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ASYMMETRIC MEMBRANES FROM AMPHIPHILIC ABC TRIBLOCK COPOLYMERS

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We report the synthesis and characterization of a new kind of amphiphilic ABC triblock copolymers consisting of a hydrophilic (polyethylene oxide)- A block a hydrophobic poly (dimethyl siloxane)-B middle block and a hydrophilic-poly (2-methyl oxazoline) C block. For certain hydrophilic to hydrophobic block length ratios these polymers self-assemble into membrane-like super-structures and vesicles in aqueous media. Due to the inherent incompatibility of the two hydrophilic blocks they segregate to different sides of the underlying membranes thus leading to asymmetric block copolymer membranes. Moreover, depending on the relative lengths of the two hydrophilic blocks we are able to control which of them is oriented toward the inner and the outer surface of the vesicles. Different water-soluble polymers are inherently incompatible and undergo phase separation in aqueous media. Hence, membranes formed by ABC triblock copolymers (with water soluble blocks A and C and a hydrophobic middle block B) are asymmetric: one side is predominantly covered by the blocks A and the other by the blocks B. These systems offer not only a convenient way to modify the inner and outer surface of vesicles with different functional groups but represent also an asymmetric matrix for the directed insertion of membrane proteins.

Keywords: amphiphilic block copolymers; asymmetric block copolymer membranes; membrane proteins matrix; self-assembling; vesicles

During the last decade self-organization of soft materials has shown to be valuable for the creation of a wide variety of nanostructures that could be used for applications in fields ranging from materials science to biology. In this context amphiphilic block copolymers are of particular interest due to their ability to self-assemble in aqueous media, their broad accessibility to different length and time scales and levels of interaction [1,2]. Similar to conventional low molar mass surfactants they may form micelles, vesicles or lyotropic mesophases. These aggregates are significantly more stable

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than those formed by low molar mass amphiphiles and additionally they can be further stabilized by a subsequent crosslinking polymerisation [2–4]. This makes, e.g., block copolymer vesicles highly interesting as transfection vectors [5] protective shells for sensitive enzymes [6] or as confined reaction vessels that allow to perform (bio-) chemistry even at a single molecular level [7]. The walls of the block copolymer vesicles are formed by membrane-like superstructures that can be regarded as mimetics of biological membranes. In fact, we were able to show that despite their enormous thickness and stability such block copolymer membranes can be used as a matrix for functional reconstitution of membrane proteins [6,8].

Most membrane proteins are asymmetric and in natural membranes oriented such that they expose an extracellular part to the outside and a cytoplasmic part to the inside of a biological cell. Artificial membranes, however, are symmetric with respect to their midplane if curvature effects are neglected. That holds, of course, also for block copolymer membranes formed by AB or ABA-type block copolymers. Therefore, during reconstitution, membrane proteins are randomly inserted without any preferred direction. Unfortunately many potential technical applications of such reconstituted systems depend on the correct orientation of the protein. This brought us to the idea to use intrinsically asymmetric membranes as a matrix. Here block copolymer chemistry offers a particularly interesting approach. Different water-soluble polymers are inherently incompatible and undergo phase separation in aqueous media. Therefore, membranes formed by ABC triblock copolymers (with water soluble blocks A and C and a hydrophobic middle block B) should be asymmetric: one side predominantly covered by the blocks A and the other by the blocks C.

In this paper we describe for the first time amphiphilic poly (ethylene oxide)-block-poly (dimethylsiloxane)-block-poly (2-methyloxazoline) (PEO-PDMS-PMOXA) triblock copolymers that self-assemble in aqueous media into asymmetric, membrane-like superstructures.

The synthesis of a representative PEO-PDMS-PMOXA copolymer is sketched in the following (see also Fig. 1). All steps were carried out under argon atmosphere. A solution of 6.2 g of poly(ethylene oxide) monomethyl ether ($M_n = 2 \times 10^3$ g/mol, $M_n/M_w = 1.03$) in dry tetrahydrofuran ($[PEO]_0 = 125$ g/L) was added to a dispersion of potassium hydride in THF. Then 18-crown-6 (19 mg, 7.11×10^{-5} mol) was added and the reaction solution was stirred for 4 h. Subsequently, 1.2 mL of 2,6 dimethylpyridine were added dropwise (50 μL/min) and the reaction stirred for another 30 min. The resulting alcoholate anion was used as an initiator for the anionic ring opening polymerization of octamethyltetracyclosiloxane (D_4) (18.7 g, 63 mM). The polymerization time was 20 h at a temperature of 55°C. The chain growth was terminated using methacryloyloxypropyldimethylchlorosilane (2.8 g, 12.6 mM). The resulting PEO-PDMS

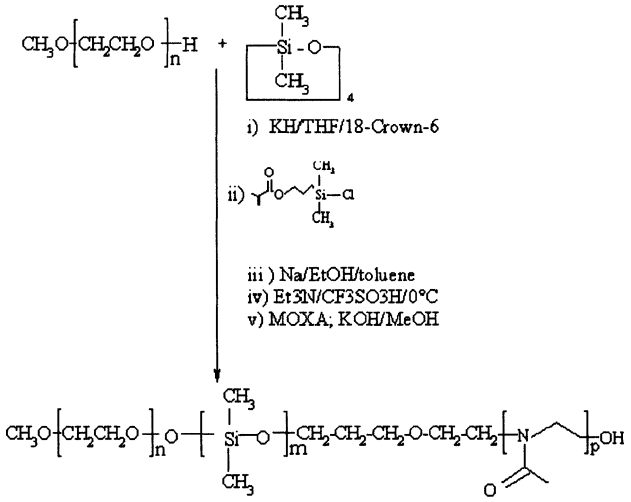

FIGURE 1 The scheme of the synthesis of PEO-PDMS-PMOXA triblock copolymer.

diblock copolymer was purified by column chromatography (THF/ Methanol (8:2, v/v)) and ultrafiltration with water. After evaporation of the solvent the pure PEO-PDMS diblock copolymer was obtained as a yellowish oil. Reduction of the ester end-group was accomplished using a modified Bouveault–Blanc method [9]. An excess (40%) of sodium in ethanol was added to a solution of the polymer in ethanol (1:1, v/v) and allowed to react for 16 h at 75°C. After dissolving the precipitated salt by addition of water, the resulting alcohol was extracted with diethyl ether and distilled under reduced pressure.

The subsequent cationic ring opening polymerization of methyl-oxazoline followed a procedure described by Hirt *et al*. [10]. The purity and the structure of the block copolymers was confirmed by ^{1}H, ^{29}Si NMR and IR spectroscopy. Their molecular weights were calculated from the molecular weight of the PEO precursor and the chemical composition.

To check whether the block copolymers form vesicles, the copolymers were dispersed in water (typically at 20 mg/mL PEO-PDMS-PMOXA). Indeed, transmission electron microscopy (TEM) and dynamic light scattering measurements revealed that this led to vesicles with sizes in the range of 60–300 nm. Figure 2 shows a representative TEM image of sphe polydispersity of the originally formed vesicles could, however, be reduced by subsequent extrusion through filters with 0.1 μm diameter pores.

To get information about the orientation of the triblock copolymers within the vesicular walls we applied an approach similar to that recently used for amphiphilic AB diblock copolymer vesicles [11]. Here we labelled

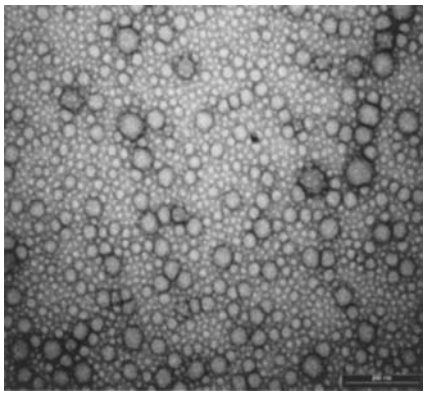

FIGURE 2 Transmission electron micrograph of negatively stained aggregates of $A_{45}B_{67}C_{346}$ (polymer weight: 7.7 %).

selectively one end of the triblock copolymers with a fluorescent dye (7-methoxycoumarin) and used quenching experiments to determine whether the labelled hydrophilic block was oriented toward the inside or the outside of the vesicles.

For that purpose 7-methoxycoumarinazide (5 mg/mL) was dissolved in chloroform. A five-fold excess of this solution was added to the $A_xB_yC_z$ triblock copolymer (with A = PEO, B = PDMS, C = PMOXA and x, y, z = number of repeat units of the respective block) and heated to 65°C for 22 h. Upon heating the azide decomposes and forms an isocyanate that reacts (in situ) with the hydroxyl groups at the end of the PMOXA blocks. Afterwards the product was separated and purified from unreacted dye by ultrafiltration with ethanol/water (7/3, v/v). ^{1}H NMR spectroscopy indicated a conversion of 30% of the terminal hydroxyl groups for $A_{45}B_{67}C_{346}$ and 12% conversion for $A_{45}B_{40}C_{97}$ under these conditions. This rather low conversions are, however, not relevant for the following experiments.

For the quenching experiments we mixed labelled ($A_{45}B_{67}C_{346}$-7-methoxycoumarin and $A_{45}B_{40}C_{97}$-7-methoxycoumarin) with the corresponding non-labelled polymers ($A_{45}B_{67}C_{346}$ and $A_{45}B_{40}C_{97}$, respectively) in molar ratios of 344 : 1 and 10 : 1. From these mixtures we prepared vesicles following the procedure described above. Interestingly light scattering proved that the diameters of the labelled vesicles were similar to those of the non-labelled (ie, 69 nm for $A_{45}B_{65}C_{346}/A_{45}B_{65}C_{346}$-coumarin vs. 80 nm for $A_{45}B_{67}C_{346}$ and 74 nm for $A_{45}B_{40}C_{97}/A_{45}$ $B_{40}C_{97}$-coumarin vs. 115 nm for $A_{45}B_{40}C_{97}$). Obviously the presence of the fluorescent dye did not disturb the self-assembly of the polymers.

To get information about the orientation of the labelled PMOXA blocks we added Co^{2+} ions to the external solution. Co^{2+} is known to quench the fluorescence of coumarin. However, due to the thickness of the block copolymer membranes (i.e., about 10 nm) and their impermeability toward Co^{2+} ions only the coumarin located at the outer surface of the vesicles can be quenched.

Figure 3 shows the results of the quenching experiments for the labelled $A_{45}B_{65}C_{346}$ and the $A_{45}B_{40}C_{97}$ systems together with data for non-labelled block copolymer vesicles. Also the non-labelled vesicles of the control experiment showed a fluorescence emission around 420 nm, however, with a ten times lower intensity than the coumarin-labelled ones. This is presumably due to traces of impurities in the block copolymers. The presence of the Co^{2+} ions influenced this 'polymer' fluorescence, however, only very little. Interestingly the data for the $A_{45}B_{40}C_{97}/A_{45}B_{40}C_{97}$-coumarin vesicles were identical with the control system within the experimental error, i.e., the fluorescence emission remains nearly unaffected by the presence of the quencher molecules (see Fig. 3). In contrast to that for the $A_{45}B_{65}C_{346}/A_{45}B_{65}C_{346}$-coumarin vesicles, with the longer PMOXA block, the data follow a Stern–Volmer relation and the fluorescence is quenched completely by Co^{2+} ions.

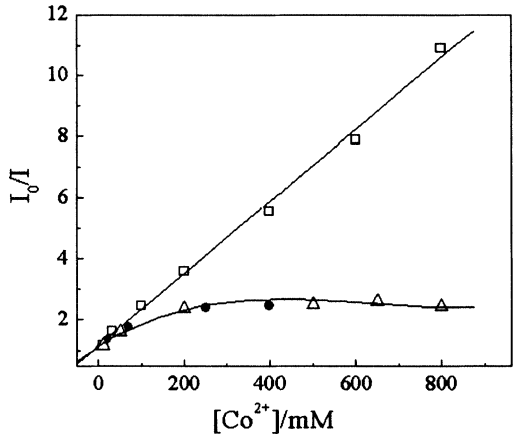

FIGURE 3 The variation of steady state fluorescence with concentration of Co^{2+} ions quencher for spherical vesicles containing (I_0: fluorescence intensity in absence of Co^{2+} ions; I: fluorecence intensity in presence of rical ABC block copolymer vesicles with a rather broad size distribution. The variable concentrations of Co^{2+}: A) $A_{45}B_{67}C_{346}/A_{45}B_{67}C_{346}$-coumarin (–□–); B) $A_{45}B_{40}CA_{97}/A_{45}B_{40}C_{97}$-coumarin (–△–); C) $A_{45}B_{67}C_{346}$ (-●-) (control with non-labelled polymer).

These results indicate that obviously in the $A_{45}B_{65}C_{346}$ system all PMOXA blocks are oriented toward the outside of the vesicles and consequently the PEO blocks toward the interior. For the shorter and less voluminous PMOXA blocks of the $A_{45}B_{40}C_{97}$ system the arrangement is reversed, ie, PEO points outwards and PMOXA toward the interior. This agrees with geometrical considerations: due to the curvature of the vesicle walls it is favourable when the hydrophilic blocks with lower volume segregate toward the interior.

A complementary study contains the assay for the electroformation of giant vesicles for this ABC amphiphilic polymers, with a diameter in a range of 2–5 μm. Using the same procedure of labeling described previously, but with tetramethylrhodamine as a dye, the confocal fluorescence microscopy indicates as well the existence of the fluorescence equally distributed at the surface of the vesicles (data not shown), proving once more that the previous theoretical assumptions are correct.

The preliminary studies concerning the biologically induced asymmetrie of ABC triblock copolymer matrix relative to the membrane proteins were promising. We are able to prove that it is a significant difference for the 'directed' orientation of a transmembrane protein, for tree different systems formed by: liposomes, ABA symmetric and ABC asymmetric triblock copolymers matrix.

An asymmetric ABC membrane matrix type allowed a physiological orientation of the membrane proteins, while for a symmetric ABA or liposomes type matrix the insertion of the protein are not following a preferred direction of this insertion [12].

Further work concerning the influence of molecular parameter of the polymers on their aggregation behaviour and studies concerning the insertion mechanism of the protein, assembled into a conformation which span the uni- and multilamellar vesicles are in progress.

Although many open questions still remain to be solved, we believe that these first results indicate clearly that the new amphiphilic ABC triblock copolymers are well-suited to prepare asymmetric membranes in aqueous media. We expect that these new artificial membrane systems could be an important breakthrough particularly for biologically inspired technical applications.

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