TOWARDS MULTI-FUNCTIONAL DRUG DELIVERY SYSTEMS FROM IONIC TRIBLOCK TERPOLYMER MICELLES

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Introduction

Since the groundbreaking work by Ringsdorf,[1] many researchers have explored the possibilities offered by polymeric drug delivery systems (DDS) in fighting various types of diseases.[2] Advances in polymer chemistry allowing for precise control of the resulting materials have helped to create more sophisticated and complex delivery vehicles.[3] Amongst those vehicles polymeric micelles are particularly appealing, because they can encapsulate hydrophobic drugs in the micellar core, offer protection against degradation and rapid clearance from the bloodstream, selectively accumulate in the target tissue, and finally release their therapeutic cargo upon a predefined trigger.[4] Commonly, core-shell type micelles from diblock copolymers are utilized in this approach, however, some examples of micelles with a higher complexity also exist. So-called multi compartment micelles (MCMs) are obtained, e.g. when triblock terpolymers of an ABC type are used for micelle formation.[5] These MCMs combine several compartments in the same particle that differ in chemical structure and consequently in their physical properties. In this manner, several different drugs or functional molecules can be simultaneously incorporated, leading to improved performance of the whole carrier system. The benefits can include synergistic effects from the combined application of two drugs, site-specific delivery through targeting molecules and a combination of therapy and imaging, i.e. "theranostics".[6] We recently reported on MCMs from polybutadiene-*block*-poly(1-methyl-2-vinyl pyridinium)-*block*-poly(methacrylic acid) (BVqMAA) triblock terpolymers as well as their interpolyelectrolyte complexes (IPECs) with double hydrophilic polycation-*block*-poly(ethylene glycol).[7] BVqMAA micelles before the addition of complexing polymer exhibited a hydrophobic core (B), a non-continuous IPEC shell (Vq + MAA) and an excess MAA corona with a negative net charge. After adding the positively charged diblock, a second, distinguishable IPEC layer forms on top of the first, increasing the number of compartments of the micelle. Furthermore, a gradual change from a MAA-corona to a PEG-corona can be achieved through complex formation. Here, we perform a first evaluation of these structures to serve as a platform for drug delivery by investigating the uptake and sub-cellular distribution of dye-incorporating BVqMAA micelles in cancer cell lines *in vitro*. Finally, a study on the interaction with biological surroundings *in vivo* in dependence of the corona composition is in preparation.

Experimental

Materials. All solvents were of p.a. quality and used without further purification. Dimethyl sulfate (99%) was delivered by Wako Chemicals, while 2,7,12,17-Tetra-tert-butyl-5,10,15,20-tetraaza-21H,23H-porphine (85%) and 2,9,16,23-Tetra-tert-butyl-29H,31H-phthalocyanine (97%) were supplied by Sigma-Aldrich. Spectra/Por dialysis membranes (MWCO 6-8kDa) from regenerated cellulose were used after short immersion in water.

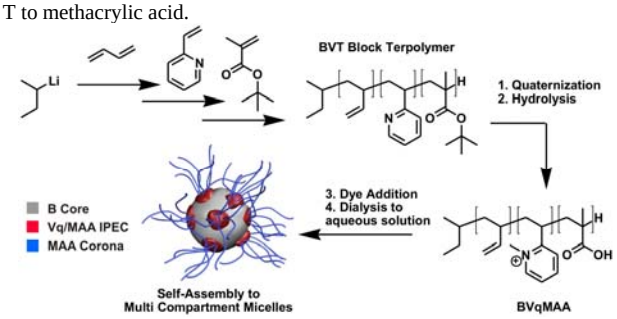
Instrumentation. Physico-chemical characterization methods and cryogenic transmission electron microscopy (cryo-TEM) conditions have been published earlier.[7] ζ-potential and hydrodynamic diameters were determined using a Malvern Zetasizer Nano ZS.

Synthesis of Polymers. Sequential living anionic polymerization in THF at low temperatures with *sec*-butyl lithium as initiator, adapted from a previously reported procedure with respect to the amounts of monomer was used for synthesizing BVT triblock terpolymers.[8] Transforming BVT to BVqMAA was achieved through quaternization with dimethyl sulfate and subsequent hydrolysis with conc. HCl under reflux with dioxane as the solvent, as previously reported.[7] Self-assembly of BVqMAA polymers to micelles was induced through direct dialysis of the mixture to aqueous solution at high pH over a period of several days. In case of dye carrying micelles, the dye was dissolved in the dioxane reaction solution before dialysis to water. After micelles had successfully formed, the solution was changed to 10mM pH 7.4 PBS including 140 mM NaCl through another dialysis step. Commercially available methoxypolyethylene glycol amine (M_n = 12,000 g/mol) was used as macroinitiator of the anionic ring opening polymerization of triflate protected lysine-NCA monomer with DMF (1 M thiourea) as the solvent. PEG-PLL was obtained from this polymer after deprotection in basic medium.[9] Complexed micelles were prepared through addition of appropriate amounts of PEG-PLL dissolved in PBS buffer to the micelle solution and shaking for 1 week. Cross-linking was achieved through EDC coupling reagent (5 fold excess to MAA units) and stirring at room temperature for 12 h, before purification with dialysis.

***In Vitro* Experiments.** The toxicity of the micelles was determined using the MTT assay with A549 cells. For the uptake study, 10,000 cells were seeded in a petri dish with glass bottom. Dye carrying micelles were added after 24h of incubation so that 20µg of dye was given to the cells. Incubation time before imaging with CLSM was 24h and the cells were stained with Lysotracker green and Hoechst 33342.

Results and Discussion

BVqMAA Precursor Micelles. For the preparation of the BVqMAA carrier system a triblock terpolymer of polybutadiene-*block*-poly(vinyl pyridine)-*block*-poly(*tert* butyl methacrylate) (BVT) was first synthesized using sequential living anionic polymerization in THF at low temperatures, as it is depicted in **Scheme 1**. By withdrawing samples at different conversions during the polymerization of the final block, we could obtain a library of polymers carrying the same degree of polymerization (DP) in the first two blocks, but differing in the DP of the third block. The polymers used in the following experiments are $B_{830}V_{180}T_{400}$ and $B_{830}V_{180}T_{1350}$, where the subscripts denote the DP of the corresponding block. In subsequent reactions BVT is rendered amphiphilic through first quaternizing V followed by the hydrolysis of T to methacrylic acid.



Scheme 1. Synthetic steps for the preparation of BVqMAA MCMs, represented as a schematic illustration, from BVT triblock terpolymer.

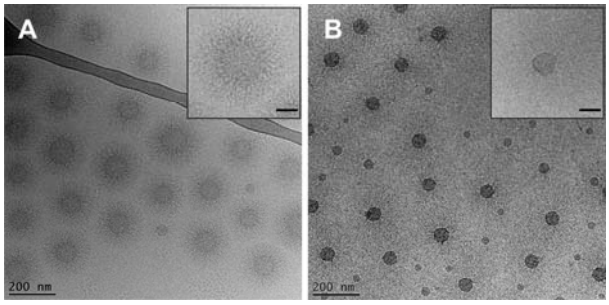


Figure 1. Cryo-TEM images of $BVqMAA_{400}$ (A, 1.2 g/L) and $BVqMAA_{1350}$ (B, 0.29 g/L) micelles in pH 10 buffer solution. Insets show single enlarged micelles with the scalebar representing 50 nm.

Upon dialysis of the reaction mixture to aqueous solution, self-assembly to BVqMAA MCMs takes place due to the insolubility and corresponding collapse of the B block (**Scheme 1**). IPEC formation between positively charged Vq and negatively charged MAA leads to the formation of a non-continuous shell on top of the B core. The micelles are stabilized in water through an excess MAA corona, because the DP of MAA is significantly larger than that of Vq. Since the two polymers used in this work only differ in the block length of the last block, we omit the explicit DPs of the other two

blocks, i.e. the sample names are shortened to $BVqMAA_{400}$ and $BVqMAA_{1350}$. The strong difference in block lengths influences the hydrophilic to hydrophobic balance in the polymers, which leads to a change in compartment-size, i.e. core diameter, corona length and overall micelle size as can be seen in the cryo-TEM images in **Figure 1**.

Dye Incorporation. Hydrophobic dyes can be incorporated into the B core of the micelles, if they are added to the solution after the hydrolysis step, while the polymer is still dissolved in a non-selective solvent (**Scheme 1**). When the solvent quality decreases during dialysis to water, the dye is forced into the core of the micelles, where it gets entrapped, but ideally retains its optical properties. Here, we used two near infrared (NIR) emitting dyes for incorporation, namely a porphine and a phthalocynanine derivate, because NIR light penetrates tissue more deeply than other wavelengths and NIR wavelength is far from the autofluorescence, which allows for *in vivo* imaging with a high signal-noise ratio. Highly colorful micellar solutions were obtained after dialysis and no significant change in the size of the micelles was observed due to the dye incorporation. The absorption spectra of the micellar solutions as well as the dye structure are given in **Figure 2**.

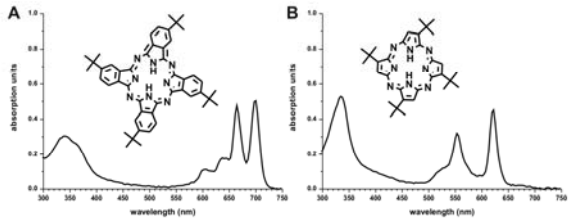


Figure 2. Absorption spectra of $BVqMAA_{1350}$ micelles incorporating phthalocyanine (A, $c_{pol.}$ = 0.84 g/L, c_{dye} = 30 µM) and porphine (B, $c_{pol.}$ = 0.75 g/L, c_{dye} = 50 µM) derivates in pH 7.4 PBS buffer. Chemical structures of the respective dyes are depicted in each spectrum.

PEG-coated Micelles. Since BVqMAA micelles have a negatively charged corona from excess MAA, we utilized this for further IPEC formation when adding polycation-*block*-PEG. With the formation of a new IPEC shell, the corona changes from MAA to PEG with increasing complexing ratio (CR) of diblock copolymer. Previously, we used quaternized poly(*N*,*N*-dimethylaminoethyl methacrylate)-*block*-PEG and observed layered complex micelles with an onion-like structure.[7] However, IPEC formation can be reversible in this case and the complex might dissociate under conditions of high salt concentration or in the presence of competing polyions, i.e. under physiological conditions. We therefore switched the double hydrophilic polymer to poly(ethylene glycol)-*block*-poly(L-lysine) (PEG-PLL), which allows to form a covalent connection through amide bond formation after cross-linking with EDC, thus fixing the structure and preventing dissociation. The overall micelle size at first decreases with increasing amount of PEG-PLL up to a CR of 50% (**Table 1**), resulting from MAA collapse during IPEC formation. At a CR of 100% the size increases again, due to increased PEG content in the micelles. Simultaneously, the ζ-potential of the micelles drastically changes from strongly negative for the precursor micelles to nearly neutral for fully complexed, indicating successful complex formation.

Table 1. Size And ζ-potential Of $BVqMAA_{1350}$ Porphine Carrying Micelles Complexed With Increasing Amount Of PEG-PLL And Cross-linked Through Amide Bond Formation. CR Is Calculated As The Molar Ratio Between Lysine And Excess MAA Units.

PEG-PLL Loading [%]	$\langle D \rangle_{z,app.}$ [nm]	$PDI_{app.}$	ζ-Potential [mV]
0	287	0.052	-31
25	280	0.073	-13
50	255	0.026	-6
100	280	0.028	-2

Toxicity and *in vitro* Uptake. Next, we investigated the toxicity of non-complexed BVqMAA micelles against human lung adenocarcinoma A549 cells by MTT assay. $BVqMAA_{1350}$ micelles without dye showed a mild toxicity with a relative viability of 75% at the highest concentration tested (0.1 mg/mL), while both the porphine and phthalocyanine carrying micelles showed relative viabilities around 15% at the same polymer concentration. The micelles themselves are nontoxic, although incorporating either type of NIR dye increases the toxicity. Therefore, the toxicity may be attributed to the inherent toxicity of the two.

After incubating A549 cells with porphine carrying micelles for 24 h, we were able to detect them co-localized with lysosomes and late endosomes stained with Lysotracker Green in the cells with confocal laser scanning microscopy (CLSM), suggesting an uptake of the micelles into the cells probably via an endocytic pathway (**Figure 3**). Also, no micelles were found freely in the cytoplasm. The micelles were expected to be stable even in the endosomes/lysosomes.

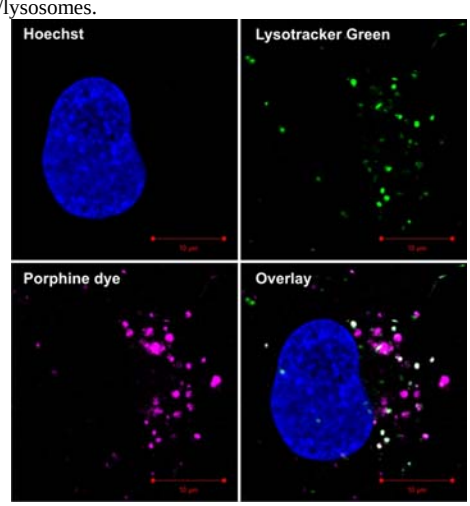


Figure 3. CLSM images of A549 cells incubated with porphine carrying $BVqMAA_{400}$ at 24 h incubation. Scalebar is 10 µm in each image.

Conclusions

We showed successful incorporation of two types of NIR dyes into MCMs from BVqMAA triblock terpolymers, which in turn are taken up by A549 cells. Micelles without dye showed little toxicity. Furthermore, the negative MAA corona could be used for complexing PEG-PLL diblock copolymer, thereby gradually changing the corona from MAA to PEG depending on the mixing ratio. We expect this change in the corona to influence the interaction of the carrier system with biological surroundings, which is currently under investigation.

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