions. The development of injectable controlled release systems based on polymeric microspheres or pegylation/acetylation of proteins can therefore be regarded as important strategies in protein delivery[1-3]. By protein pegylation or acylation, the protein is chemically attached to poly(ethylene glycol) or fatty acids thereby improving the circulation time in blood. In systems based on polymeric microspheres, proteins are encapsulated in a polymeric matrix and gradually released over time as a result of diffusion, swelling and degradation of the matrix. By slowly releasing the protein, 'infusion-like' drug release profiles can be obtained.

In this thesis, dextran based microspheres are investigated as controlled delivery system for proteins. In previous work carried out in our Department on dextran based systems, it was demonstrated that biodegradable dextran hydrogels were obtained by polymerization of dextran derivatized with hydroxyethyl methacrylate (dex-HEMA) [5-8]. Microspheres were prepared by polymerization of dex-HEMA in an aqueous two-phase system of dex-HEMA and PEG [9]. Due to the presence of hydrolytically sensitive carbonate ester groups linking HEMA to dextran, these gels degrade under physiological conditions. It was also shown that the degradation rate of these hydrogels and the release of proteins and small particles such as liposomes from these hydrogels could be tailored by the degree of methacrylate substitution (DS; the number of methacrylates per 100 glycopyranose residues) of the polymers and the initial water content of the gel[6-11]. Moreover, implantation studies in rats carried out by Cadée et al. showed that hydrogels and microspheres were well tolerated and degrade *in vivo*[12,13]. In another study, the therapeutic efficacy of IL2-loaded dex-HEMA microspheres was demonstrated[14]. In this thesis, several important aspects (e.g. in vivo in vitro correlation, protein release, encapsulation and degradation of microspheres) in the development of these dex-HEMA based microspheres as controlled delivery system of protein pharmaceuticals were further investigated.

In **Chapter 1**, an introduction to controlled release systems for proteins/small protein pharmaceuticals was given with a special emphasis on dextran based microspheres studied in this thesis. Moreover, the contents of this thesis are outlined in this chapter.

In **Chapter 2**, a controlled release formulation for human growth hormone (hGH) based on hydroxy ethyl methacrylated dextran (dex-HEMA) microsphere was developed for preclinical and

clinical studies. HGH is a small 22 kDa protein used to treat short stature caused by growth hormone deficiency, Turner's syndrome, or chronic renal failure. For formulation development, the effects of formulation parameters such as potassium peroxodisulfate (KPS; an initiator of radical polymerization reactions) concentration, protein loading and degree of hydroxyethyl methacrylate substitution on hGH release were investigated. By rational design, a microsphere formulation with an hGH release time of about 7 days, a high loading efficiency (≥90 %) and a low burst release (≤10% of the encapsulated protein) was identified. This formulation was evaluated both in Pit-1 deficient Snell dwarf mice and in healthy human volunteers. Subcutaneous administration in the mice of a single dose hGH-loaded dex-HEMA microspheres resulted in a dose dependent and significant increase in body length and weight. Importantly, a good correlation between *in vitro* hGH release and *in vivo* effects was obtained. This indicates that hGH, released from dex-HEMA microspheres has mainly retained its bioactivity. A pharmacokinetic study of dex-HEMA hGH microspheres in healthy volunteers demonstrated also a good *in vivo in vitro* correlation. The serum concentrations calculated using a one-compartment linear model were in good agreement with the measured hGH serum concentrations. Furthermore, subcutaneous administration of an hGH dex-HEMA microsphere formulation in man increased insulin-like growth factor-I (IGF-I) and IGF binding protein response-3 (IGFBP-3) levels. This indicates that hGH released from dex-HEMA microspheres is bioactive. A good *in vitro in vivo* correlation is important in the development of a controlled drug delivery system since such correlation enables the prediction of the therapeutic effects in a clinical situation. The possibility of translating *in vitro* release data into *in vivo* release characteristics is therefore an important advantage in the development of dex-HEMA based microspheres as a parenteral protein delivery system.

To optimize and predict the release of proteins from dex-HEMA microspheres, a fundamental understanding of the mechanisms controlling their release is very important. For that purpose, in **Chapter 3** a mathematical model was developed to describe the release of proteins from dex-HEMA microspheres. A kinetic Monte Carlo scheme for the degradation of a small domain inside the microsphere was developed. The results from this model were used in a second kinetic Monte Carlo scheme to model the diffusion and the subsequent release of proteins. The general effects of diffusion, crosslink density, protein loading, and clustering of proteins on the release were investigated. The model crosslink density (X_{model}) and the model diffusivity (D_{model}) were fitted to experimental release data of BSA monomer from dex-HEMA microspheres. By using the experimental release curves of liposomes and BSA monomer, it was found that *(1)* the model crosslink density (X_{model}) scales with the hydrodynamic diameter (d_h) as $d_h^{1.64}$ and *(2)* the diffusivity of the protein (D_{model}) scales approximately with $1/d_h$ (as expected from the Stokes-Einstein

equation). By using these scaling relations, quantitative predictions of the release curves of BSA dimer, immunoglobulin G and hGH were shown to be possible. Therefore, this model may play an important role in the optimization, understanding and prediction of the release of various proteins from degradable hydrogels in general.

The dex-HEMA microspheres studied in this thesis were prepared by a water-in-water emulsion polymerization process. The polymerization reaction was initiated by KPS and catalyzed by *N,N,N',N'*-tetramethyl ethylene diamine (TEMED). In **Chapter 4**, the effects of the initiator concentration, reaction temperature and pH on the mechanical properties of the microspheres were investigated. The size and size distribution of the microspheres, equilibrium water content, and methacrylate conversion were also determined. The mechanical properties of single microspheres were measured by a micromanipulation technique (technique to measure the mechanical properties of a single microsphere) and the rheological characteristics of macroscopic hydrogels of the same composition as the microspheres were determined by a controlled stress rheometer. The results showed that the Young's moduli of the microspheres and of macroscopic hydrogels measured by these two methods were in good agreement. Higher KPS concentrations resulted in a more rapid polymerization with a shorter gelation and lag time, and a higher Young's modulus of the gels. An increase in temperature also resulted in a more rapid polymerization with a shorter gelation and lag time. However, the Young's modulus of the gels decreased with an increase in polymerization temperature. The pH had no significant effect on the mechanical properties of the microspheres. This study demonstrates that the network properties of dex-HEMA hydrogels and microspheres can be tailored by the polymerization conditions, which opens the possibility to modulate the release rate of entrapped compounds.

In **Chapter 5**, the possibility was investigated to modulate the encapsulation efficiency and release hGH-loaded dex-HEMA microspheres by using excipients. Microspheres were prepared by polymerization of dex-HEMA (DS 16) in an aqueous two-phase system of dex-HEMA and PEG. Excipients (tween 80, pluronic F68, sucrose, NaCl, urea or methionine) were added and dissolved in the aqueous phases prior to the polymerization of dex-HEMA. High hGH encapsulation efficiencies (50-70 %) were obtained for microspheres prepared without excipients and with tween 80, NaCl or methionine. Substantially lower encapsulation efficiencies (27 and 19%, respectively) were obtained for microspheres prepared in the presence of sucrose and urea. A biphasic release profile was observed for microspheres prepared without excipients from which hGH, after a delay time of 2 days, was gradually released over a period of 6 days. In contrast, for microspheres prepared with tween 80, pluronic F68, sucrose, NaCl and urea, hGH was gradually released without delay over a period of 6-8 days. Microspheres prepared with methionine showed a methionine

concentration-dependent delay time varying from 0 to 2 days followed by zero order release for 6 days. It was demonstrated that the excipients, except methionine, had no effect on the hydrogel matrix. Furthermore, it was shown that hGH precipitates in an aqueous solution of dex-HEMA, and these hGH precipitates are likely entrapped in the hydrogel matrix. Moreover, it was demonstrated that smaller hGH precipitates were formed in the presence of excipients, which explains the difference in protein release profiles of microspheres prepared with and without these additives. This finding is consistent with model discussed in **Chapter 3**, which showed that with increasing protein-cluster size and thus restricted mobility of proteins inside a cluster, the release delay time was prolonged. Once sufficient crosslinks are hydrolyzed to allow diffusion of a protein molecule, first the precipitate needs to dissolve before the dissolved protein molecules can be released. Likely, protein dissolution is a slow process in a matrix with a high concentration of dextran, and consequently, the delay time increased. The reason for the lower encapsulation efficiency for proteins in microspheres prepared with urea and sucrose might be ascribed to (small) conformational changes or denaturation of the protein. This results in exposure of the relatively hydrophobic core of the protein to the solvent which in turn results in an increased partitioning over the PEG phase. Consequently, this results in a decreased concentration of the protein in the dex-HEMA phase, which explains the observed lower encapsulation efficiency of hGH in dex-HEMA microspheres.

The aim of the study presented in **Chapter 6** was to investigate the *in vitro* degradation of dex-HEMA microspheres. Microspheres were incubated in phosphate buffer pH 7.4 at 37 ^{o}C, and the dry mass, mechanical strength and chemical composition of the microspheres were monitored in time. The amount and nature of the formed degradation products were established for microspheres with different crosslink densities by FT-IR, NMR, mass spectrometry, SEC analysis and XPS (X-ray photoelectron spectroscopy). Dex-HEMA microspheres (DS 12) incubated at pH 7.4 and 37 ^{o}C showed a continuous mass loss as a result of the release of water-soluble degradation products, leaving after 6 months a residue of water-insoluble degradation products of about 10 % (with respect to the initial solids content). NMR, mass spectrometry and SEC showed that the water-soluble degradation products consisted of dextran, low molecular weight pHEMA (M_n ~ 15 kg/mol), and small amounts of unreacted HEMA and HEMA-DMAP (intermediate reaction product of the Baylis-Hillman reaction of HEMA with DMAP (4-dimethyl aminopyridine)). Microscopy revealed that the water-insoluble residue consisted of particles with a similar shape and size as non-degraded microspheres. However, these particles had lost their mechanical strength as evidenced from rheological data obtained using micromanipulation technique. FT-IR and XPS revealed that these particles consisted of pHEMA of which a small fraction was low-molecular-weight material

(M_n ranging between 27-82 kg/mol) soluble in methanol. The remaining methanol-insoluble material likely consisted of lightly crosslinked pHEMA. In conclusion, *in vitro* degradation of dex-HEMA microspheres results in the formation of water-soluble degradation products (mainly dextran), leaving a small water-insoluble residue mainly consisting of pHEMA.

It was reasoned that hydrogels based on dextran derivatives with a more hydrophilic polymerizable groups as compared to HEMA would result in fully soluble degradation products. In **Chapter 7**, a series of dextrans with hydrophilic polymerizable groups were synthesized to investigate whether hydrogels based on these derivatized dextrans indeed degrade under physiological conditions into fully water-soluble degradation products. These polymerizable dextrans include dextran derivatized with HPMAm (N-(2-hydroxypropyl)methacrylamide), lac-HPMAm (N-(2-hydroxypropyl)methacrylamide-monolactate), lac_2-HPMAm (N-(2-hydroxypropyl)methacrylamide-dilactate) and HEMAm (N-(hydroxyethyl)methacrylamide. It was demonstrated that the degradation of hydrogels based on dex-HEMAm and dex-HEMA resulted in the formation of both water-soluble and water-insoluble degradation products. In contrast, the degradation of both macrogels and microspheres based on dex-($lac_{1,2}$-)HPMA resulted in the formation of only water-soluble degradation products. Therefore, swelling and degradation kinetics of hydrogels based on these derivatives were investigated. The degradation time depended on the degree of substitution (DS, number of polymerizable side groups units per 100 glucose units) and nature of the polymerizable side group, and increased according to the following order: dex-lac_2-HPMAm < dex-lac-HPMAm < dex-HEMA < dex-HPMAm. It was demonstrated that microspheres loaded with bovine serum albumin (BSA) based on dex-($lac_{1,2}$-)HPMA could be obtained of which the release profile was dependent on the number of lactic acid units in the crosslinks and the DS. Most attractive for further investigation are microspheres based on dex-lac_2-HPMA, as BSA-loaded microspheres based on this polymerizable dextran showed high encapsulation efficiency (86%), low burst release (2% of the encapsulated BSA) and a sustained release for 5 days resulting in a quantitative release of the encapsulated protein. Moreover, as observed for macroscopic hydrogels, the microspheres degraded into in fully water-soluble degradation products.

Perspectives

The work performed in this thesis demonstrates that dex-HEMA based microspheres are very suitable for the controlled delivery of proteins. Importantly, it was demonstrated that the biological activity of a pharmaceutically relevant protein (hGH) released from these microspheres

was mainly retained and that the *in vitro* release corresponds with the *in vivo* effects (**Chapter 2**). In **Chapter 3** it was demonstrated that the *in vitro* protein release from dex-HEMA microspheres can be predicted from the hydrodynamic radius of the entrapped protein, the crosslink density of the microspheres and the hydrolysis rate of the crosslinks. If, in addition, its *in vivo* absorption rate (from the subcutaneous site of application), elimination rate (from the circulation), bioavailability from the subcutaneous site (which are data that can often be easily found in literature) is known, theoretically, for any protein and dex-HEMA microsphere of any DS, the plasma concentration time profile can be predicted. This is an important advantage in the development of the dex-HEMA microspheres as controlled drug delivery system, since it is possible to rationally select a certain matrix on beforehand, and to reduce the number of costly experiments. Nevertheless, there are still a number of issues that need further investigation.

One of these issues is upscaling. For the *in vivo* studies described in **Chapter 2**, microspheres were prepared at one kilogram emulsion scale, resulting in approximately 9 g of dry microspheres. To produce sufficient material for clinical testing and, ultimately, commercialization, further upscaling of the manufacturing process is required. Figure 2 in the introduction summarizes the process steps involved in the manufacturing of dex-HEMA microspheres. Basically, in the manufacturing procedure of dex-HEMA based microspheres 4 main steps can be distinguished: 1) emulsification, 2) polymerization, 3) washing of the formed microspheres to remove PEG, unreacted products and non-encapsulated protein and 4) drying of the microspheres. The duration and capacity of each process step determines the final throughput of the process.

One way to increase the capacity in the first step would be the use of a higher dex-HEMA/PEG ratio in the emulsion. All microsphere batches prepared in this thesis were prepared with a dex-HEMA/PEG volume ratio of 1/40. Calculations based on the phase diagram PEG/dex-HEMA reveal that an increase in volume ratio to e.g. 1/30 or 1/20 would imply an increase in microsphere yield (based on their dry weight) of 30 or 90 %, respectively. It is important to realize, that without increasing the stirring speed a higher dex-HEMA/PEG ratio also will result in larger particles[9]. Moreover, one should realize that proper mixing of KPS and TEMED through an emulsion with a larger number of dex-HEMA droplets is also challenging, because mixing of these initiators through the emulsion by stirring might also result in much more coalescence of particles. During polymerization this can result in the formation of large lumps of aggregated microspheres (unpublished results). Another possibility to improve capacity is to use a larger bioreactor. Advantages of this approach are similarities with processes at small scale and relatively straightforward rules for upscaling. Disadvantages are the huge equipment costs and the need for larger production areas. Alternatively, to increase throughput a continuous process can be designed

with in line static mixers to prepare the dex-HEMA in PEG emulsion. Due to the high flow rates applied in such systems generally large throughputs can be obtained. Application of such system requires extensive testing and investigation of the process parameters affecting protein encapsulation, release characteristics and microspheres size to find optimum processing conditions.

Reduction of the polymerization time is an option to reduce manufacturing time. Previously, Stenekes et al. demonstrated that maximum methacrylate conversion of microspheres was reached within 30 minutes, when incubating the dex-HEMA in PEG emulsion with the initiators at 21°C [18]. In agreement with these findings, in **Chapter 4** of this thesis, the gelation of dex-HEMA hydrogels at 21°C was found to be complete (as reflected by a constant G') within 30 minutes. This implies that, based on the above mentioned results, it can be concluded that in future small improvements in process time can be obtained by reducing the polymerization time from 60 (used in all general microsphere preparation procedures in this thesis) to 30 minutes.To make this feasible, further reduction of the polymerization time is preferred especially when designing a continuous process (inline emulsification, polymerization etc) for manufacturing of microspheres. The current polymerization requires a residence time of 30-60 minutes. Although reactors with such long residence time are available (e.g. plug flow reactors), long residence times may affect the particle size distribution (e.g. aggregation of polymerizing microspheres).

Finally, improvements in the washing procedure or the freeze-drying process can also add up to further reduction of the total processing time. At a small scale, sequential washing steps are used to remove PEG, non-encapsulated protein, as well as KPS (plus its degradation products) and TEMED. At larger scale, efficient removal of PEG, non-encapsulated protein, and KPS and TEMED was achieved using tangential flow filtration instead (**Chapter 1**). Optimization of the filters and flow rates, would further reduce the total duration of the wash steps. Substantial improvements in terms of processing time can be obtained in the freeze-drying step. To achieve an acceptable shelf life, microsphere formulations are often freeze-dried. Generally, freeze-drying of proteins is a time consuming process. Freeze-drying generates both freezing and drying stresses which can induce conformational changes and irreversible damage to the protein and aggregation of the protein. The presence of aggregated protein in the microspheres can finally result in incomplete release of the protein. Moreover, aggregated proteins are often inactive, and can be toxic or immunogenic[15] and aggregation should therefore be prevented. To protect proteins against these stresses both cryo- and lyoprotectants can be added. Most cryo/lyoprotectants are small molecules, which will not be co encapsulated in the microsphere matrix. Therefore, in **Chapter 2** excipients (e.g. mannitol) were added to the wash buffer prior to freeze-drying. Nevertheless, freeze-drying of those microspheres process took almost 2 days. Further insight into the effect of the freeze-drying

and the use of cryo and lyoprotectants on the protein inside a microsphere would yield important information for optimization of freeze-drying times. Possible techniques for probing the structural changes of proteins within dex-HEMA microspheres after freeze-drying or rehydration of the microspheres are differential scanning calorimetry and FT-IR. Circular dichromism and/or fluorescence spectroscopy, gel electrophoresis, mass spectrometry and bioactivity assays are recommended techniques to investigate the integrity of proteins after release from freeze-dried microspheres after rehydration.

As briefly mentioned in the previous section, another major issue in developing controlled-release systems for protein pharmaceuticals is preservation of the protein structure during micro-encapsulation. In the preparation procedure of dex-HEMA microspheres, shear is applied to the dex-HEMA/PEG two-phase system, to obtain the dex-HEMA in PEG emulsion. It is well known that shear applied on hGH solutions can induce aggregation [16-18], which can result incomplete release of the protein from the microspheres should therefore be prevented. The in vitro results **Chapter 2** demonstrate that quantitative release of protein (except when high shear stresses were applied when using a microfluidizer) was obtained. Furthermore, in vivo data demonstrate that the released hGH had maintained its bioactivity. This suggests that the general manufacturing procedure for hGH-loaded microspheres does not induce significant aggregation of this sensitive protein.Changes in process parameters or manufacturing equipment (e.g static mixer) might induce more shear stress on the emulsified dex-HEMA droplets which might induce protein unfolding/aggregation, and therefore, their effect on protein integrity should be carefully investigated.

Previous work has demonstrated that KPS can induce oxidation of in particular methionine residues in proteins[20]. Since protein oxidation is unwanted, reducing the KPS concentration is an obvious strategy to reduce the oxidation of proteins. It should be mentioned that changing the KPS concentration can have effects on the properties of the formed network. In **Chapter 4** it was shown that reduction of KPS concentration resulted in a lower Young's modulus and increase of pore size, which in turn might result in a faster protein release (**Chapter 5**). Possibilities to prevent protein oxidation include the addition of anti-oxidants such as methionine[20], and controlled precipitation of proteins in the dex-HEMA phase before addition of KPS/TEMED. **Chapter 5** demonstrates that proteins may precipitate in the dex-HEMA. Oxidation of solid proteins is much slower than oxidation of dissolved proteins. Also demonstrated in this chapter was that the size of these precipitates can be influenced by the addition of excipients, which will finally result in changes in the release profiles. In **Chapter 1** it was suggested that processing time might affect the size of the precipitate. To take full advantage of the possibility to prevent protein oxidation by controlled

precipitation of proteins, the mechanism of protein precipitation and the dissolution process of such precipitates within a dex-HEMA phase require further investigation. In addition, more information on the dissolution of protein precipitated in dex-HEMA can be used to further optimize the model developed in **Chapter 3** and increase its predictive power.

In further studies, the possibility to modify particle size and obtain narrower particle size distributions should be investigated. Batches of microspheres obtained via the current emulsification technique using a vortex have a broad size distribution (1-30 μm)[9]. For certain application particles with a small size range (e.g. 1-10 μm for vaccine delivery) are requested. These small microspheres may be targeted to various cells of the immune system[21].On the other hand, for local and systemic protein as well as vaccine delivery, large microspheres (>10 μm) are more suitable. In stirred vessels, particle size and size distribution depend on processes such as break-up and coalescence of emulsion droplets. The average size and particle size distribution depend on the interplay between these processes. Generally, increasing stirring times generates narrower particle size distributions. Particle size can be modulated by shear stresses. E.g smaller particles are formed at high shear (e.g. using of an ultra turrax) whereas low shear results in the formation of larger particles. Increasing or decreasing the viscosity of the PEG or dex-HEMA phase can used to modify particles size. To obtain small particles, increasing the concentration or molecular weight of PEG, and decreasing the concentration or molecular weight of dex-HEMA are obvious options for further investigation. On the other hand, it is expected that larger particles are formed by decreasing the concentration or molecular weight of PEG and increasing the concentration or molecular weight of dex-HEMA. Adjusting these formulation parameters, may results in changes of the water content of the microspheres or may cause problems in mixing of KPS/TEMED through the emulsion. Further it was expected that addition of surfactants can be used to decrease the particle size. In contrast, introductory experiments using tween 80 (1% (w/w)) have shown that the size of microspheres prepared with this excipient at this concentration was slightly larger than the size of microspheres prepared without tween. Microscopic analysis revealed that small PEG droplets were included in the microspheres. The addition of surfactants on dex-HEMA/PEG water-in-water emulsion requires further investigation and effect on particles size Finally, filters/sieves of specific mesh size can also be used to obtain microspheres with a preferred size range. It should be noticed that due to the broad size distribution, sieving of microsphere batches will lead to (substantial) loss of material and therefore loss of the (expensive) active.

A more elegant way to obtain microspheres with more narrow size distributions has been recently investigated by de Geest et al[22], who used a microfluidic device in which the size and the breakup rate can be controlled by the flow conditions. As droplets are formed at constant rate in

mineral oil and remain at a uniform distance from each other by which coalescence of particles is avoided, after polymerization using a photoinitiator and UV irradiation, microspheres are obtained with a very narrow particle size distribution (e.g. 9.3 and 10.7 μm[22]). Further optimization of these systems is required as with the current droplet frequencies only 0.01-02 mg microspheres per hour can be produced[22]. Moreover, currently this method is only tested for the formation of microspheres with relatively high water contents (≥ 70%). For manufacturing of hGH-loaded microspheres in **Chapter 2** a water content of 50 % is preferred.

For the application of dex-HEMA microspheres in man, a low level of impurities is required and complete removal of the degradation products at the site of administration is preferred. **Chapter 6** showed that *in vitro* degradation of dex-HEMA microspheres results in the formation of HEMA-DMAP and a small amount of water-insoluble degradation products. To avoid the formation of HEMA-DMAP, the synthesis of dex-HEMA should be optimized. In a recent study it was shown that dex-HEMA can be synthesized without the addition of DMAP. Furthermore, additional studies are required to investigate the fate of insoluble degradation products at site of application. Marbilleau et al have investigated the *in vivo* degradation of pHEMA by J774.2 macrophages[23]. Their results revealed that the degradation of highly crosslinked pHEMA particles by macrophages is a slow process. In the same study it was shown that loosely crosslinked material appeared to be degradable by macrophages. Similar studies need to be performed to investigate the in vivo degradation of the water-insoluble pHEMA degradation products with predegraded microspheres. An interesting technique to investigate the intracellular degradation of dex-HEMA microspheres or the water-insoluble pHEMA degradation products is Raman spectroscopy[24]. Disadvantage of this technique is that *in vitro* cell viability is limited and consequently degradation can only be followed for limited period of time. Alternatively, the in vivo fate of the water-insoluble residues of the microspheres can be studied using dex-HEMA labeled with radio-isotopes.

To circumvent the issue of the water-insoluble degradation products, in **Chapter 7** dextrans derivatized with better water-soluble polymerizable groups than HEMA were synthesized and evaluated. Very attractive alternatives for dex-HEMA are dex(-$lac_{1,2}$)-HPMAm. It was demonstrated that release of an entrapped model protein (BSA) could be tailored and importantly, the degradation yielded only water-soluble degradation products. Interestingly, hydrogels based on dex-lac_2-HPMAm showed extensive swelling, within a short period of time, which was attributed to the increase in hydrophilicity of the gels due to the conversion of pHPMAm-dilactate (cloud point at 13°C)[25] into pHPMAm-monolactate and pHPMAm (fully water-soluble). Dex-lac_2-HPMAm might be an interesting alternative for dex-HEMA applied in self-rupturing microcapsules which

are currently under investigation by the group of Professors De Smedt and Demeester of the Ghent University[26-28].

To complete the evaluation of the feasibility of the new polymerizable dextrans synthesized in **Chapter 7**, one of the main issues is the analysis of molecular weight of the degradation products. High molecular weight degradation products can not be excreted from the body, and must therefore be avoided. To prevent the formation of high molecular weight degradation products the use of multi-arm chain transfer agents can be investigated. The addition of a multi-arm chain transfer agents[29] containing hydrolyzable groups during the preparation of dex-($lac_{1,2}$)-HPMAm microspheres may result in the formation of dextran microspheres with sufficient crosslink density to obtain suitable release profiles and which result after degradation in water-soluble low molecular weight degradation products.

In conclusion, this thesis demonstrates that dextran based microspheres are attractive systems for the controlled release of pharmaceutically active proteins such as hGH. Focusing future development efforts on upscaling, optimization of particles size and the formation of low molecular weight water-soluble degradation products would further enhance their potential application in humans as controlled release system for a wide variety of protein pharmaceuticals.

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Appendix A

Achtergrond en samenvatting

Achtergrond

Eiwitten spelen een belangrijke rol bij veel fysiologische processen zoals o.a. energievoorziening, zuurstoftransport, groei en ontwikkeling. Wanneer de eiwitvoorziening door bepaalde omstandigheden, zoals bijvoorbeeld door beschadiging van bepaalde organen, geheel of gedeeltelijk wegvalt worden deze processen ernstig verstoord. Behandeling met eiwitten is dan noodzakelijk. De aflopen drie decennia zijn er door recombinant DNA en hybridoma technologie een groot aantal pharmaceutisch actieve eiwitten op grote schaal beschikbaar gekomen voor therapie. Echter, de efficiënte toediening van therapeutisch eiwitten is niet eenvoudig gebleken. Redenen hiervoor zijn de snelle afbraak in het maag-darmkanaal en het feit dat deze eiwitten vaak nauwelijks kunnen worden opgenomen door aanwezigheid van natuurlijke biologische barrières. Daarom worden dergelijke medicijnen vaak via parenterale weg toegediend bijvoorbeeld via een intraveneuze, subcutane of intramusculair injectie. Omdat vele van deze eiwitten binnen korte tijd weer geklaard worden, is ook deze methode niet optimaal en moeten deze continu (d.m.v infuus) of zeer frequent (meerder injecties per dag/week) worden toegediend. Dergelijke therapieën zijn erg belastend voor patiënten. Het terugdringen de doseringsfrequentie van dagelijkse naar weekelijkse of maandelijkse toediening zou de patiënt-vriendelijkheid van dergelijke therapieën aanzienlijk verbeteren.

De afgelopen decennia is er dan ook veel onderzoek gedaan naar formuleringen waarmee het eiwit gedurende een langere periode op de juiste plaats en in de juiste concentratie actief blijft. Om een verlengde werking te bewerkstelligen zijn o.a. eiwitten chemisch gemodificeerd door bijvoorbeeld polyethyleenglycol (PEG) te koppelen aan het eiwit. Door de aanwezigheid van dit polymeer wordt het eiwit minder snel door het lichaam geklaard. Een andere veelbelovende benadering is om het eiwit te verpakken in polymere systemen die geleidelijk het eiwit vrij kunnen geven. In dit proefschrift richten we ons op de laatste strategie. Door zwelling en degradatie van de polymeermatrix kunnen de eiwitten geleidelijk worden afgegeven. Veel polymere afgiftesystemen zijn gebaseerd op biodegradeerbare polymeren die tijdens en na het vrijgeven van het eiwit geleidelijk afbreken. Hierdoor hoeft er na afgifte geen materiaal operatief verwijderd te worden. Een voorbeeld hiervan zijn biodegradeerbare systemen gebaseerd op poly (D,L-lactic-co-glycolic acid) (PLGA). Hoewel er al jaren onderzoek naar systemen gebaseerd deze polymeren wordt gedaan, zijn er maar weinig eiwit beladen systemen op de markt gekomen. Eén van de redenen hiervoor is dat vaak burst release en incomplete afgifte van het eiwit plaatsvindt als gevolg van aggregatie of degradatie van het eiwit. Vergelijkbare problemen zijn ook opgetreden bij gebruik van andere (hydrofobe) biodegradeerbare polymeren zoals poly(orthoesters) en poly(anhydrides). Door

het hydrofobe karakter van deze polymeren zijn formuleringstechnieken noodzakelijk zoals het gebruik van organische oplosmiddelen of verhitting om microsferen te bereiden. Deze technieken zijn vaak schadelijk voor het eiwit. Om deze schade te voorkomen is er een grote belangstelling voor meer eiwit-vriendelijkere systemen. Een goed voorbeeld hiervan zijn hydrogelen. Hydrogelen zijn chemisch of fysische vernette polymeren die een grote hoeveelheid water op kunnen nemen. Door deze eigenschap zijn deze systemen vaak zeer compatibel met zowel het in te sluiten eiwit als het levend weefsel.

Samenvatting

In dit proefschrift wordt het potentieel van dextraan gebaseerde microsferen onderzocht voor het gebruik als gecontroleerd afgiftesysteem voor farmaceutisch actieve eiwitten. In eerder werk uitgevoerd in de Biofarmacie en farmaceutische technologie groep in Utrecht is aangetoond dat hydrogelen verkregen kunnen worden door polymerisatie van hydroxyethyl methacrylate gesubstitueerd dextraan (dex-HEMA). Microsferen (de injecteerbare vorm voor hydrogelen) die gebaseerd zijn op dex-HEMA kunnen bereid worden door polymerisatie van de dex-HEMA druppels in een waterige emulsie van dex-HEMA in PEG. Door de aanwezigheid van de hydrolyseerbare bindingen tussen HEMA en dextraan zijn deze gelen hydroyseerbaar onder fysiologische omstandigheden. Door vóór polymerisatie een eiwit aan de dex-HEMA oplossing toe te voegen kunnen microsferen verkregen worden waarin het eiwit ingesloten zit. Het is gebleken dat de afgifte van dit eiwit kan worden gestuurd door de vernettingsgraad ('crosslink' dichtheid) van de microsferen aan te passen door een andere DS (aantal HEMA groepen per 100 glycopyranose eenheden in dextraan) of watergehalte te kiezen. De dichtheid van het netwerk wordt hierdoor veranderd; een hogere DS of een lager watergehalte leidt tot kleinere gaten in het netwerk. Dit resulteert in een langzamere afgifte, omdat er meer crosslinks verbroken moeten worden voordat de gaten in het netwerk groot genoeg om de diffusie van het eiwit toe te laten. Daarnaast is er met *in vivo* experimenten aangetoond dat hydrogelen en microsferen gebaseerd op dex-HEMA biocompatibel zijn en ook *in vivo* afbreken. Bovendien is aangetoond dat een eenmalige injectie van dextraan microsferen met een geleidelijke afgifte van rhIL-2 therapeutisch zeer effectief is.

In dit proefschrift worden verschillende aspecten (zoals *in vivo in vitro* correlatie voor hGH beladen dex-HEMA microsferen, eiwit afgifte en incapsulering en degradatie van de microsferen) bestudeerd die van belang zijn voor de verdere karakterisering en ontwikkeling van dex-HEMA microsferen als gereguleerd afgiftesysteem voor eiwitten.

In **Hoofdstuk 1** wordt een overzicht gegeven van gecontroleerde afgiftesystemen voor eiwitten in het algemeen en van dextraan microsferen als gecontroleerd afgiftesysteem in het bijzonder. Bovendien worden in dit hoofdstuk de opbouw en doelen van het werk in dit proefschrift uiteengezet.

In **Hoofdstuk 2** is een gecontroleerd afgiftesysteem ontwikkeld voor humaan groeihormoon (hGH) gebaseerd op dex-HEMA microsferen voor gebruik in preklinische en klinische studies. HGH is een klein 22 kDa eiwit dat gebruikt wordt bij de behandeling van patiënten met een groeiachterstand veroorzaakt door groei hormoon deficiëntie (GHD), het syndrome van Turner of chronische nierinsufficiëntie. Om een formulering te ontwikkelen gebaseerd op dextraan microsferen werden in eerste instantie de effecten van formuleringsparameters zoals de concentratie kalium peroxodisulfaat (KPS, een initiator voor de polymerisatiereacties), de eiwitbelading en de substiutiegraad van dex-HEMA op het afgifteprofiel uit de microsferen onderzocht. Er is een geschikte formulering gevonden met een afgifte gedurende 7 dagen, een hoge encapsuleringsefficiëntie en een lage initiële 'burst'. Deze formulering is getest in zowel 'Pit-1 deficient Snell' dwerg muizen als in gezonde vrijwilligers. Subcutane toediening van één enkele dosering resulteerde in een dosis-afhankelijke, significante toename in lengte en gewicht van deze muizen. Een belangrijk resultaat is dat er een goede correlatie is gevonden tussen de *in vitro* hGH afgifte uit de microsferen en het effect *in vivo* (toename in lengte en gewicht). Dit is een bewijs dat de bioactiviteit van de eiwitmoleculen afgegeven uit dex-HEMA microsferen in belangrijke mate behouden is gebleven. In de farmacokinetische studie uitgevoerd met gezonde vrijwilligers is ook een goede *in vitro in vivo* correlatie gevonden. De hGH concentraties in het serum berekend met behulp van een één-compartiment farmacokinetisch model komen overeen met de gemeten hGH concentraties in het serum van deze vrijwilligers. Bovendien resulteerde de éénmalige toediening van deze hGH microsfeerformulering in een toename van de 'insulin-like growth factor-I (IGF-I)' en 'IGF binding protein response-3 (IGFBP-3)' spiegels. Dit is wederom een bewijs dat de hGH dat vrijkomt uit de dex-HEMA microsferen bioactief is. Deze goede *in vitro in vivo* correlatie is belangrijk voor de ontwikkeling van gecontroleerde afgiftesystemen, omdat het een voorspelling van de het therapeutisch effect geeft. De mogelijkheid om de vertaalslag te maken van de *in vitro* naar de *in vivo* afgifte is een belangrijk voordeel van dex-HEMA microsferen bij hun ontwikkeling als parenteraal afgiftesysteem voor eiwitten.

Om de afgifte van eiwitten uit dex-HEMA microsferen verder te optimaliseren en te voorspellen is het belangrijk om een fundamenteel inzicht te hebben in de mechanismen die de afgifte bepalen. Daarom is in **Hoofdstuk 3** een model ontwikkeld dat de afgifteprofielen van eiwitten uit dex-HEMA microsferen beschrijft. Een Kinetische Monte Carlo (KMC) simulatie is

ontwikkeld voor de afbraak van een klein domein in een microsfeer. Het resultaat van deze simulatie is gebruikt in een tweede KMC simulatie om de diffusie en de afgifte van een eiwit uit de microsfeer te beschrijven. De algemene effecten van diffusie, 'crosslink' dichtheid, eiwitbelading en clustering van eiwitten zijn onderzocht. Met behulp van experimentele afgiftedata van BSA uit dex-HEMA microsferen zijn de modelwaarden voor de 'crosslink' dichtheid (X_{model}) en diffusiecoëfficiënt (D_{model}) bepaald. Met behulp van experimentele data voor de afgifte van BSA monomeer en liposomen uit dex-HEMA microsferen is gevonden dat de gemodelleerde 'crosslink' dichtheid (X_{model}) en de gemodelleerde diffusiecoëfficiënt respectievelijk schalen met de hydrodynamisch diameter van het eiwit (d_h) tot de macht 1.64 ($d_h^{1.64}$) en met $1/d_h$ (in overeenstemming met de Stokes-Einstein vergelijking voor diffusie). Verder is er aangetoond dat met behulp van deze schalingsrelaties de afgifteprofielen van BSA dimeer, IgG en hGH goed voorspeld konden worden. Hierdoor kan dit model een belangrijke rol spelen bij het verkrijgen van meer inzicht in de optimalisatie van de afgifteprofielen van eiwitten uit degradeerbare hydrogelen.

Zoals eerder aangegeven worden de dex-HEMA microsferen in dit proefschrift bereid in een waterige emulsie. De polymerisatie van de dex-HEMA in de emulsiedruppels wordt geïnitieerd door KPS en gekatalyseerd door *N,N,N',N'*-tetramethyl ethylene diamine (TEMED). In **Hoofdstuk 4** worden de effecten van de initiatorconcentratie, de temperatuur en de pH op de mechanische eigenschappen van de microsferen onderzocht. Daarnaast zijn de grootte en grootteverdeling van de microsferen, het watergehalte en de methacrylaatconversie van deze microsferen bepaald. De mechanische eigenschappen van een enkele microsfeer zijn gemeten met de zogenoemde 'micromanipulatie' techniek (m.b.v deze techniek kunnen de mechanische eigenschappen van één microsfeer gemeten worden). De mechanische eigenschappen van macrogelen van dezelfde samenstelling zijn gemeten m.b.v. een 'controlled stress' reometer. Er is aangetoond dat de Young's modulus van microsferen en van macrogelen met elkaar overeenkomen. Een hogere KPS concentratie resulteerde in een sneller polymerisatie, een kortere geleringstijd en een hogere Young's modulus. De pH had geen significante invloed op de Young's modulus. De Young's modulus neemt wel af met een toename in de polymerisatietemperatuur. De resultaten uit deze studie tonen aan dat het netwerk van dex-HEMA macrogelen en microsferen kan worden gestuurd door aanpassing van de polymerisatiecondities. Mogelijk is dit ook een geschikte methode om de afgifteprofielen van geincapsuleerde eiwitten te sturen.

In **Hoofdstuk 5** is onderzocht hoe de incapsuleringsefficiëntie en afgifteprofielen gestuurd kunnen worden door toevoeging van excipiënten tijdens de bereiding van hGH beladen microsferen. De microsferen zijn bereid volgens de eerder beschreven methode met dit verschil dat excipiënten zoals tween 80, pluronic F68, sucrose, NaCl, ureum of methionine opgelost werden in

beide fasen vóór de vorming van de emulsie en de polymerisatie van de dex-HEMA. Hoge incapsuleringsefficiënties werden gevonden voor microsferen bereid zonder excipiënten en voor microsferen bereid met tween 80, NaCl of methionine. De incapsuleringsefficiëntie was veel lager voor microsferen bereid met sucrose en ureum. Microsferen bereid zonder excipiënten vertoonden een bifasische afgifteprofiel voor hGH waarbij gedurende een periode van 2 dagen vrijwel geen eiwit werd afgegeven (de zogenaamde 'delay time') met daarna een periode van 6 dagen waarin het eiwit geleidelijk werd afgegeven. Microsferen bereid met één van de excipiënten tween 80, pluronic F68, sucrose, NaCl en urea de hGH vertoonden een geleidelijke afgifte van hGH over een periode van 6-8 dagen (zonder 'delay time'). De afgifte van hGH uit microsferen bereid met methionine vertoonde een concentratie-afhankelijke 'delay time' die varieerde tussen 0 en 2 dagen gevolgd door een geleidelijk afgifte van hGH over een periode van 6 dagen. Verder is aangetoond dat behalve methionine geen van de andere excipienten effect heeft op de hydrogelnetwerk. Uit precipitatie-experimenten is gebleken dat hGH precipiteert in een oplossing van dex-HEMA. Hoogstwaarschijnlijk wordt dit precipitaat ingesloten in de hydrogelmatrix tijdens de bereiding van de microsferen. Daarnaast is aangetoond dat in aanwezigheid van zowel dextraan als van de excipiënten een veel fijner precipitaat gevormd en ingesloten wordt in de hydrogel matrix tijdens de bereiding. Het verschil in precipitaatgrootte vormt een verklaring voor het verschil in afgifteprofiel tussen microsferen bereid met en zonder excipiënten. Dit komt overeen met de resultaten voor het effect van clustergrootte op de afgifte volgens het model van **Hoofdstuk 3**. In dit hoofdstuk is aangetoond dat bij grotere clusters (en daardoor de gereduceerde mobiliteit van eiwitten in het cluster) de 'delay time' in het afgifteprofiel toeneemt. Dit wordt veroorzaakt door het feit dat de eiwitmoleculen in een precipitaat eerst moeten oplossen voordat ze afgegeven kunnen worden uit de microsfeer. Door de hoge concentratie dextraan in een microsfeer zal de oplossnelheid laag zijn, met als gevolg dat de 'delay time' toeneemt. De lagere incapsuleringsefficiëntie voor microsferen bereid met ureum en sucrose kan toegeschreven worden aan (kleine) verandering in de conformatie of denaturatie van het eiwit. Hierdoor komen er meer hydrofobe groepen aan het oppervlak van het eiwit te liggen. Een toename in de hydrofobiciteit van het eiwit verschuift de verdeling van het eiwit tussen de PEG en dex-HEMA fase meer naar de PEG-fase. Dit heeft tot gevolg dat de eiwitconcentratie in de dex-HEMA fase en daardoor de uiteindelijke incapsuleringsefficientie afneemt.

Het doel van het onderzoek beschreven in **Hoofdstuk 6** was om de afbraak van dex-HEMA microsferen *in vitro* in kaart te brengen. Hiervoor werd de verandering in (droge) massa, de mechanische sterkte en de chemische samenstelling gevolgd van microsferen die geïncubeerd werden in een fosfaatbuffer van pH 7.4 bij 37 °C. De hoeveelheid en de chemische samenstelling

van deze afbraakproducten van microsferen met verschillende 'crosslink' dichtheden werden onderzocht met FT-IR, NMR, massaspectrometrie, SEC en XPS (X-ray fotoelectron spectroscopie). Dex-HEMA microsferen (DS12) die geïncubeerd werden in een fosfaatbuffer van pH 7.4 bij 37 °C vertoonden een geleidelijke afname van (droge) massa door de afgifte van water-oplosbare afbraakproducten. Dit resulteerde in een residu van ongeveer 10% (van de initiële hoeveelheid droge massa) water-onoplosbare afbraakproducten na 6 maanden. Met NMR, massaspectrometrie en SEC werd aangetoond dat de water-oplosbare afbraakproducten voornamelijk bestonden uit dextraan, poly-HEMA met een laag molecuulgewicht (M_n ~ 15 kg/mol), en kleine hoeveelheden HEMA en HEMA-DMAP (tussenproduct van de Baylis-Hillman reactie van HEMA met DMAP (4-dimethyl aminopyridine)). Microscopisch onderzoek aan de water-onoplosbare afbraakproducten toonde aan dat deze afbraakproducten bestaan uit deeltjes met met een vergelijkbare ronde vorm en grootte als de microsferen die verkregen worden direct na polymerisatie. Ondanks dat verse microsferen na hydrolyse dezelfde vorm en grootte hadden, bleek uit metingen m.b.v. de 'micromanipulation' techniek dat de mechanische sterkte van de deeltjes na hydrolyse veel minder was. Uit FT-IR, XPS en SEC metingen is gebleken dat deze deeltjes bestaan uit onoplosbaar poly-HEMA en een klein deel poly-HEMA dat oplosbaar is in methanol (M_n ~ 27-82 kg/mol). Waarschijnlijk is het onoplosbare poly-HEMA in lichte mate gecrosslinked. Samengevat blijkt uit deze experimenten dat de *in vitro* afbraak van deze microsferen resulteert in de vorming van water-oplosbare (voornamelijk bestaande uit dextraan) en een kleine hoeveelheid water-onoplosbare afbraakproducten (bestaande uit pHEMA).

Omdat in **Hoofdstuk 6** werd aangetoond dat een deel van de poly-HEMA afbraakproducten van dex-HEMA microsferen niet water-oplosbaar waren, werd in **Hoofdstuk 7** een serie gesubstitueerde dextranen gesynthetiseerd met meer hydrofielere zijgroepen. Van deze dextranen werden hydrogelen gemaakt en er werd onderzocht of de afbraak van deze gelen (onder fysiologische omstandigheden) inderdaad resulteerde in uitsluitend water-oplosbare afbraakproducten. De geteste dextranen bestonden uit dextraan gederivatiseerd met HPMAm (N-(2-hydroxypropyl)methacrylamide), lac-HPMAm (N-(2-hydroxypropyl)methacrylamide-monolactaat), lac_2-HPMAm (N-(2-hydroxypropyl)methacrylamide-dilactaat) en HEMAm (N-(hydroxyethyl)methacrylamide. Het is aangetoond dat de afbraak van hydrogelen gebaseerd op dex-HEMAm and dex-HEMA resulteerde in de vorming van zowel water-oplosbare als water-onoplosbare afbraakproducten. Desalniettemin resulteerde de afbraak van hydrogelen bereid met dex-($lac_{1,2}$-)HPMA uitsluitend in water-oplosbare afbraakproducten. Daarom werd de zwelling- en degradatiekinetiek voor hydrogelen gebaseerd op uitsluitend deze dextranen verder onderzocht. Hieruit is gebleken dat de afbraaksnelheid afhankelijk is van de substitutiegraad (DS, aantal of

polymeriseerbare groepen per 100 glucose eenheden), de samenstelling van de polymeriseerbare groep (mono of di lactaat substitutie) en toeneemt volgens: dex-lac$_2$-HPMAm < dex-lac-HPMAm < dex-HEMA < dex-HPMAm. In dit onderzoek is verder aangetoond dat het afgifteprofiel van BSA uit microsferen gebaseerd op dex-(lac$_{1,2}$-)HPMA ook afhankelijk was van het aantal lactaat eenheden in de crosslinks en de DS. Hieruit blijkt dat voor verder onderzoek polymeren gebaseerd op dex-lac$_2$-HPMA het meest interessant zijn, omdat met deze polymeren microsferen gemaakt kunnen worden met *(1)* een hoge incapsuleringsefficiëntie (86% voor BSA), *(2)* afgifteprofielen met een lage burst (2% voor BSA) en *(3)* een gereguleerde en kwantitatieve afgifte over een periode van 5 dagen (BSA). Daarnaast is het belangrijk dat de afbraakproducten van deze microsferen volledig wateroplosbaar zijn.

Conclusie

Dit proefschrift toont aan dat dextraan microsferen erg aantrekkelijk zijn als gecontroleerd afgiftesysteem voor farmaceutisch actieve eiwitten zoals hGH. Er is een goede relatie tussen de *in vitro* en *in vivo* afgifte waarbij de *in vitro* afgifte grotendeels voorspeld kan worden aan de hand van een model. Om dit systeem in de toekomst verder geschikt te maken voor de klinische toepassing van een grotere groep farmaceutische eiwitten zullen verder studies zich moeten richten op *(1)* de optimalisatie van de water-oplosbaarheid van de afbraakproducten (met laag molecuulgewicht), *(2)* de optimalisatie van de deeltjesgrootte (grote deeltjes voor m.n. lokale en systemische toepassing, kleine deeltjes voor bijvoorbeeld ontwikkeling van vaccins) en *(3)* de optimalisatie van het bereidingsproces op grotere schaal.

Appendix B

List of abbreviations and list of symbols

List of abbreviations

AIBN	2,2'-Azobisisobutyronitrile
ATM1,2	Animal trial material 1 or 2
BSA	Bovine Serum Albumin
CDI	1,1'-carbonyldiimidazole
CTM	Clinical trial material
D[1,0]	Number weight mean particle diameter
D[3,0]	Volume weight mean particle diameter
Dex	Dextran
Dex-HEMA	Dextran derivatised with HEMA
Dex-HPMAm	Dextran derivatised with HPMAm
Dex-$lac_{1,2}$-HPMAm	Dextran derivatised with HPMAm containing 1 or 2 lactate groups as hydrolysable spacers
DMAP	4-(N,N-dimethylamino) pridine
DMSO	Dimethyl sulfoxide
DP	Degree of polymerization
DS	Degree of substitution (number of monomer (eg. HEMA, HPMAm) groups per 100 glucopyranose residues)
FT-IR	Fourier transformed infrared spectroscopy
GPC	Gel permeation chromatography
(^{1}H-)NMR	(Proton) Nuclear magnetic resonance spectroscopy
HCl	Hydrochloric acid
HEMA	Hydroxyethyl methacrylate
HEMA-CI	Hydroxyethyl methacrylate activated with CDI
HEMAm	Hydroxyethyl metacrylamide
HEMA-CI	Hydroxyethyl metacrylamide activated with CDI
HPMAm-CI	Hydroxypropyl methacrylamide activated with CDI
HPMAm- $lac_{1,2}$	Hydroxypropyl methacrylamide (mono, di)lactate activated with CDI
HPMAm-$lac_{1,2}$-CI	Hydroxypropyl methacrylamide-$lac_{1,2}$ activated with CDI
rhGH-190	Recombinant Human growth hormone consisting of 190 amino acids
IGF-I	Insulin growth factor I
IGFBP-3	Insulin growth factor binding protein 3
IgG	Immunoglobulin G

KBr	Potassium bromide
KPS	Potassium peroxodisulfate
LC/MS/MS	Liquid Chromatography/Mass Spectrometry/Mass Spectrometry
MA	Methacrylate
MAA	Methacrylic acid
NaCl	Sodium chloride
NaI	Sodium iodide
NaOH	Sodium hydroxide
PBS	Phosphate-buffered saline
PDI	Poly dispersity index
PEG	Poly(ethylene glycol)
pHEMA	Poly(2-hydoxyethyl methacrylate)
pHPMAm	Poly(2-hydoxypropyl methacrylamide)
PL(G)A	Poly(lactic acid) or poly(lactic-co-glycolic acid)
(RP)-HPLC	Reversed Phase) High Performance Liquid Chromatography
SD	Standard deviation
SEM	Standard error of the mean
SEM	Scanning Electron Microscopy
TEMED	*N,N,N',N'*-Tetramethylethylenediamine
XPS	X-ray photoelectron spectroscopy

List of symbols

A_1	Amount of protein present subcutaneously
A_2	Amount of protein present in serum
B	Bioavailability
D_{model}	Rate at which a molecule jumps to a neighboring *open* lattice site
d_h	Hydrodynamic diameter
E	Elasticity (or compression) modulus
G'	Elastic modulus
G''	Visous modulus

X_{model}	Model crosslink density; number of neighboring crosslinks that needs to be broken to open a lattice site. Note that X_{model} depends on two experimental parameters: the actual degree of substitution of dextran and the hydrodynamic diameter (d_h) of the protein.
r	Reaction rate
$I(E_{dextran,\ pHEMA})$	Signal intensities of dextran dn pHEMA, E is bindings energy of electron
k	Reaction rate constant: also hydrolysis rate constant
k_{obs}	$k_{observed}$, overall hydrolysis rate constant
k_a	Absorption rate constant
k_{el}	Elimination rate constant
K	Number of events
u	Uniformly distributed random number between 0 and 1
M	Number of cycles
M_c	Molecular weight between cross-links
M_n	Number average molecular weight
M_w	Weight average molecular weight
N_{model}	Model parameter describing the maximum number of crosslinks per lattice site. In all simulations reported here, N_{model}=2000 (see Chapter 3 section 2.1.1)
$P(t)$	Probability that a lattice site is open at time t
$\theta(t)$	Heaviside step function
P	Protein loading
R	Gas constant
T	Absolute temperature
$t_{1/2,\ a}$	Absorption half life
$t_{1/2,\ el}$	Elimination half life
V_d	Volume of distribution
W_0	Initial weight of a gel
W_t	Weight of a gel at time t

Appendix C

Curriculum Vitae

en

Publicatielijst

Curriculum Vitae

14 Maart 1976	Geboren te Zevenaar.
1988 – 1994	Atheneum, Liemers College, Zevenaar.
1994 - 1999	Doctoraal Farmacie en Technische Farmacie, Rijksuniversiteit Groningen. Afstudeeronderzoek: *'Material properties of microcrystalline cellulose granules.'* Begeleiders: Dr. J. Ramaker, Dr. P. Vonk.
1999 - 2001	Onderzoeker bij vakgroep Complexe Vloeistoffen, Faculteit Technische Scheikunde, Universiteit van Amsterdam: *'Wetting and dewetting of thin liquid crystalline films.'* Begeleider: Prof. Dr. B. Jérôme.
2001 - 2005	Promotie-onderzoek vakgroep Biofarmacie en Farmaceutische technologie, Faculteit Farmacie, Universiteit Urecht / OctoPlus N.V., Leiden: *'Dextran-based microspheres as controlled delivery systems for proteins.'* Promotoren: Prof. Dr. Ir. W.E. Hennink Prof. Dr. D.J.A. Crommelin Prof. Dr. W. Jiskoot Co-promotor: Dr. R. Verrijk Dit onderzoek heeft geleid tot het verschijnen van dit proefschrift.
2005 – 2007	Scientist/Pojectleader Drug delivery OctoPlus N.V., Leiden.

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Appendix D

Dankwoord

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