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Photo-crosslinked and pH sensitive polymersomes for triggering the loading and release of cargo†

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Crosslinkable and pH-sensitive amphiphilic block copolymers are promising candidates to establish pH-stable and permeable vesicles for synthetic biology. Here, we report the fabrication of crosslinked and pH-stable polymersomes as swellable vesicles for the pH-dependent loading and release of small dye molecules.

A large number of biological processes takes place within natural compartments, so-called vesicles.¹ Those vesicles are called liposomes,² since they are lipid-based. In a synthetic approach to biology it is of great interest to create artificial variants of these vesicles for a better understanding of their fundamental features in natural processes.^{1–3} Since liposomes have restrictions concerning their mechanical and chemical stability, polymersomes are attractive alternatives to yield vesicles of higher stability.^{4,5}

Polymersomes consist of amphiphilic block copolymers, possessing a defined block length ratio.^{6,7} Using vesicles as drug-delivery systems,^{4–8} various stimuli-responsive vesicles were created.⁵ Generally, the hydrophobic part switches to hydrophilic and, thus, disassembly of the amphiphilic block copolymer occurs to initiate the delivery of the cargo.⁷ Here, redox,^{9,10} thermal,^{11,12} hydrolysis^{13,14} and pH-sensitive^{15–18} polymers are known to be feasible for this application. However, if one rebuilds natural compartments, the vesicles should remain stable, yet still be open for diffusive exchange at certain conditions. Currently, pore or channel proteins have to be included into the membrane to ensure membrane traffic of small bio-active molecules,^{16,19} since polymer membranes are considerably thicker than lipid-based ones.^{4–7} Diffusion would be possible if the hydrophobic part could be switched to hydrophilic, while the vesicle remained stable. Therefore, crosslinking is the key step to establish stable and permeable vesicles for the diffusive exchange of smaller/larger (bio-)molecules, as a prerequisite for using them in future synthetic biology research and applications.

A combination of pH sensitive and crosslinkable monomers within the hydrophobic block should lead to crosslinked vesicles with an increased pH stability over the complete

pH range. Once the pH-sensitive and hydrophobic part in the vesicle switches to hydrophilic, permeable vesicles with different pore sizes can be prepared for loading them with cargoes of different size dimensions. However, loading and release (< pH 7) have to occur at the same pH range, where, in opposite, the transport or the entrapment of the cargo in the vesicle has to be realized in another pH range (> pH 7). To achieve our goal of loading and release of small molecules, we adapted the well-known pH-sensitive poly(ethyleneglycol)₄₅-block-poly(diethyl-aminoethylmethacrylate)₈₁ (PEG₄₅-*b*-PDEAEM₈₁)²⁰ in our synthetic ATRP approach (Scheme 1). Here, we also integrated the polymerizable photo-crosslinker 3,4-dimethyl imido-ethyl methacrylate²¹ (DMIEM) in the hydrophobic part of the block copolymer (Scheme 1). Starting from PEG macroinitiator (Scheme 1) under ATRP conditions, statistical copolymerization of the two chemically similar monomers DMIEM and DEAEM was performed to realize the desired PEG₄₅-*b*-PDEAEM-*s*-PDMIEM structure. Thus, the hydrophobic blocks contained 0, 2, 5, 10 and 15 mol% of DMIEM, respectively (Scheme 1, Table 1 and ESI†). The molecular structure of the various PEG₄₅-*b*-PDEAEM-*s*-PDMIEM was proven using ¹H NMR study. Here, methylene groups in the block copolymer (labeled as a, b and c in Scheme 1) were used to evaluate the required block

Scheme 1 ATRP of the final polymer, using a PEG-Br macroinitiator available after a method by Armes *et al.*²² Integral ratios of ¹H NMR signals of the various CH₂-groups (a, b and c) within the polymer were used to define the composition of the polymer (ESI†).

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Table 1 Overview on synthesized polymers and their properties

No.	Ratio hydrophilic/hydrophobic ^a	Mol% crosslinker ^a	PDI ^b (polymer)	M_n^a /g mol ⁻¹	Diameters of vesicles ^c /nm	PDI ^c (vesicles)
1	1 : 2.0	0	1.33	18 500	90	0.2
2	1 : 2.1	2	1.30	19 500	100	0.2
3	1 : 1.8	5	1.33	17 500	90	0.2
4	1 : 1.9	10	1.30	18 000	120	0.2
5	1 : 1.9	15	1.34	18 500	135	0.2

^a Calculated using mentioned ratio of integrals in ¹H-NMR spectra (ESI†). ^b Determined via GPC. ^c Diameters from DLS, intensity averaged.

length ratio and the desired incorporation of DMIEM in the pH-sensitive block (Table 1 and ESI†). Further characterization of the polymers was performed by GPC and DLS measurements (Table 1, Fig. 2 and 3 and ESI†).

The next step was to study the formation of polymersomes using the different block copolymers. Furthermore, the block ratio between hydrophilic and hydrophobic part was adjusted to about 1 : 2 (Table 1) that is ideal for polymersome formation.²⁰ Thus, two methods were carried out to evaluate which size dimensions are available: 1. simple dissolving in acidic aqueous solution, followed up by the addition of aqueous NaOH (final pH 9–10); 2. using electroformation which is well-established for the formation of liposomes.^{23,24} By the first route, that is, changing the pH from low to high values, all block copolymers undergo the desired and reproducible formation of polymersomes of about 100 nm in size (Table 1). This showed us that there is no negative influence of the DMIEM content on the polymersome formation of the various block copolymers (Table 1). In contrast to this, large, upper μ m scaled vesicles (Fig. 1) are available using the electroformation technique after evaluating the right conditions, such as frequency and growth time (ESI†).

In electroformation, growth of the vesicles was only observed from a thin film on an indium tin oxide (ITO) surface²⁵ (Fig. 1a). Laser scanning confocal microscopy (LSCM) was used to visualize the membrane of the vesicles by adding a hydrophobic green fluorescent dye (Fig. 1b) and a hydrophilic red fluorescent dye (Fig. 1c). While the hydrophobic dye was added to the polymer prior to vesicle formation, the hydrophilic dye was

added afterwards. This proved the non-permeability of the hydrophobic membrane for the diffusion of the hydrophilic dye into the inner compartment of the vesicles under basic conditions (Fig. 1c). In both cases, no diffusion of the dyes into the vesicles was observable, but only localization in the membrane or at the interface between the membrane and outer shell (Fig. 1d).

Despite electroformation being a promising approach to achieve large vesicles, the adhesive force of the polymers to the ITO crystal is too high to detach them. Due to this adhesion, only half-shell structures could be obtained. Thus, all further experiments were carried out using the polymersomes (Table 1: 90–135 nm) created without electroformation.

Achieving the aforementioned goal of loading and release of cargo from pH-sensitive and crosslinked vesicles, the necessary amount of photo crosslinker (0, 2, 5, 10 and 15 mol%) was investigated. Hence, unfiltered light of a mercury lamp was irradiated up to 2 h on formed polymersomes in basic solution, followed up by an immediate acidification step (Fig. 2 (top), ESI†). Final stability of the resulting crosslinked polymersomes was studied by DLS (Fig. 2 (top)). Due to the protonation of the nitrogen in the PDEAEM, non-crosslinked and not sufficiently crosslinked samples turned hydrophilic and disassembled upon acidification ($\varnothing \leq 10$ nm; Fig. 2 (top)). However, the desired stability was observed with polymersomes consisting of

Fig. 1 Microscopic images of polymersomes created using electroformation; (a) bright field image (all other LSCM images), (b) image after vesicle formation, green hydrophobic dye, (c) same spot, now red hydrophilic dye visible, (d) overlay of both images revealing vesicles being sealed towards diffusion and perfect overlay of both dyes, (e) giant vesicle (over 100 μ m) with overlay of both dyes, (f) the shape of the vesicle is indicated as inset in the image (a).

Fig. 2 Crosslinking and pH sensitive behaviour of vesicles with 0, 2, 5, 10 and 15 mol% crosslinker DMIEM: (top) sizes detected after time-dependent UV irradiation and subsequent acidification of samples studied by DLS; (down) changes in size upon acidification from pH 10 to pH 3 studied by *in situ* DLS.

polymers containing 10 and 15 mol% crosslinker (> 100 nm in size dimension) after at least 80 minutes of UV radiation.

Furthermore, the pH-stability and pH dependent swelling and phase transition behavior of the photo-crosslinked polymersomes over a wide pH range, in comparison to those polymersome without photo-crosslinker DMIEM, was of interest. Therefore, subsequent pH titration experiments starting from pH 10 to final pH 3 were carried out, using *in situ* DLS to depict the change in size of the corresponding polymersomes (Fig. 2 (bottom)). Surprisingly, a crosslinking density dependent step-wise size increase around pH 7 is given for the crosslinked samples, indicating swelling. In opposite to this, the non-crosslinked sample reveals a sharp drop in size at pH 7, indicating an immediate disassembly, forming small micelles of dimensions below 10 nm at lower pH values (Fig. 2 (bottom)). Furthermore, the crosslinked samples show the desired definite and reversible swelling (ESI^+) and the required stability of the vesicles ($\varnothing$ 150–170 nm; Fig. 2 (bottom)) after switching to lower pH values. Due to the higher crosslinking density in vesicles with 15 mol% of crosslinker DMIEM, lower swelling is recognizable. Generally, the swelling of the vesicles is easily explainable by the repulsive character of the cationic chains of PDEAEM in the crosslinked vesicles upon the protonation at lower pH values.

After successful photo-crosslinking of the polymersomes, stable and reversibly swellable vesicles in the acidic state (Fig. 2 (top), ESI^+) are available. Due to their stability at low pH, the polymersomes possess the required hydrophilic and permeable membrane to load the inner compartment of the vesicles with hydrophilic cargo. The permeability of the cross-linked vesicles strongly depends on the crosslinking density in the vesicles, and its resulting swelling behavior allows the diffusion/penetration of nm-sized cargo through the hydrophilic membrane. To check the diffusion of the cargo through the membrane of the crosslinked vesicles with 10 and 15 mol% of DMIEM, rhodamine B as hydrophilic dye was used as first cargo. Swellable vesicles were exposed to the cargo under acidic state for 5 hours, followed by a dialysis step under basic conditions in order to remove residual cargo. Thus, vesicles can successfully be loaded with the pure dye after entrapment in the inner compartment of the vesicle (Fig. 3). Subsequent release of the dye from the vesicle was successfully achieved by changing the pH from high to low values, resulting in similar UV-Vis monitoring, as presented in Fig. 3a.

Fig. 3 UV-Vis monitoring of crosslinked vesicle with 10 mol% crosslinker before and after the dialysis of pure rhodamine B in the presence of the vesicle under acidic condition (left) and dialysis of rhodamine B entrapped in the vesicle after changing from low to high pH value (right).

In summary we were able to form pH-stable and pH dependent swellable vesicles by effective photo-crosslinking. This pH stability allowed a controlled loading of the vesicles with cargo, as well as a triggered release of the cargo under acidic conditions. Thus, those UV-crosslinked polymersomes make perfect carrier candidates for effective transport and repeated uptake and release.

In further studies, we will address still existing challenges, like the reduction of the radiation time for effective cross-linking, and explore the possibility for diffusion of small as well as larger bio-active molecules through permeable vesicles. This may provide us at the end with tailor-made vesicles, which are highly feasible for synthetic biology.

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