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Anthracene functionalized thermosensitive and UV-crosslinkable polymeric micelles†

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An anthracene-functionalized thermosensitive block copolymer was synthesized, which formed micelles by heating its aqueous solution above the lower critical solution temperature (LCST). The micelles were subsequently crosslinked by UV illumination at 365 nm with a normal handheld UV lamp. The micelles showed a small size (30 nm) and high loading capacity (16.0 ± 0.1%) for paclitaxel and released paclitaxel for more than ten days.

1. Introduction

Amphiphilic block copolymers form polymeric micelles in aqueous solutions above the critical micelle concentration (CMC).[1] Polymeric micelles, composed of a hydrophilic corona and a hydrophobic core, have been extensively studied for drug delivery purposes.[2–7] Block copolymers based on a permanently hydrophilic PEG block and a thermosensitive block self-assemble in aqueous solution into micelles by simply increasing the temperature above the lower critical solution temperature (LCST) of the thermosensitive block.[8] Thermosensitive polymeric micelles are attractive systems as their facile preparation is an obvious advantage since the use of organic solvents is avoided for their preparation. On the other hand, polymeric micelles are dynamic systems that are prone to dissociation due to the shift of the equilibrium between micelles and unimers, in case of massive dilution of the system or removal of the unimers.[9,10] The instability of micelles hampers their application as targeted delivery systems when aiming for passive or active targeting strategies.

To stabilize polymeric micelles, various chemical/physical crosslinking methods have been exploited and indeed shown to increase the stability of polymeric micelles.[11–13] Among different crosslinking methods, photo-crosslinking is a facile method with advantages including mild reaction conditions, minimum side-product formation and fast curing times.[14–17] The [4 + 4] cycloaddition of anthracene (An) groups has been applied for fabrication and crosslinking of polymer films and self-assemblies,[14,18–20] without the aid of photo-initiators which could be potentially toxic.[14] Inspired by that work, in the present study, An was introduced into a thermosensitive micelle-forming block copolymer and the [4 + 4] cycloaddition of An was shown to be a facile method for the crosslinking of the micelles. Apart from the function of crosslinking, we anticipated that the aromatic An pendant groups attached to the polymer chains would provide strong interaction with (and high loading of) aromatic drugs.

Thus, in this article, we report the synthesis of a new anthracene functionalized HPMAm monomer (HPMAm-An) and a thermosensitive block copolymer by copolymerizing HPMAm-An with the monolactate ester of (2-hydroxypropyl) methacrylamide (HPMAm-Lac) initiated by a PEG-modified azo initiator. Polymeric micelles were prepared by simply heating the aqueous polymer solution to above its LCST, and subsequently crosslinked by illumination at 365 nm (Fig. 1). The crosslinking efficiency was studied using UV spectroscopy and HPLC analysis. The stability of the micelles was studied by lowering the temperature below the LCST of the polymer. Furthermore, paclitaxel (PTX) was encapsulated in the polymeric micelles and the compatibility of PTX with the crosslinking was assessed. Finally, the impact of UV crosslinking of the polymeric micelles on the PTX retention in the micelles was studied.

2. Results and discussion

A new photo-reactive monomer HPMAm-An was synthesized (characterizations given in ESI,† section 1) and copolymerized with HPMAm-Lac (molar ration of 15/85) by an established method using PEG-modified 4,4′-azobis(4-cyanopentanoic acid) (ABCPA) as a macroinitiator.[3,21] The polymer was obtained in a yield of 65% and the mol% of HPMAm-An in the obtained copolymer was 13% (^{1}H NMR analysis (see ESI,† section 2)), which is close to that of feed (15%). The number

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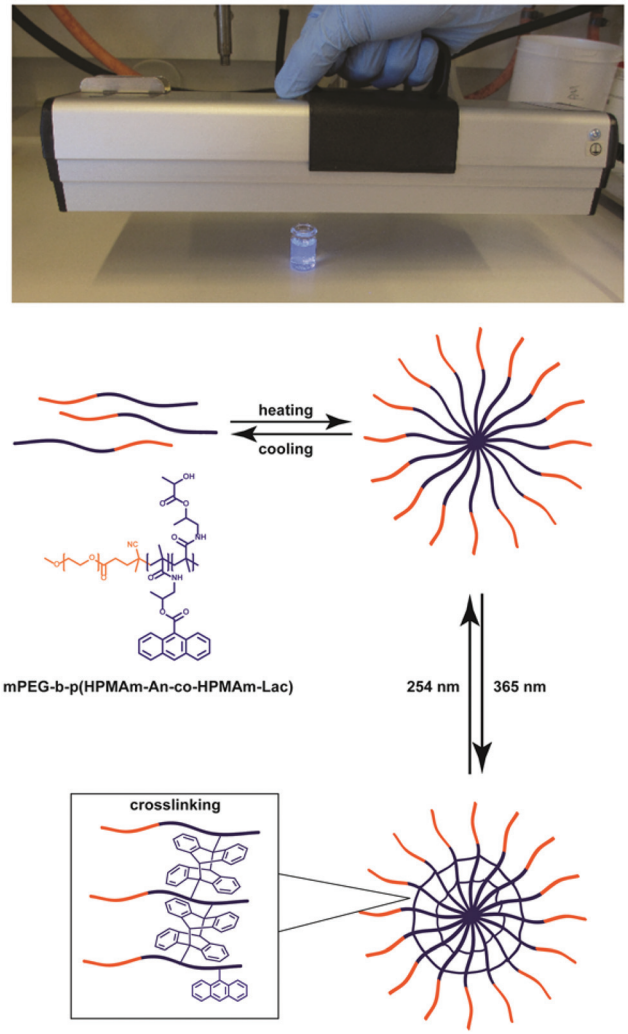

Fig. 1 Crosslinking of polymeric micelles under a normal handheld low power UV lamp for laboratory use (upper) and schematic illustration of the formation and UV crosslinking (365 nm) of mPEG-*b*-p(HPMAm-An-*co*-HPMAm-Lac) based micelles (lower).

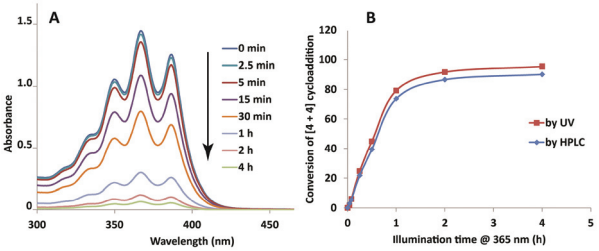

Fig. 2 A: UV spectra of the micelles after UV illumination at 365 nm for different times; B: Conversion of [4 + 4] cycloaddition of An groups in the micelles under UV illumination at 365 nm by the UV and HPLC method, respectively.

average molecular weight (M_n) of the polymer was 13 kDa by ^{1}H NMR, which is close to that measured by GPC (14 kDa, PDI = 1.7). This polymer had a LCST of 12 °C (Fig. 3). With other polymer compositions, *e.g.*, 5 mol% of HPMAm-An, the polymer had a too high LCST (29 °C) that is not convenient to work with, and the polymer with 20 mol% of HPMAm-An was not thermosensitive and not soluble in water at 0 °C. Therefore, for further studies the polymer with 13 mol% of HPMAm-An was selected.

To prepare micelles, the polymer solution at 0 °C was rapidly heated by placing the samples in a water bath at 50 °C with vigorous shaking for one minute.[6] Dynamic light scattering (DLS) measurement showed that the micelles had a small hydrodynamic diameter of 30 nm with a low polydispersity index (PDI) of 0.10 (Experimental section 4.5), which are potentially beneficial for *in vivo* application of polymeric micelles for tumor-targeted drug delivery.[22]

The mPEG-*b*-p(HPMAm-An-*co*-HPMAm-Lac) micelles were illuminated by a normal handheld low power UV lamp for laboratory use (ENF-280C/FE, 8 W) at 365 nm (± 7 nm), which is the specific wavelength for the anthracene groups (Experimental section 4.8).[18,19] The UV spectra of the micelles were recorded after different illumination times. A substantial decrease of the UV absorption of anthracene between 300 and 430 nm is clearly observed (Fig. 2A), which indicates that [4 + 4] cycloaddition of the anthracene groups and thus crosslinking of the micelles had occurred. According to eqn (1) (Experimental section 4.9) the conversion was around 80% during the first hour, and the final conversion of ~90% was achieved in 2 hours (Fig. 2B). To confirm An [4 + 4] cycloaddition, the micelles illuminated for different times were hydrolysed (3 M NaOH at 60 °C for 48 hours), which resulted in release of non-reacted An groups. The concentration of An in the different samples was quantified by HPLC analysis and the conversion of the [4 + 4] cycloaddition was calculated according to eqn (2) (Experimental section 4.9). Fig. 2B shows similar kinetics and conversion as observed by the UV method. The size of the crosslinked micelles after two hours UV illumination was 32 nm with a low PDI of 0.05. The constant size of the micelles after crosslinking means that intermicellar crosslinking hardly occurred.

The thermal stability of the crosslinked and non-crosslinked micelles was studied by DLS. The non-crosslinked micelles showed a continuous decrease of both the size and light scattering intensity (LSI) from 25 to 2 °C (Fig. 3, left), indicating gradual dissociation of the micelles due to hydration of the thermosensitive block. On the contrary, the LSI and size of the crosslinked micelles were constant while cooling, demonstrating that indeed intermolecular covalent bonds were formed between the blocks present in the core of the micelles due to [4 + 4] cycloaddition of the An groups.

Polymeric micelles are used as carrier systems for hydrophobic drugs. mPEG-*b*-p(HPMAm-An-*co*-HPMAm-Lac) based micelles were loaded with paclitaxel (PTX), a hydrophobic chemotherapeutic drug, to evaluate the loading capacity (LC) and release. Due to thermosensitivity of the polymer, PTX could be loaded into the polymeric micelles by the fast heating method (Experimental section 4.4).[21] The encapsulation efficiency (EE)

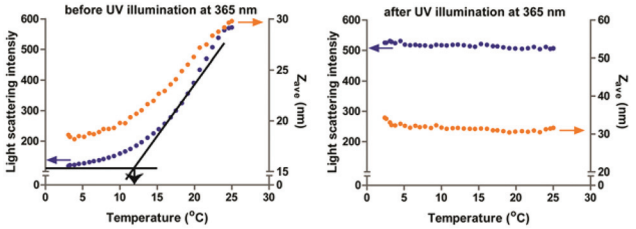

Fig. 3 Size (Z_{ave}) and light scattering intensity of the micelles before and after UV illumination for two hours at 365 nm, upon cooling from 25 to 2 °C.

and LC of the micelles for PTX were 85.5 ± 0.2% and 16.0 ± 0.1%, respectively, at a feed concentration of PTX of 2 mg mL^{-1} and a polymer concentration of 9 mg mL^{-1} (Experimental section 4.6). The size of the PTX loaded micelles was 51 nm with a PDI of 0.05 as measured by DLS. Significant increase of the size of polymeric micelles after loading with PTX was reported previously.[23,24] This phenomenon can be attributed to interference of hydrophobic drug molecules with the micellation process of amphiphilic polymers, which may cause a less dense packing of the polymer chains and in an increase of the micellar size. After loading with PTX, the micelles were exposed to UV illumination at 365 nm for two hours for crosslinking. The efficiency of [4 + 4] cycloaddition was 82% (UV spectroscopic analysis), which was close to that of non-loaded micelles. Previously it was found that PTX can undergo photolysis when exposed to UV illumination (350–450 nm, light intensity was ~210 mW cm^{-2}) for 10 min.[2] However, UPLC analysis showed that the amount of PTX in the micelles after UV illumination for crosslinking was identical to that before UV illumination, demonstrating that no detectable photolytic degradation of PTX occurred during the crosslinking of the micelles by UV illumination at 365 nm, which can be ascribed by the fact that the light intensity applied for crosslinking was rather low.

As shown in Fig. 4, around 60% of the loaded PTX was released from the non-crosslinked micelles in ten days at pH 7.4 and 37 °C, which is substantially lower than that from

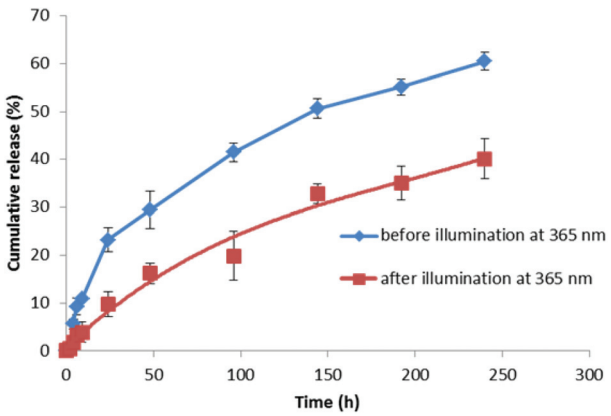

Fig. 4 PTX release from the non-crosslinked and UV crosslinked micelles at 37 °C. Mean ± SD (n = 3).

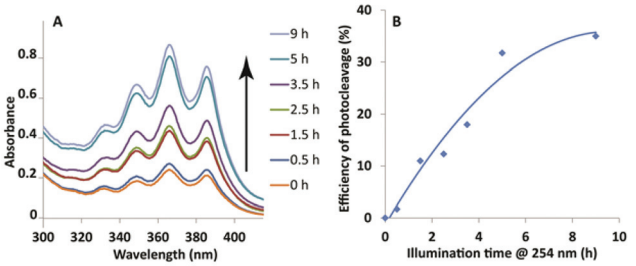

Fig. 5 A: UV spectra of the crosslinked micelles after UV illumination at 254 nm for different times; B: Kinetics of the photocleavage of the dianthracene in the micelles upon UV illumination at 254 nm (UV spectroscopic analysis).

mPEG-*b*-p(HPMAm-dilactate) micelles.[21] However, the PTX release rate from the crosslinked micelles was significantly slower, *i.e.*, 40% in 10 days (Experimental section 4.7). Release of PTX from the polymeric micelles is likely driven by diffusion,[25] which can be retarded by crosslinking of the polymeric micelles.[26] As a result, PTX release from the crosslinked polymeric micelles is slower than that from the noncrosslinked ones. Therefore, it points to the fact that more stable retention of PTX in thermosensitive polymeric micelles can be achieved by crosslinking of the micelles by anthracene [4 + 4] cycloaddition, which can result in a better stability for *in vivo* application of the PTX-loaded polymeric micelles.

To study the reverse photocleavage of the dianthracene in the micellar core, the crosslinked micelles were illuminated at 254 nm (Experimental section 4.8).[14] Fig. 5A shows that UV absorbance of the micelles between 300 and 400 nm increased in time when the crosslinked micelles were exposed to 254 nm illumination. Fig. 5B shows that the photocleavage of the dianthracene in the crosslinked micelles can induce partial decrosslinking with a conversion of 33% after five hours of illumination at 254 nm (Fig. 5B). Similar photocleavage kinetics of dianthracene or coumarin have been reported.[14,27]

TEM images of the noncrosslinked polymeric micelles, crosslinked polymeric micelles and those after UV illumination at 254 nm are shown in Fig. 6. The size of the micelles by TEM analysis was smaller than that obtained by DLS measurement, which was observed previously.[28,29] Furthermore, TEM analysis showed that aggregation of the micellar particles did not occur before and after illumination at 254 nm (B and C).

The cytocompatibility of the (non)crosslinked micelles was tested on human umbilical endothelial cells (HUVECs).

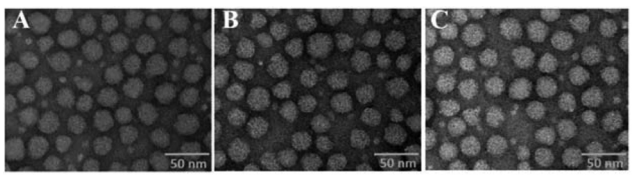

Fig. 6 TEM images of empty polymeric micelles. A: noncrosslinked micelles; B: crosslinked micelles; C: crosslinked micelles after UV illumination at 254 nm.

The cells retained their viability (>85%) at a concentration of the (non)crosslinked polymeric micelles up to 1 mg mL^{-1}, which shows that the (non)crosslinked micelles have a good cytocompatibility.

3. Conclusions

mPEG-*b*-p(HPMAm-An-*co*-HPMAm-Lac) is a novel thermosensitive block copolymer that self-assembles into polymeric micelles in water above its LCST. The micelles were efficiently crosslinked by UV illumination at 365 nm due to the [4 + 4] cycloaddition of the anthracene groups in the micellar core. The micelles showed high loading capacity for PTX and the loaded PTX molecules did not hinder the crosslinking of the micelles. Furthermore, the chemical integrity of PTX was preserved during UV illumination. Drug release study showed that the PTX release rate from the micelles was significantly reduced by crosslinking the micelles, which is potentially a benefit for better *in vivo* stability of the PTX-loaded polymeric micelles. The (non)crosslinked micelles showed high cytocompatibility. These beneficial properties warrant further *in vivo* applications of the crosslinkable micelles for delivery of PTX.

4. Experimental section

4.1. Materials

N-(2-Hydroxypropyl) methacrylamide (HPMAm) was purchased from Zentiva, Czech Republic. 9-Anthracenecarboxylic acid (AA), *N*,*N*-dimethylacetamide (DMAc) and *N*,*N*′-dicyclohexylcarbodiimide (DCC) were purchased form Sigma-Aldrich. Acetonitrile (ACN), dichloromethane (DCM), diethyl ether, ethyl acetate, tetrahydrofuran (THF) and *N*,*N*-dimethylformamide (DMF) were obtained from Biosolve BV. Paclitaxel (PTX) was supplied by LC Laboratories. 4-(Dimethylamino)pyridinium 4-toluenesulfonate (DPTS) was synthesized according to Moore *et al.*[30] HPMAm-Lac and the PEG macroinitiator based on 5 kDa PEG were synthesized as previously published.[31]

4.2. Synthesis and characterizations of *N*-(9-anthranoyloxypropyl) methacrylamide (HPMAm-An)

HPMAm-An was synthesized by the DCC assisted esterification of HPMAm and 9-anthracenecarboxylic acid (Scheme 1, ESI†). Briefly, a two-necked flask was dried at 180 °C overnight and cooled down to room temperature under a nitrogen stream. Next, 1.43 g (0.01 mol) HPMAm, 2.22 g (0.01 mol) anthracene-9-carboxylic acid and 0.87 g (0.0028 mol) DPTS were weighed and transferred into the flask and 70 ml of THF/DCM (3/4, v/v) was added under nitrogen atmosphere. After dissolution of the solids, 6.0 g (0.03 mol) of DCC was transferred into the flask immersed in an ice bath. The reaction mixture was stirred for 24 hours at room temperature. Next, the formed precipitates were removed by filtration, the solvent was removed by evaporation under reduced pressure and the product was purified by silica column chromatography (260 g) with an eluent of hexane/ethyl acetate (1/1, v/v). The fractions that contained the compound with R_f of 0.6 (hexane/ethyl acetate (1/1, v/v)) were collected and the solvents were removed under reduced pressure. The final product was collected as a dark yellow powder with a yield of 1.46 grams (or 42%). The compound was characterized by melting point, ^{1}H NMR spectroscopy and HPLC and the results are shown in section 1, ESI.†

4.3. Synthesis of ω-methoxy poly(ethylene glycol)-*b*-(*N*-(9-anthranoyloxypropyl) methacrylamide)-*co*-(*N*-(2-lactoyloxypropyl) methacrylamide) (mPEG-*b*-p(HPMAm-An-*co*-HPMAm-Lac))

The block copolymers were synthesized by radical polymerization initiated by the mPEG-modified azo initiator (Scheme 1, ESI†). The feed molar ratio of the comonomers HPMAm-Lac and HPMAm-An was between 95/5 to 80/20, and that of total monomers to macroinitiator was 150/1. The total monomer concentration was 0.3 g mL^{-1} in DMAc. The solution was degassed by flushing with nitrogen for 30 minutes and the polymerization was conducted at 70 °C for 24 hours under a nitrogen atmosphere. Next, the polymer was purified by precipitation in diethyl ether for three times and then dialyzed against reverse osmosis water at 4 °C for 24 h. The polymer was collected as a pale-yellow fluffy powder after freeze-drying with a yield of 65%. The polymer was characterized by ^{1}H NMR spectroscopy and GPC (section 2, ESI†).

4.4. Preparation and characterizations of the (PTX-loaded) polymeric micelles

Polymeric micelles were prepared by rapidly heating an aqueous solution of mPEG-*b*-p(HPMAm-An-*co*-HPMAm-Lac).[21,32] In short, the polymer was dissolved in pH 5.0 ammonium acetate buffer (AAB, 120 mM) at a concentration of 10 mg mL^{-1} at 0 °C. Next, the polymer solution was heated in a water bath at 50 °C for one minute with vigorous shaking. To prepare PTX-loaded micelles, one volume of PTX solutions in ethanol was mixed with nine volumes of the polymer solution prior to heating. Subsequently, the micellar dispersion was stored overnight at room temperature and filtered through 0.45 μm nylon membrane to remove non-entrapped (precipitated) drug. Transmission electron microscopy (TEM) images of the micelles were taken using a previously reported method.[21]

4.5. Measurement of the size of the polymeric micelles by dynamic light scattering (DLS)

DLS was performed using a Malvern 4700 system (Malvern Ltd., Malvern, U.K.) consisting of an Autosizer 4700 spectrometer, a pump/filter unit, a model 2013 air-cooler argon ion laser (75 mW, 488 nm, equipped with a model 2500 remote interface controller, Uniphase) and a water bath, and a computer with DLS software (PCS, version 3.15, Malvern). Autocorrelation functions were analyzed by the cumulants method (fitting a single exponential to the correlation function to obtain the mean size and the polydispersity) and the CONTIN routine (fitting a multiple exponential to the correlation

function to obtain the distribution of particle sizes). The measurement angle was 90°.[21]

4.6. Quantification of PTX loaded in the (non)crosslinked polymeric micelles

The PTX loaded micelles were 10-fold diluted with ACN and vortexed to dissolve PTX. The obtained solutions were centrifuged at 12.000*g* for 10 min to remove possible particles/aggregates in the samples prior to analysis by a Waters ACQUITY UPLC System. Eluent A: ACN–water = 45/55 (v/v) with 0.1% formic acid; eluent B: ACN–water = 90/10 (v/v) with 0.1% formic acid. A gradient method was run with the volume fraction of eluent B increasing from 0 to 100% from 4.5 to 7 minutes and decreasing to 0% from 7.5 minutes to 10 minutes. An ACQUITY UPLC HSS T3 column was used and the detection wavelength was 227 nm. Seven μL of the supernatant was injected and the PTX concentration was calculated by a calibration curve with PTX standards prepared in ACN in a concentration range of 0.2 to 500 μg mL^{-1}. The loading capacity (LC) and encapsulation efficiency (EE) are calculated as follow:

$$\mathrm{LC} = \frac{\text{concentration of PTX measured}}{\text{concentration of (PTX measured + polymer added)}} \times 100\%$$

$$\mathrm{EE} = \frac{\text{concentration of PTX measured}}{\text{concentration of PTX added}} \times 100\%$$

4.7. PTX retention in the (non)crosslinked micelles

The retention of PTX in the (non)crosslinked micelles was performed as previously reported.[21] Briefly, drug retention in the (non)crosslinked micelles at pH 7.4 and 37 °C was evaluated by measuring the remaining drug content in the micellar dispersion in time. PTX-loaded (non)crosslinked micelles were prepared as described in section 4.4 and the pH was adjusted to 7.4 by diluting 5-fold with 500 mM phosphate pH 7.4 buffer. The released PTX crystallized and precipitated due to its low water solubility (0.3 μg mL^{-1}). The micellar dispersions were incubated at 37 °C with constant shaking, and aliquots were taken and centrifuged at 5000 *g* for 10 min to spin down the precipitated drug. Next, the PTX content in the micellar dispersion was quantified by UPLC analysis as described above. Due to overnight evaporation of ethanol after micellar preparation and 5 times of dilution of the micellar dispersion in the release medium, the final concentration of ethanol in the drug retention study was very low, which likely had limited impact on the PTX release rate from the micelles.

4.8. Core-crosslinking of the micelles and photocleavage of the dianthracene by UV illumination at 365 and 254 nm

Core-crosslinking of the mPEG-*b*-p(HPMAm-An-*co*-HPMAm-Lac) micelles was achieved *via* UV induced [4 + 4] cycloaddition of the anthracene side groups attached to the thermosensitive block of the polymer. The micelles prepared according to section 4.4 were irradiated by a Spectroline® E-series UV lamp (ENF-280C/FE, 8 W) at 365 nm (±7 nm). The distance between the micellar suspension and the UV lamp was 2 cm and the height of the liquid was 1 cm. The crosslinking was performed at room temperature (22 °C) and there was a slight increase of the temperature (to ~26 °C) during the procedure. The weight loss of water in the micellar dispersion was calculated to be less than 5% after 6 hours of irradiation. The photocleavage of the dianthracene in the crosslinked micelles was performed in the same way as the crosslinking, under the UV illumination at 254 ± 5 nm by a Spectroline® E-series UV lamp (ENF-280C/FE, 8 W).

4.9. Efficiency of the crosslinking by UV spectrometric and HPLC analysis

The conversion of the anthracene side groups was evaluated by UV spectroscopy and HPLC, respectively.

UV method: Micellar samples were taken at different time points of irradiation and the samples were diluted 10 times in AAB. UV spectra of the samples were recorded on a Shimadzu 2450 UV/Vis spectrometer. The efficiency of the crosslinking was calculated according to eqn (1) based on the absorbance of anthracene groups at 365 nm.[14,27]

HPLC method: Micellar samples were taken at different time points and diluted 2 times in NaOH solution (final NaOH concentration was 3 M). The samples were incubated at 60 °C for 48 hours to hydrolyze and released anthracen-9-carboxylic acid (AA) was subsequently quantified by the aforementioned HPLC system with a Prevail™ Organic Acid column. The samples were neutralized with HCl before injection. The conversion was calculated according to eqn (2) based on the concentration of AA which was determined by a calibration curve with AA standards prepared in eluent A in a concentration range of 2 to 200 μg mL^{-1}. The conversion of the micelles was calculated according to the following equation:

$$\text{UV method}: \ \text{efficiency} = \frac{\text{Absorbance}_{0h} - \text{Absorbance}_{xh}}{\text{Absorbance}_{0h}} \times 100 \quad (1)$$

$$\text{HPLC method}: \text{efficiency} = \frac{\text{Concentration}_{0h} - \text{Concentration}_{xh}}{\text{Concentration}_{0h}} \times 100 \quad (2)$$

4.10. Cytocompatibility of the polymeric micelles before and after crosslinking

The *in vitro* cytocompatibility of the mPEG-*b*-p(HPMAm-An-*co*-HPMAm-Lac) micelles before and after crosslinking was studied using human umbilical endothelial cells (HUVECs).[33] The cells were cultured in Ham's F-12K media containing 10% FBS, heparin (100 μg mL^{-1}), ECGS (40 μg mL^{-1}), and 1% penicillin-streptomycin solution, and in a 5% CO_2 humidified atmosphere at 37 °C. The cells were seeded into 96-well plates at a density of (5×10^3 cells per well) and incubated for 24 hours at 37 °C in a 5% CO_2 humidified atmosphere. The

micelles before and after crosslinking were prepared in PBS 7.4 with an initial polymer concentration of 10 mg ml^{-1} (crosslinking was performed for two hours). Then, the micellar dispersions were diluted with the cell culture medium to reach concentrations ranging from 1 ng to 1 mg ml^{-1}. The viability of the cells was measured by XTT assay after 48 hours of incubation with the samples at 37 °C and 5% CO_2.

Acknowledgements

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