Hybrid materials from amphiphilic block copolymers and membrane proteins

Corinne Nardin, Wolfgang Meier*

Institut für Physikalische Chemie, Universität Basel, Klingelbergstrasse 80, 4056 Basel, Switzerland

Abstract

Self-assembly of reactive amphiphilic block copolymers is used to prepare nanostructured hydrogels with exceptional permeability properties, vesicular structures and planar, freestanding membranes in aqueous solution. Although the underlying block copolymer membranes are two–three-fold thicker than conventional lipid bilayers, they can be regarded as mimetic of biological membranes and can be used as a matrix for membrane-spanning proteins. Surprisingly, the proteins remain functional, despite the extreme thickness of the membranes and even after polymerization of the reactive block copolymers. The unique combination of block copolymers with membrane proteins allows the preparation of mechanically stable, defect-free membranes and nanocapsules that have highly selective permeability and/or specific recognition sites. This is documented by some representative examples. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Amphiphilic block copolymer; Hydrogel; Membrane; Membrane protein

1. Amphiphilic block copolymers

A current topic in materials science is to create nanometer-sized, well-defined structures. One key step is to develop preparative procedures to precisely control the formation and morphology of these structures. Self-assembled superstructures of surfactants, and especially of amphiphilic block copolymers, have proven to be valuable tools. They provide a compartmentalization at the nanometer level, which may be used as a structural template for the newly formed materials.

Amphiphilic block copolymers consist of at least two parts with different solubility, causing their self-assembly into superstructures in the sub-micrometer range with cores consisting of their insoluble parts, surrounded by a corona of their soluble parts (Tuzar and Krachtovil, 1993; Chu, 1995; Alexandridis, 1996; Moffitt et al., 1996; Förster and Antonietti, 1998). This self-organization of block copolymers is based on the same underlying principles as for typical low
molecular-weight amphiphiles, such as surfactants or lipids in water. Block copolymers consisting of hydrophilic and hydrophobic blocks behave like conventional surfactants in water: similar to the latter, they self-assemble in water into micelles of various shapes, and at higher concentrations into lyotropic liquid-crystalline phases. Their aggregation is controlled by hydrophobic interactions and their lyotropic phase behavior by the packing constraints for hard, spherical objects (Wanka et al., 1994; Förster et al., 1996; Svensson et al., 1999). However, it is the macromolecular nature of the block copolymers that determines their special features.

The high diversity of block copolymer chemistry allows, for example, variation of the chemical constitution (e.g. nature and sequence of the repeat units), the length and structure of the different blocks, and even the molecular architecture of the whole polymer (e.g. block, graft, star, multiblock copolymers). As a consequence, block copolymers cannot only be tailored to lead to micelles of a pre-defined size, but their critical micelle concentration can also be controlled and pushed to extremely low limits. The resulting micelles are then stable against dilution, are very monodisperse in size and display extremely low dynamics. In contrast to conventional surfactant micelles that have typical lifetimes in the millisecond range, for block copolymer micelles, this can easily be adjusted to be in the second–hour region.

The equilibrium shape of amphiphilic aggregates is related to the molecular geometry of the underlying molecules. The relation between molecular geometry and the resulting local curvature within an aggregate is readily estimated for low molecular-weight surfactants by the concept of the packing parameter (Israelachvili et al., 1976). This parameter describes the shape of a surfactant molecule in terms of the hydrophobic chain volume, the preferred length of the chains and the area covered by the hydrophilic headgroups. Despite their larger size and higher flexibility, similar considerations must hold for the amphiphilic block copolymers. In fact, decreasing the length of the hydrophilic blocks at constant hydrophobic block length causes a transition from spherical to worm-like micelles, and finally to vesicular structures (Zhang and Eisenberg, 1995; Hajduk et al., 1998; Discher et al., 1999; Won et al., 1999). This tendency towards the formation of less-curved aggregates with decreasing hydrophilic block length corresponds to the observation with low molecular-weight non-ionic oligo(ethylene oxide monoalkyl ethers) when the number of ethylene oxide units is reduced (i.e. their packing parameter increases) (Mitchell et al., 1983). A similar influence is also observed in the lyotropic phase behavior of the block copolymers at higher concentrations. The extent of the lamellar phase grows at the expense of spherical and hexagonal phases of higher local curvature with decreasing length of the hydrophilic blocks (Maassen et al., 1990; Yang et al., 1992; Yang and Wegner, 1992a,b; Wanka et al., 1994; Hajduk et al., 1998).

As a result of their broad accessibility to different length scales, levels of interaction and time-scales, amphiphilic block copolymers become increasingly attractive for applications. Amphiphilic block copolymers substitute low molecular-weight surfactants in numerous applications as emulsifiers, dispersants, foaming agents, thickeners or compatibilizers. Recently, their self-assembled superstructures have also been used as a matrix for the controlled preparation of nanostructured inorganic and organic materials. In the latter case, this may lead to well-defined nanostructured hydrogels (Maassen et al., 1990; Yang et al., 1992; Yang and Wegner, 1992a,b; Wanka et al., 1994; Hajduk et al., 1998; Hentze et al., 1999), worm-like rubber micelles (Won et al., 1999), nanocapsules (Ding and Liu, 1998a; Huang et al., 1999; Stewart and Liu, 1999; Nardin et al., 2000a), nanospheres (Wooley, 1997; Ding and Liu, 1998b; Büttin et al., 1999; Hang et al., 2000; Rheingans et al., 2000) or ultrathin films (Goedel and Heger, 1998; Nardin et al., 2000b), depending on the initial structure.

2. Nanostructured hydrogels

Interestingly, the phase behavior of amphiphilic block copolymers in water is not only controlled
by their chemical constitution (e.g. nature and sequence of the repeat units), the length and structure of the different blocks and the molecular architecture of the whole polymer (e.g. block, graft, star or multiblock copolymers). The molecular weight distribution of the individual blocks also has significant influence on the phase behavior of such systems. In this context, we recently introduced a new type of amphiphilic ABA triblock copolymer. The polymer consists of a flexible, hydrophobic poly(dimethylsiloxane) (PDMS) middle block and two water-soluble poly(2-methyloxazoline) (PMOXA) side blocks. Additionally, the ends of this PMOXA–PDMS–PMOXA triblock copolymer carry methacrylate groups, which allow cross-linking polymerization, i.e. ‘freeze in’ of the self-assembled superstructure of the system. Recently, we could show that with increasing polydispersity of these polymers, bicontinuous cubic phases with a complex structure of mutually interwoven hydrophobic and water-swollen hydrophilic channels (see Fig. 1) are stabilized at the expense of the more classical spherical, hexagonal or lamellar mesophases, which possess interfaces of nearly constant curvature (Nardin and Meier, 2001). This phenomenon can be explained by the fact that the polymers of higher polydispersity can better accommodate the associated curvature variations in the topologically complex superstructures of the bicontinuous phase. In this case, the polymer molecules can simply distribute themselves over the regions of variable curvature according to their respective chain length.

It has to be emphasized that in general, a subsequent polymerization of the reactive end groups of the polymers did not lead to any measurable changes in the structure or the phase behavior of these systems. This is in contrast to reactive low molecular-weight amphiphiles, in which it has often been observed that polymerization may disturb the delicate balance that controls the morphology of the system, and may therefore lead to structural transformations. Obviously, for the block copolymers this is prevented by their rather slow dynamics with respect to phase transitions or separation in comparison to the rate of polymerization. The resulting covalently cross-linked hydrogels then combine solid-state properties, such as elasticity and shape persistence, with the special superstructure of the respective lyotropic mesophase. For example, PMOXA–PDMS–PMOXA hydrogels with a bicontinuous cubic structure have simultaneous high permeability for oxygen and aqueous electrolytes, which can diffuse through the PDMS or the water-swollen hydrophilic channels, respectively. Therefore, such systems can be used as a new type of contact lens that allows good supply to the cornea of the human eye through the lens material, as membranes for separation processes, or as biomaterials for tissue engineering.

### 3. Block copolymer vesicles

For a given composition of such block copolymers (Zhang and Eisenberg, 1995; Hajduk et al., 1998; Discher et al., 1999; Won et al., 1999; Nardin et al., 2000a) (e.g. for PMOXA–PDMS–PMOXA triblock copolymers with $M_n/PMOXA = 1800$, $M_{n,PDMS} = 5400 \text{ g mol}^{-1}$; $M_{n,PDMS}/M_n = 1.7$), the phase behavior in water is similar to that of typical bilayer-forming lipids, such as lecithin (Lasic, 1993; Nardin et al., 2000c). For example, for this triblock copolymer the basic morphological units are lamellae with a hydrophobic PDMS core and a hydrated PMOXA
corona over the whole composition range. Similar to conventional lipids, such polymers may form vesicular structures in dilute aqueous solution, which consist of spherically closed block-copolymer membranes. Depending on the preparation method applied, the amphiphilic block copolymers may be converted into vesicles with diameters in the range of 50 nm up to approximately 100 μm.

Giant vesicles with diameters ranging from approximately 1 to 100 μm can be prepared by standard swelling procedures or electroformation (Discher et al., 1999, 2000). For the electroformed vesicles, a thin film of polymer deposited on adjacent electrodes (Bucher et al., 1998) or conductive glass (Angelova et al., 1992) is phoresed by alternating current into the aqueous solution. Fig. 2 shows representative block copolymer vesicles with diameters between 5 and 50 μm that have been prepared by this technique. Giant vesicles are convenient systems for studying fundamental properties of bilayer membranes. Recently, micromanipulation experiments have shown that block copolymer vesicles are, for example, almost an order of magnitude tougher and sustain far greater areal strain before rupture in comparison to conventional lipid bilayers. In addition, the polymer membrane has been shown to have a 10-fold lower permeability to water than common lipid analogues (Discher et al., 1999).

Similar to conventional lipid vesicles, small, unilamellar, block copolymer vesicles with sizes in the sub-micrometer range can be prepared, for example by injection and extrusions methods (Zhang and Eisenberg, 1995; Ding and Liu, 1998a,b; Jenekhe and Chen, 1998) (Fig. 3). Interestingly, the average size and size distribution of the resulting vesicles depend not only on the details of the preparation procedure, but also on the polydispersity of the block copolymer molecules. In fact, there is experimental evidence that in block copolymer vesicles, the polymer molecules with shorter hydrophilic block lengths segregate to the inside of the vesicles, and long hydrophilic chains to the outside. This segregation increases repulsion between hydrophilic blocks on the outside of the vesicles relative to that on the inside and provides thermodynamic stabilization of the curvature (Luo and Eisenberg, 2001) (see Fig. 4 for a schematic representation). As a result, the vesicles adapt a certain equilibrium size that mainly depends on the molecular weight distribution of the polymers. Moreover, such segregation leads to intrinsically asymmetric

Fig. 2. Differential interference contrast (DIC) micrograph of giant vesicles formed by poly(2-methyloxazoline)–poly(dimethylsiloxane)–poly(2-methyloxazoline), PMOXA–PDMS–PMOXA triblock copolymer.

Fig. 3. Transmission electron micrograph (TEM) of small, unilamellar triblock-copolymer vesicles obtained with PMOXA–PDMS–PMOXA triblock copolymer vesicles.
membranes with chemically different inside and outside walls of the vesicles, which may have useful applications (e.g. for reconstitution of membrane proteins).

4. Nano- and microcapsules from amphiphilic block copolymers

The formation of vesicular aggregates from block copolymers is generally a result of non-covalent interactions, and hence is reversible (even though block copolymer aggregates may be significantly more stable than those formed from low molecular-weight amphiphiles). This is, for example, directly reflected in the occurrence of a critical aggregation concentration (cac), below which the vesicles begin to disintegrate and dissolve as individual block copolymer molecules (Nardin et al., 2000a). In the case of the reactive PMOXA–PDMS–PMOXA triblock copolymers, cross-linking polymerization of the methacrylate end groups of the underlying polymers can be performed within the vesicles. The particles are then additionally held together by a covalently cross-linked polymer network structure. As a consequence, the cac vanishes upon polymerization and the resulting nano- and microcapsules possess solid-state properties, such as shape persistence. Therefore, they are able to preserve their hollow sphere morphology, even after their isolation from the aqueous solution. It has to be emphasized that during the past few years, extensive efforts have been devoted to the preparation of hollow polymer particles. This is due to their potential for applications in fields such as medicine, cosmetics and pharmacology, or as containers for (bio-) chemistry performed with single molecules (Chiu et al., 1999). In the context of such applications, their high stability and shape persistence could be particularly interesting. It allows, for example, loading of preformed capsules with guest molecules in an organic solvent, isolation of the loaded polymer shells and subsequent release of the encapsulated material in an aqueous medium.

5. Ultrathin films

As already mentioned, block copolymer vesicles and the underlying block copolymer membranes are considerably more stable than conventional lipid bilayers. In addition, they can be further stabilized by interconnecting the individual block copolymers within the vesicle-forming bilayers via covalent bonds to form a giant ‘supermacromolecule’.

The mechanical and viscoelastic properties of such membranes can be quantified by micromanipulation of giant vesicles (see above). Complementary information can be obtained by electroporation experiments, i.e. by applying controlled forces via short electric-field pulses onto planar freestanding films with a thickness of a few nm and area of up to 1 mm², so-called ‘black polymer membranes’ (the block copolymer analogues of the well-known ‘black lipid membranes’). These electric field pulses charge the membranes, causing an internal electric stress (Benz and Bauer, 1988). Above a critical voltage, rupture of the membrane is induced and a fast discharge across the defect is observed. An analysis of the time course of the associated voltage gives, for example, information about the energy required to form a defect in the membrane, the intramembrane mobility of the underlying molecules, or the kinetics of defect widening and the underlying physical forces.

Such experiments clearly confirm the enormous
mechanical stability of the ca. 10-nm-thick block copolymer membranes, which further increases upon cross-linking polymerization. Interestingly, we could show that the PDMS middle blocks of our reactive triblock copolymers preserve a certain mobility within the membrane, despite the polymerization (Nardin et al., 2000b), which covalently links together all the individual triblock copolymer molecules. This can be explained by the location of the polymerizable groups at the very ends of the hydrophilic blocks of the polymers, which decouples, at least partially, their hydrophobic blocks from the newly formed polymer network structure. In contrast to conventional (fluid-like) lipid membranes, such cross-linked block copolymer membranes behave like thin rubber films due to their cross-linked nature. As a result, they can be elastically (i.e. reversibly) deformed by applying a slight overpressure on one side of the membrane, similar to an inflating balloon.

6. Reconstitution of naturally occurring membrane proteins

Another interesting aspect of the freestanding films is that there is direct access to both sides of the membrane, which allows investigation of transmembrane transport processes. In fact, conventional black-lipid membranes are frequently employed as model systems to reconstitute membrane proteins responsible for translocation. It is obvious that the tendency of the PMOXA–PDMS–PMOXA triblock copolymer to form membrane-like superstructures in water closely resembles the behavior of lipid molecules. Hence, the block copolymer membranes can be regarded as mimetic of biological membranes, however, with considerably greater thickness (i.e. approx. 10 nm and more compared to 3–5 nm) and stability. Therefore, we found it quite tempting to try to reconstitute such membrane proteins in our black polymer membranes. We expected the resulting new combination of the broadness of polymer science with the richness of natural or genetically modified proteins to create new types of hybrid materials with unique properties.

As a model system, we used naturally occurring membrane proteins (i.e. porins) extracted from the outer cell wall of Gram-negative bacteria, which contains a multitude of unspecific and specific protein channels with a broad range of different transport properties for substrates (Nikaido, 1992).

Porins form trimeric water-filled channels that allow passive diffusion of small solutes, such as ions, nutrients or antibiotics, across the membrane. Therefore, the incorporation of the channels into planar freestanding films could be directly monitored using conductivity measurements (Benz and Bauer, 1988; Winterhalter, 1999). For example, the protein maltoporin forms very narrow channels with a conductance of 150 pS per trimer in 1 M KCl. In addition, it has stereo-specific binding sites for maltooligosaccharides inside the aqueous channels, which enhance the diffusion of the sugars across membranes (Nikaido, 1992; Schirmer et al., 1995; Dutzler et al., 1996; Wang et al., 1997; Winterhalter, 1999). Therefore, not only the incorporation of maltoporin into the polymer membranes, but also its binding to maltooligosaccharides could be monitored. Upon titration, the sugar is driven into the channels in a concentration-dependent manner and causes closure of the channels. Hence, the binding constants between sugars and the proteins can be calculated from the concentration dependence of the conductivity data. Interestingly, the sugar affinity constants for maltoporin within the triblock copolymer membranes were the same and in good agreement with previous investigations on maltoporin in conventional lipid membranes (Benz and Bauer, 1988; Winterhalter, 1999). This indicates that the proteins remain fully functional, despite the fact that their hydrophobic–hydrophilic pattern is naturally optimized with respect to the thinner biological membranes, and that the block copolymer membranes are considerably thicker than conventional lipid bilayers due to the larger size of the underlying block copolymer molecules (Discher et al., 1999; Nardin et al., 2000c,d). It seems, however, that the high flexibility and conformational freedom of the polymer molecules allow a block copolymer membrane to adapt to the specific geo-
metric and dynamic requirements of membrane proteins without considerable loss of free energy. As already mentioned, the hydrophobic PDMS middle block preserves a certain mobility within the membrane, even after cross-linking polymerization of the reactive block copolymers (Nardin et al., 2000b). As a result, the binding affinity for the sugars remains unchanged, even after polymerization of the block copolymer–protein hybrid membranes. Since the binding affinity of the protein is known to be very sensitive towards conformational changes, the conformation of the protein is obviously not affected by the artificial surrounding within such a polymerized triblock copolymer membrane and its functionality is fully preserved.

It has to be emphasized that these results should be regarded as a representative example of this new type of polymer–protein hybrid material. In fact, nature provides many more membrane or membrane-associated proteins that can be reconstituted in the same way. This opens the possibility to benefit from the enhanced stability and diversity of the block copolymer aggregates, which might be particularly interesting for the development of new biosensors or devices for rapid drug screening.

7. Nanoreactors

It is obvious that such membrane proteins cannot only be reconstituted in planar membranes, but also in the shells of block copolymer vesicles or the polymerized block copolymer capsules. Recently, we demonstrated that this could be used to prepare a new type of stable nanoreactor with controlled permeability (Nardin et al., 2000d, 2001; Nardin and Meier, 2001). Incorporation of membrane proteins into the shell of (polymerized) triblock copolymer vesicles allows the selective harvest or separation of specific molecules, as well as their release on demand. The shell can protect encapsulated enzymes against a hostile environment and the channels in the shell can be used for ‘pre-filtering’ the substrates to enhance the sensitivity of the enzyme (Nardin et al., 2000d, 2001; Nardin and Meier, 2001).

To demonstrate this, we incorporated the porin OmpF into the membranes of triblock copolymer vesicles to control the permeability of their shells (Fig. 5). It is known that molecules with a molecular weight above 600 g mol\(^{-1}\) are sterically excluded from these channels (Nikaido, 1992). As a representative example, we encapsulated the enzyme \(\beta\)-lactamase \(M_w\ 50,000\ g\ mol^{-1}\) in the aqueous core domain of the nanocapsules. \(\beta\)-Lactamase hydrolyzes \(\beta\)-lactam antibiotics, such as ampicillin \(M_w\ 349\ g\ mol^{-1}\). In contrast to ampicillin, the product of the hydrolysis, ampicillinoic acid, can reduce iodine to iodide. Therefore, the activity of the enzyme can be readily monitored by iodometry, i.e. via the decolorization of a starch–iodine complex (Novick, 1962; Zimmermann and Rosselet, 1977). It has to be emphasized that subsequent polymerization of the nanoreactors did not change their activity within experimental error.

Interestingly, for a given ampicillin concentration outside the nanoreactors, a steady state is rapidly established, at which the rates of antibiotic diffusion through the OmpF channels and the \(\beta\)-lactam hydrolysis are equal, thus resulting in a constant ampicillinoic acid release (Nardin et al., 2000d, 2001; Nardin and Meier, 2001). This makes the nanoreactors interesting as drug deliv-
ery systems. In such systems, constant drug release over an extended period of time is often required.

Furthermore, the OmpF protein has the interesting property of being closed if a transmembrane voltage above a critical value of approximately 100 mV is applied (Lakey et al., 1991; Nikaido, 1992; Van Gelder et al., 2000). It seems that the cells from which the protein has been isolated have evolved this mechanism to protect themselves against drastic changes in their environment. Recently, we could show that this gating transition could be used to switch on or off the nanoreactors via external stimuli (Nardin and Meier, 2001; Nardin et al., 2001). The possibility of triggering the activation (or deactivation) of such systems is highly interesting for applications, since it allows local and temporal control of the uptake and release of substrates and products. The block copolymer–protein hybrid shells of the nanoreactors can be regarded as a semi-permeable membrane separating their internal volume from the external solution. Large molecules above 600 g mol\(^{-1}\) are excluded. This property opens a convenient approach to triggering the gating transition of OmpF. Large polyelectrolyte ions, such as poly(styrene sulfonate), do not permeate, and therefore their sodium counterions must be distributed inside and outside the nanocapsules according to a Donnan equilibrium, i.e. their concentration should be different on both sides of the nanocapsules wall, giving rise to a Donnan potential. If this potential exceeds the critical value necessary for closure of OmpF, the substrates can no longer enter the interior of the nanoreactors, i.e. the reactors are deactivated (Nardin and Meier, 2001; Nardin et al., 2001). The closure is a reversible process, and decreasing the potential below 100 mV reactivates the nanoreactors. This could be carried out by diluting the system with buffer or by increasing the Na\(^+\) concentration in the system. In both cases, the nanoreactors regain full activity after reactivation.

This principle of using the protective ability of nano or micro containers in combination with controlled permeability, either by natural or genetically modified channels or pumps, has potential for many future applications in areas such as medicine, pharmacology or diagnostics. For example, the encapsulation could be extended to antibodies to decrease the immunogenicity of an enzyme when injected in plasma. This would allow the use of enzymes designated to have a therapeutic role from various sources.

8. Conclusions

Reactive, amphiphilic block copolymers provide a convenient basis for the preparation of a wide variety of nanostructured polymeric materials, ranging from hydrogels to nanoreactors and biomimetic membranes.

In particular, the novel block copolymer–protein hybrid materials open a whole new area, taking advantage of the many possibilities that polymer chemistry offers, e.g. the use of electrically neutral polymers as a matrix for membrane proteins. Moreover, the unique combination of block copolymers with membrane proteins allows the preparation of mechanically stable, defect-free membranes and nanocapsules that have highly selective permeability and/or specific recognition sites. The proteins remain fully active, even after cross-linking polymerization of the underlying reactive block copolymers, which leads to a considerable mechanical stabilization of the whole system. Moreover, in the context of the reconstitution of membrane proteins, a particularly interesting aspect of small, nanometer-sized block-copolymer vesicles arises from their inherently asymmetric walls, due to block length-dependent segregation of short and long hydrophilic blocks as described above. In contrast to conventional lipid vesicles, in which membrane proteins are mostly inserted with random orientation, such asymmetric block-copolymer membranes allow a directional insertion with the periplasmic side of the proteins oriented towards the interior of the vesicles and their extracellular part towards the outside.

Furthermore, it is important to note that despite their intriguing properties, in the past, membrane proteins were not accessible as material components, since they are difficult to produce
and to purify on a large scale. Only recently have larger quantities of, for example, porins been produced (some g compared to a few µg), so that our approach can be regarded as one example of their multitude of possible future applications in polymer and materials science.

References


Ding, J., Liu, G., 1998b. Polystyrene-block-poly(2-cinnamoyl-
lethyl methacrylate) nanospheres with cross-linked shells. Macromolecules 31, 6554–6558.


