Triblock Copolymer as an Effective Membrane-Sealing Material

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Abstract

An intact cell membrane serves as a permeable barrier, regulating the influx and efflux of ions and small molecules. When the integrity of the membrane is compromised, its barrier function is also disrupted, threatening the survival of the cell. Triblock copolymer surfactants of the form poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) have been shown to help seal structurally damaged membranes, arresting the leakage of intracellular materials.

In order to understand how this particular family of triblock copolymers helps seal damaged membranes, model lipid monolayer and bilayer systems have been used to unravel the nature of the lipid/copolymer interaction. The copolymer surfactant is found to selectively insert into structurally compromised membranes, thus localizing its sealing effect on the damaged regions. The inserted polymer is "squeezed out" when the lipid packing density is increased, suggesting a mechanism for the cell to be rid of the polymer when the membrane integrity is restored.

Keywords: *biological, cellular, diffusion, polymer.*

Barrier Function of the Cell Membrane

The cell membrane separates the interior of the cell from the outside and regulates the molecular and ionic content of the intracellular medium. The majority of the energy required to sustain cellular function is expended in maintaining large differences in ion concentrations across the cell membrane. The lipid bilayer provides the ionic diffusion barrier that makes it energetically possible to maintain large transmembrane ion concentration gradients. The bilayer serves this role remarkably well by establishing a nonpolar region through which an ion must pass in order to cross the membrane. The high energy needed for such a passage is a strong impediment to passive ion diffusion across the bilayer.[1] However, cell membranes consist typically of 30% protein; many of these proteins facilitate and regulate membrane ion transport. Roughly, these protein effects combine to make the cell membrane approximately 10^6 times more conductive to ions than the pure lipid bilayer.[2]

The cell membrane is in essence a two-dimensional structured fluid, held intact only by van der Waals, hydrophobic, hydrogen-bonding, and screened electrostatic interactions. Occasionally, small separations in the lipid packing order occur, producing transient structural defects with lifetimes on the order of nanoseconds. The lifetime and size of these transient pores are influenced by external factors such as temperature and electric field strength.

Loss of Membrane Integrity

Many forms of trauma can disrupt the barrier function of the cell membrane. Loss of membrane integrity occurs in tissues at supraphysiologic temperatures (i.e., thermal burns), with very intense ionizing radiation exposure, in frostbite, in barometric trauma, and with exposure to strong electrical forces. Resuscitated victims of major trauma often experience ischemia-reperfusion injury in which the integrity of the cell membrane is compromised due to oxidative damage to the membrane lipids.[3] Reactive-oxygen-mediated membrane breakdown is the mechanism of acute necrosis induced by high-dose radiation.[4] Under freezing conditions, ice nucleation can lead to mechanical disruption of the membrane,[5] while sudden changes in very strong barometric pressures can result in acoustic disruption of the membrane. Electrical shock is the paradigm for necrosis mediated primarily by membrane permeabilization.

The ability of an electric field to disrupt membrane integrity has been exploited for the delivery of genes and drugs.[6–8] The first demonstration of gene transfer into rat cells using electroporation was made in 1982,[9] and the first clinical study on the use of electroporation to increase uptake of a chemotherapeutic agent in tumors was reported in 1993.[10] As in the case of electric-shock trauma, electroporation occurs when an applied external field exceeds the capacitance of the cell membrane. This leads to the formation of what one hopes will be transient pores, allowing the cell to be loaded with different types of molecules, from radiotracers[11] and drugs[12,13] to RNA[14] and DNA,[9,15] through either simple diffusion or electrophoretically driven processes. Depending on the particular cell type and the type of molecule to be delivered, some systems require the use of high electric fields and/or long pulses to achieve reasonable loading yield, but at the expense of survivability of the cell due to the inability of the membrane to reseal.

Under these scenarios, ion pumps cannot keep pace with the increased diffusion of ions across the membrane. As a result, the cell's metabolic energy is quickly exhausted, leading to biochemical arrest and necrosis. Defects formed in the membrane can be further stabilized by membrane proteins anchored in the intra- or extracellular space. Chang and Reese[16] have demonstrated that stable defects—"pores" in the range of 0.1 μm—occur in electroporated membranes. In other cases, the translateral motion of lipids, normally restricted by anchored proteins, may cause the membrane to bleb, or form a sac-like structure that protrudes towards the extracellular space, due to the expansion of electroporated membranes. This compromises the local lipid packing and leads to enhanced permeability.

While trauma-induced membrane permeabilization and disruption constitute a

cause of tissue injury, the same process can be used therapeutically for effective gene and drug delivery. The commonality between the two suggests that the rescue of cells whose membranes are structurally damaged in the case of trauma, and the enhancement of cell survival in electroporation-mediated gene and drug delivery, can be achieved by the design and administration of a biomaterial capable of sealing structurally compromised membranes.

Surfactant Sealing of Cell Membranes

Under normal circumstances, fusogenic proteins, which are capable of fusing lipid membranes together, induce membrane sealing following exocytosis, the process whereby intracellular materials are transported to the extracellular space by the formation of vesicles and by vesicles pinching off the cellular membrane. This is done by creating a low-energy pathway for phospholipids to flow across the defect or to induce fusion of transport vesicles to plasma membranes. When the cell is subjected to insults like electric shock, this normal pathway may be compromised and the natural sealing process can be impeded. It has been demonstrated that membrane sealing can be accomplished by surfactants such as poloxamers and poloxamines. Poloxamers and poloxamines belong to a class of water-soluble triblock copolymers often abbreviated as PEO-PPO-PEO, with PEO and PPO representing poly(ethylene oxide) and poly(propylene oxide), respectively. The PEO chains are hydrophilic, due to their short carbon unit between the oxygen bridges, whereas the PPO center is hydrophobic, due to the larger propylene unit (see Structure **1**). Commercially available poloxamers and poloxamines have both PEO chains of similar length in a particular copolymer. The lengths of the hydrophilic and the hydrophobic chains and their length ratios can vary tremendously, forming a large group of copolymers widely used in industrial applications as emulsifying, wetting, coating, stabilizing, lubricat-

ing, and foaming agents.[17] The poloxamer series covers a range of liquids, pastes, and solids, with molecular weights varying from 1100 to about 14,000. The poloxamine series is slightly different from the poloxamer series in that the hydrophobic center consists of two tertiary amino groups each carrying two PPO chains of equal length and each followed by a PEO chain. It is therefore much bulkier than poloxamer.

Poloxamer 188 (P188) has been widely used in medical applications since 1957, mainly as an emulsifier and anti-sludge agent in blood.[18] P188 has an average molecular weight of about 8400 and is prepared from a 1750 average molecular weight hydrophobe (the first two numbers in P188 reflect the 1.8 kDa molecular weight of this unit), and its hydrophile comprises about 80% (reflected in the last digit, 8) of the total molecular weight. Thus, most investigations on the sealing capabilities of surfactants have focused on P188 because of its established medical safety record.

The first demonstration of sealing showed that P188 could prevent cells from losing carboxyfluorescein dye after electroporation.[19] Low-molecular-weight (10 kDa) neutral dextran was unsuccessful in producing the same effect. P188 can seal membrane pores in skeletal muscle cells after heat shock[20] and enhance the functional recovery of lethally heat-shocked fibroblasts.[21] P188 was also shown to protect against glutamate toxicity in rat brain cells,[22] protect embryonic hippocampal neurons against death due to neurotoxic-induced loss of membrane integrity,[23,24] and reduce the leakage of normally membrane-impermeant calcein dye as well as prevent acute necrosis in high-dose-irradiated muscle cells.[4,25] Poloxamine 1107 (P1107), with two hydrophilic blocks linked to the central hydrophobic block on each side, has been shown to reduce testicular ischemia-reperfusion injury,[26] hemoglobin leakage from red blood cells after ionizing radiation,[27] and propidium iodine uptake of white blood cells after high-dose ionizing irradiation.[28] In all of these cases, the observed phenomena have been attributed to surfactant sealing of permeabilized membranes. The effect of P188 infusions in reducing the duration and severity of acute painful episodes of sickle cell disease is currently explained by beneficial surfactant–erythrocyte membrane interactions.[29] Other poloxamers have been shown to increase the uptake of cancer drugs into tumor cells.[30]

Surfactant–Lipid Interactions

This class of triblock copolymers clearly constitutes an effective biomaterial for

membrane sealing. Elucidation of the mechanism by which these polymers help seal damaged membranes should aid the design of suitable polymers for therapeutic purposes. From a design point of view, an ideal sealant should selectively interact with damaged membranes without interfering with intact ones. Moreover, when the membrane structural integrity is restored, there should be an exit mechanism in place for the polymer to depart from the membrane so as not to interfere with the cell healing process. As the native cell membrane is complex, model lipid systems provide excellent platforms for elucidating the molecular mechanism of membrane sealing.

Insertion of Poloxamers into Membranes with Compromised Lipid Packing Density[31]

Although a monolayer at the air–water interface represents only one of the two lipid monolayers, or leaflets, that make up a lipid bilayer, using it to mimic the outer leaflet of the cell membrane provides an effective way to assess how various attributes of the lipid and the copolymer surfactant can affect their interactions. For an intact membrane, the lipids are packed in a way similar to the lipid packing found in a monolayer at about 30 mN/m. Using an insertion assay, where a lipid monolayer is compressed to the bilayer equivalent pressure and the pressure is held constant via a feedback mechanism, we monitor the association of poloxamer with the lipid film. If the poloxamer inserts into the monolayer, the surface pressure will increase; in order to hold the pressure constant, the area has to increase to reverse the effect. Likewise, if poloxamer desorbs from the surface, the surface pressure will drop; an effort to maintain constant surface pressure therefore results in the decrease of the surface area.

At the bilayer equivalent pressure, no insertion is observable for either zwitterionic dipalmitoyl phosphatidylcholine (DPPC) or anionic dipalmitoyl phosphatidylglycerol (DPPG) monolayers.[31] The loss of membrane integrity is mimicked by systematically lowering the lipid packing density (reducing the surface pressure by 2 mN/m at a time). No sign of insertion is observed until the threshold surface pressure of ~24 mN/m is reached,[31] at which point the change in area per molecule (indicative of insertion) is small but significant. When the surface pressure is further reduced to 22 mN/m, there is rapid insertion of P188 into both monolayers.[31] Figure 1 shows such an insertion isotherm for DPPC; the insertion isotherm for DPPG looks similar. Together, these re-
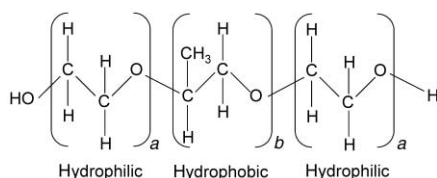
*Structure **1**. Chemical structure of poloxamers. The series of different poloxamers is constituted through varying numbers and ratios for* a *and* b.
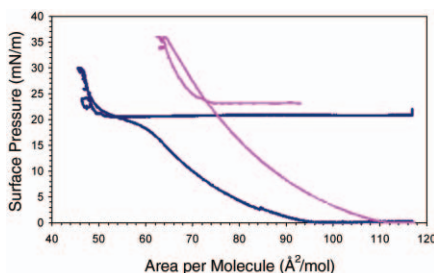
Figure 1. Step-down curves for
dipalmitoyl phosphatidylcholine (DPPC)
(blue curve) and dioleoyl phospha-
tidylcholine (DOPC) (magenta curve)
monolayers in the presence of
Polaxamer 188 (P188). Monolayer films
were compressed to 30 mN/m before
P188 was injected into the subphase.
As no changes in the area per molecule
were observed at 30 mN/m after
10 min, the pressure was lowered by
2 mN/m and held at the reduced
pressure for 10 min to allow for insertion.
This surface-pressure-lowering
procedure was continued until insertion
was noted. P188 inserts into the lipid
films below a critical surface pressure,
and the critical pressure for DOPC is
higher than that for DPPC.[31,32]

sults suggest that P188 only interacts with compromised membranes where the local lipid packing density is reduced, and does not non-specifically insert into membranes whose lipid packing is intact.[31] Moreover, as similar injection results are obtained for DPPC and DPPG monolayers, poloxamer insertion is not influenced by the electro-statics of the lipid head group.[31] Insertion assays using unsaturated zwitterionic di-oleoyl phosphatidylcholine (DOPC) show similar results (see Figure 1), except that insertion occurs at a slightly higher surface pressure, likely arising from the looser packing of the acyl chains in the unsatu-rated lipid.

"Squeeze-Out" of Poloxamer upon Restoration of Membrane Structural Integrity

With the poloxamer inserted into the structurally compromised membrane, what then is the fate of the polymer when the membrane integrity is reestablished (e.g., by cell healing processes)? To address this, we compare compression isotherms for a pure DPPG monolayer, a DPPG monolayer pretreated with 50 μL of P188, and another DPPG monolayer pretreated with 200 μL of P188.[31,32] In the pretreated cases, the lipid monolayer is spread at a low surface density, and the polymer is in-troduced to the subphase when the sur-face pressure π = 0 mN/m. In both P188-pretreated cases, the surface pressure

rises from 0 mN/m to around 20 mN/m upon administration of the poloxamer. The three isotherms, however, are essen-tially identical when the surface pressure of 26 mN/m is reached (Figure 2). This suggests that at higher surface pressure (or tighter packing density), P188 is "squeezed out" of (i.e., excluded from) the film. As the polymer leaves the lipid film, forming most likely micellar structures in the water subphase, the monolayer is left with almost purely DPPG molecules.[31,32] X-ray reflectivity data for lipid monolay-ers with or without P188 in the subphase show identical reflectivity curves at high pressures, further corroborating the "squeeze-out" of P188.[33,34] This interesting phenomenon points to the ability of the cell to eliminate the poloxamer sealant after the normal membrane lipid packing density is regained.

Lipid Corralling by Poloxamers

The immediate increase in the surface pressure from 0 mN/m to 20 mN/m upon the introduction of P188 in the subphase (see Figure 2) indicates the surface activity of the polymer.[31] By physically occupying part of the surface area, the adsorbed poloxamers leave the lipid molecules a smaller surface area to span, and hence help tighten their packing. This tightening of the lipid packing by poloxamer inser-tion is further confirmed using grazing in-
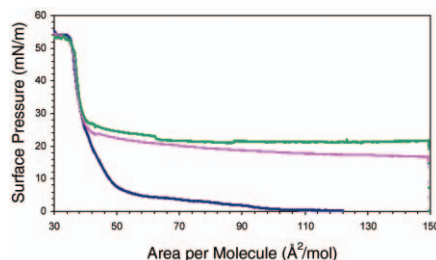

Figure 2. Isotherms of pure DPPG (blue
curve); DPPG pretreated with 50 μL of
P188 (magenta curve); and
DPPG pretreated with 200 μL of
P188 (green curve).[31,32] DPPG is
dipalmitoyl phosphatidylglycerol. The
films were spread at a high area per
molecule. For the P188-treated
systems, the polymer was injected into
the subphase. The surface activity of
the polymer resulted in a rise of the
surface pressure from 0 mN/m to
~20 mN/m upon its introduction into
the subphase. When the films were
compressed to surface pressures of
25 mN/m and greater, the isotherm of
the P188-treated systems overlapped
with that of the pure lipid, indicating
that P188 is "squeezed out" of the film
at surface pressures equal to 25 mN/m
or greater.

cidence x-ray diffraction. Figure 3 shows that Bragg peaks, signifying lipid order-ing, are present at a much larger nominal lipid area when P188 is present in the subphase.[33,34]

Tunability of Poloxamer Insertion Capability

With the three blocks constituting the poloxamer, its ability to insert into mem-branes can be tuned by varying the num-ber of monomers in each block. To test whether the size of the PPO subunit regu-lates the polymer's insertion capabilities, we have investigated the effect of sister poloxamers P108, P238, and P338 with identical PPO/PEO weight percentages but different overall molecular weights.[35] While the higher-molecular-weight poloxa-mers have a larger number of PPO sub-units, the bulkiness of the hydrophobic block limits their ability to insert into the lipid monolayer. Once inserted, however, their large hydrophobic subunits help them maintain their positions in the mono-layer.[35] Together, these results suggest that
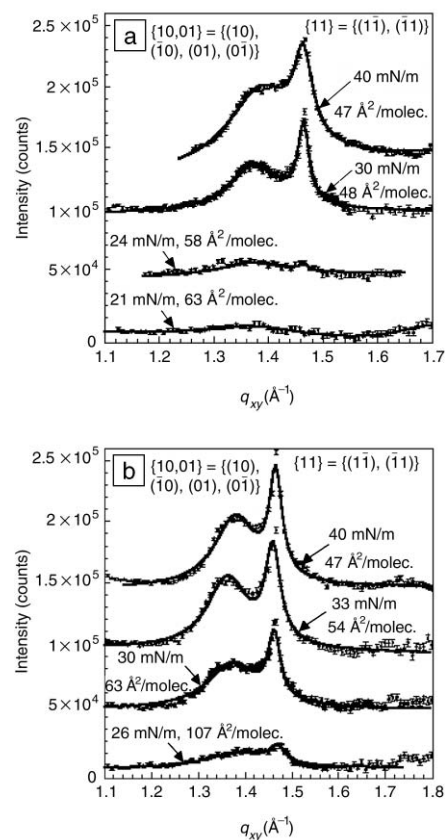

Figure 3. Bragg peaks from grazing
incidence x-ray diffraction on water at
30°C for (a) DPPC and (b) DPPC/P188.
The presence of the polymer forces the
lipids to pack more tightly.[33,34]

the energy barrier for poloxamer insertion is different from that for poloxamer squeeze-out. Increasing the hydrophobic: hydrophilic ratio while keeping the overall molecular weight constant, on the other hand, enhances the poloxamer's ability to insert into lipid membranes.

Conclusion

Poloxamer has the ability to insert into the damaged region of a membrane where the local lipid-packing density is reduced. By doing so, it helps increase the local packing density and thus re-establish the barrier function of the membrane. The incapability of the poloxamer to remain in the lipid film when the normal bilayer packing density is restored provides a graceful exit mechanism for the polymer.

The results discussed here are only the first step toward understanding lipid/poloxamer interactions in the context of membrane sealing. Many questions regarding the molecular mechanism of this sealing action remain largely unknown. A thorough understanding of the structure–activity relations of these polymers is clearly needed in order to better design and develop them as sealing agents. Not only will these engineered copolymers be important materials for therapeutic treatment in medical trauma, they will also be beneficial for improving cell survivability in the delivery of genes or drugs into cells using electroporation.

Acknowledgments

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