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PEO-PPO diblock copolymers protect myoblasts from hypo-osmotic stress *in vitro* dependent on copolymer size, composition, and architecture

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Abstract

Ploxamer 188, a triblock copolymer of poly(ethylene oxide) (PEO) and poly(propylene oxide) (PPO), protects cellular membranes from various stresses. Though numerous block copolymer variants exist, evaluation of alternative architecture, composition, and size has been minimal. Herein, cultured murine myoblasts are exposed to the stresses of hypotonic shock and isotonic recovery, and membrane integrity was evaluated by quantifying release of lactate dehydrogenase. Comparative evaluation of a systematic set of PEO-PPO diblock and PEO-PPO-PEO triblock copolymers demonstrates that the diblock architecture can be protective *in vitro*. Short PPO blocks hinder protection with >9 PPO units needed for protection at 150 μ M and >16 units needed at 14 μ M. Addition of a *tert*-butyl end group enhances protection at reduced concentration. When the end group and PPO length are fixed, increasing the PEO length improves protection. This systematic evaluation establishes a new *in vitro* screening tool for evaluating membrane-sealing amphiphiles and provides mechanistic insight to guide future copolymer design for membrane stabilization *in vivo*.

Abstract

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SUPPORTING INFORMATION

NMR, SEC, MALDI-TOF, DLS, and LDH release at initial isotonic buffer solution. The supporting information is available free of charge on the ACS publications website.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Disclosure

J.M.M. is on the scientific advisory board of and holds shares in Phrixus Pharmaceuticals Inc., a company developing novel therapeutics for heart failure.

Keywords

diblock copolymer; poly(ethylene oxide); poly(propylene oxide); poloxamer; membrane sealant; hypo-osmotic stress

1. Introduction

Amphiphilic block copolymers consist of covalently bonded hydrophilic and hydrophobic blocks. Their amphiphilic nature provides these molecules with remarkable properties and potential for biomedical and pharmaceutical applications. A representative class of amphiphilic block copolymers is triblock copolymers formed from poly(ethylene oxide) (PEO) and poly(propylene oxide) (PPO), also known as Pluronics or poloxamers¹. Poloxamers have been used as versatile biomaterials such as thermosensitive hydrogels^{2,3}, drug delivery carriers^{4,5}, and chemosensitization agents for cancer therapy^{6–8}. Poloxamer 188 (P188), an 8400 g/mol PEO-PPO-PEO triblock copolymer comprising two segments of 75 PEO units on either side of a segment with 30 PPO units, has demonstrated membrane stabilization characteristics in multiple scenarios.

In dystrophic mice, P188 improved ventricular geometry and protection from acute cardiac failure⁹, cardiac function¹⁰, respiratory tidal volume and minute volume¹¹ and protection from cardiac ischemia/reperfusion injury¹² as well as improved isometric contraction force¹³ and protected skeletal muscles from mechanical stress *in vivo*¹⁴. P188 infusion to dystrophic dogs reduced myocardial fibrosis, prevented left-ventricular remodeling, and reduced elevation of serum cardiac troponin and natriuretic peptide¹⁵. In dysferlin-deficient mice, P188 reduced muscular atrophy¹⁶ and protected from ischemia/reperfusion injury¹². P188 reduced membrane leakage, apoptosis, and loss of mitochondrial polarization, but had no impact on lipid peroxidation, upon simulated ischemia/reperfusion in cardiac myocytes¹². P188 reduced infarct volume, motor deficits, and death after ischemia/reperfusion in mice¹⁷; improved recovery to myocardial infarction¹⁸ and cardiac arrest in pigs;¹⁹ prolonged survival and reduced tissue damage upon hypotensive resuscitation in rats;²⁰ and enhanced survival time after severe hemorrhage in pigs.²¹ In mice, P188 reduced intracerebral hemorrhage injury volume and reduced neurological symptoms;²² improved blood-brain barrier integrity after traumatic brain injury;²³ and resealed cells in the cortex and hippocampus after controlled cortical impact.²⁴ In culture, P188 reduced necrosis and apoptosis²⁵ as well as improved viability, reduced enzyme leakage, and improved integrity of mitochondrial and lysosomal membranes following mechanical stress or ischemia.^{26,27} After traumatic mechanical impact, P188 preserved glycosaminoglycan content in rabbits²⁸

and explanted cartilage;²⁹ improved chondrocyte viability in rabbits³⁰ and bovine explants;³¹ and preserved anatomic spinal cord integrity in guinea pigs.³² In rats, P188 also reduced alveolar cell death upon ventilation;³³ aided viability of skeletal muscle cells after ionizing radiation exposure;^{34,35} aided membrane integrity of electroporated muscle;^{36,37} and improved response to second-degree burns.³⁸

There is tremendous opportunity for mechanistic insight and enhanced performance because this array of protective effects has been achieved with limited comparative evaluation of polymers varying in composition, size, or architecture, despite the multitude of alternatives³⁹. A PEO-PPO-PEO triblock copolymer with the same 80% PEO content as P188 but 74% higher molecular weight exhibited improved protection of dystrophic myofibers in culture and an alternative optimal delivery (intraperitoneal) for *in vivo* protection.¹⁴ In resealing cortical and hippocampal cells after controlled cortical impact, P188 was superior to a 70% PEO, 13 kDa PEO-PPO-PEO triblock copolymer but inferior to 60% vinylpyrrolidone – 40% vinylacetate random copolymer VA64.²⁴ Conversely, the 70%, 13 kDa PEO-PPO-PEO triblock copolymer was superior to P188 in maintaining cell viability after transient photoacoustic permeabilization for molecular delivery.⁴⁰

Physical analysis of model monolayer and bilayer lipid systems has been pursued to provide insight. A subset of poloxamers with different lengths of PEO and PPO blocks were used to vary overall hydrophilicity and molecular weight. Hydrophilic triblock copolymers were likely to adsorb on the surface of lipid bilayer, while hydrophobic counterparts appeared to be inserted into the bilayer^{41,42} and increased the permeability of the lipid membrane^{43–46}. Similar results were obtained from molecular dynamics simulations^{46,47}. At fixed PEO/PPO ratio, it was experimentally observed that increasing molecular weight enhanced interaction between the polymer and lipid, retarding the lipid diffusion in turn^{48,49}. Conformations of the poloxamers interacting with lipid membranes have been studied by synchrotron X-ray scattering^{50,51} and computational simulation⁵². When the PPO length is comparable with lipid bilayer thickness (*i.e.* 39 monomer units), the block copolymer was fully inserted and spanned the bilayer. In contrast, poloxamers having PPO length less than the bilayer thickness interacted only weakly with the lipid membrane^{50,51}. A phase diagram of conformational states as a function of PPO length and hydrophilicity of the poloxamer chain was obtained by generic Monte Carlo simulation⁵².

As most of the previous studies were restricted to commercially available triblock copolymers, systematic and detailed study – to understand the role of block copolymer architecture on membrane protection – has been limited. Recently, we discovered that a PEO-PPO diblock copolymer, with a composition and size corresponding to essentially one half of P188, can protect a model lipid membrane⁵³ as well as skeletal muscles from mechanical stress *in vivo*⁵⁴. Also, PEO-poly(butylene oxide) diblock copolymers (73% hydrophilic; 2700 g/mol) were shown to effectively interact with 1,2-dipalmitoyl-sn-glycero-3-phosphocholine monolayers with surface activity increasing with longer poly(butylene oxide) segments.⁵⁵ These results suggest that membrane protection is not limited to only triblock copolymer, but there is large room to tailor molecular architecture to improve the protection efficacy of block copolymers. Systematic biological evaluation of molecules varying in composition, size, and architecture could enlighten the molecular

mechanism of membrane interaction and protection. Such systematic analysis requires efficient synthesis of copolymer variants, which would be facilitated by the use of a diblock, rather than a triblock, architecture. Furthermore, measuring the membrane protection ability with various block copolymers requires an efficient evaluation method that can be correlated with *in vivo* biological studies described above. One such method is an *in vitro* cellular assay where a cell membrane is damaged by hypo-osmotic stress and isotonic recovery, both of which are efficient methods to provide membrane stress⁵⁶. Herein, we comparatively evaluate a series of PEO-PPO and PEO-PPO-PEO copolymers for protection of murine myoblasts from hypo-osmotic stress and isotonic recovery.

2. Materials and Methods

2.1. Materials

Poloxamer 188, 338, 237, and 407 (Pluronic F68, F108, F87, and F127 respectively) were generously provided by BASF (Wyandotte, MI). PEO₇₅PPO₁₆-methyl (E₇₅P₁₆m) was purchased from Polymer Source (P1862-EOPO, Montreal, Quebec). PEO 8K homopolymer was purchased from EMD Millipore (Billerica, MA) and PEO 4K was purchased from Alfa Aesar (Ward Hill, MA). Methoxy polyethylene glycols (3300 g/mol purchased from Polymer Source and 2000 and 5000 g/mol from Sigma) were used to make PEO-PPO diblocks with hydroxyl end group on PPO block. All polymers were used as received. High purity (>99.0%) monomers (propylene oxide and ethylene oxide), potassium *tert*-butoxide, and 18-crown-6 ether for anionic polymerization were purchased from Sigma. Salts for buffer solution (sodium chloride, potassium chloride, calcium chloride, magnesium chloride, and HEPES; bioreagent grades for all) and Triton X-100 (bioreagent grade) were also purchased from Sigma.

2.2. Block copolymer synthesis

Polyethylene oxide-*b*-polypropylene oxide (PEO-PPO) were prepared by anionic polymerization. Experimental setup for anionic polymerization and monomer purification is described elsewhere^{53,57,58}. All reactions were performed under inert conditions (air and water free) and alumina column-dried tetrahydrofuran (THF) was used as the solvent. To synthesize a diblock copolymer with a *tert*-butyl end group on PPO, the PPO block was first synthesized by initiating and propagating propylene oxide using potassium *tert*-butoxide as the initiator at room temperature. 18-crown-6 ether, at 2:1 molar ratio to the initiator, was added to the reaction to narrow dispersity and increase conversion of propylene oxide^{59,60}. After 48 hours, the reaction was terminated with acidic methanol (1:10 37 w/w% hydrochloric acid:methanol). Potassium and crown ether complexes were removed by iterative filtration, solvent removal, and dissolution in fresh THF until a clear solution was obtained. The PEO block was grown by reinitiating the PPO block with potassium naphthalenide at 40 °C. The reaction was terminated with acidic methanol after 20 hours. After termination, diblock copolymer was repeatedly dissolved in fresh solvent and filtered, then further purified by dialysis.

To synthesize PPO with a hydroxyl end group, methoxy-PEO was reinitiated with potassium naphthalenide followed by propagation of propylene oxide at 35 °C. 18-crown-6 ether was

added to increase conversion and narrow dispersity. After 45 hours of reaction, the reaction was terminated with acidic methanol. The final diblock copolymer was purified by the same procedure for the diblock copolymer with *tert*-butyl end group on PPO.

2.3. Molecular Characterization

Molecular weight and block composition of the polymers were determined from peak analysis and integration on the ^1H NMR spectrum of polymers in deuterated chloroform. Spectra were obtained on a 400 MHz Bruker Avance III HD nanobay AX-400. ^1H NMR spectra along with peak assignments are shown in Figure S1. Dispersities of the polymers were obtained from size exclusion chromatography (SEC), which was pre-calibrated with polystyrene standards, with THF as the effluent. Molecular weights and dispersities of PEO homopolymers were measured with matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer (TOF 5800, Sciex, Washington, DC) using α -cyano-4-hydroxycinnamic acid (Sigma) as a matrix. SEC chromatograms and MALDI-TOF spectra of the polymers are shown in Figure S2. Obtained molecular weights, numbers of monomer units in each block and dispersity are summarized in Table 1.

Dynamic light scattering (DLS) measurements were performed on a Brookhaven BI-200SM DLS (Brookhaven Instruments Corporation, Holtsville, NY) at 90° scattering angle using a 633 nm HeNe laser operating at 20 mW. Block copolymers in iso-osmotic buffer were incubated for 30 minutes at 37°C before the measurements. Scattering data were collected for 2 minutes from 600 ns to 0.1 s delay times at 37°C . Hydrodynamic radius and dispersity were obtained by fitting autocorrelation function by the method of cumulant.

2.4. Cell culture

Mouse muscle myoblasts (C2C12; American Type Culture Collection, Manassas, VA) were grown in proliferation media containing high-glucose DMEM (Gibco Invitrogen, Grand Island, NY), 20% fetal bovine serum (Gibco Invitrogen, Grand Island, NY), and 1% penicillin/streptomycin (Gibco Invitrogen, Grand Island, NY) at 37°C humidified atmosphere of 5% CO_2 . Before cells reached 70% confluency, they were split in order to prevent partial differentiation caused by cell-cell contact. The cell culture media was replaced every two days. Myoblasts after more than 10 passages that became resistant to differentiation were used for hypo-osmotic stress and isotonic recovery assay.

2.5. Hypo-osmotic stress and isotonic recovery assay

Approximately 10^4 cells were added to each well of a 96-well plate and grown to 70–90% confluence. When ready to use, the cells were transferred from the growth media to a 330 mOsm isotonic buffer (140 mM NaCl, 5 mM KCl, 2.5 mM CaCl_2 , 2 mM MgCl_2 , and 10 mM HEPES). Using a method modified from Martindale *et al.*¹² and Houang *et al.*¹⁴, after incubating for 30 min, the cells were subjected to hypo-osmotic stress by changing the iso-osmotic buffer with 134 or 74 mOsm hypotonic buffer (composition equivalent to 330 mOsm solution but with NaCl reduced to 50 or 20 mM) for 50 min. The cells were subsequently re-equilibrated in an isotonic buffer for 30 min, named as isotonic recovery. After isotonic recovery, cells were lysed with 0.01% Triton X-100 to obtain the remaining amount of lactate dehydrogenase (LDH) per well. To evaluate polymer protection ability, the

polymers were added in every step during the whole protocol except the lysis step with Triton X-100. In every solution exchange step, the solution removed from the well was collected and stored at 4 °C before being used for enzyme release assay. The LDH release at each step was normalized to the total amount of LDH per well. The fractional LDH release in the presence of the polymer was further normalized to the fractional LDH release without the polymer (non-treated).

2.6. Enzyme release assay

LDH release has been extensively validated as a metric for membrane integrity and loss of cellular viability.^{61,62} The amount of LDH in solution was measured by incubating sample aliquots with lactate and nicotinamide adenine dinucleotide (LDH Assay, Pointe Scientific Inc., Canton, MI) at 37 °C. The rate of generation of reduced nicotinamide adenine dinucleotide was determined by measuring absorbance at 340 nm.

2.7. Statistics

All results are expressed as mean $\pm$ standard error. Multigroup comparison for LDH release experiments were assessed using one-way analysis of variance with Tukey *post hoc* test and $p < 0.05$ considered to be statistically different. All statistical analysis was carried out using Prism (GraphPad Software).

3. Results

3.1. Cellular assay

A cellular assay, using C2C12 murine myoblasts, assessed LDH release resulting from two types of injury, namely hypo-osmotic stress and isotonic recovery (Figure 1). In a control experiment using iso-osmotic buffer throughout, only moderate LDH release is observed (Figure 1(B)). Conversely, when the osmolality of the solution is decreased, the LDH release increased nominally ($p > 0.05$) to $10 \pm 0.9\%$ for 134 mOsm and significantly ($p < 0.001$) to $23 \pm 1\%$ for 74 mOsm, due to hypo-osmotic stress (Figure 1(C) and (D)) in contrast to the control experiment with the iso-osmotic buffer ($5 \pm 0.7\%$). After isotonic recovery, more LDH released than when under hypo-osmotic stress itself (i.e., $32 \pm 1\%$ for 134 mOsm and $38 \pm 2\%$ for 74 mOsm vs. $12 \pm 0.6\%$ for iso-osmotic; $p < 0.001$ for both hypo-osmotic vs. iso-osmotic comparison) (Figure 1(C) and (D)). This increase of LDH release indicates that cell membranes were more compromised after isotonic recovery than hypo-osmotic stress itself. We can exclude the possibility that incubating long time in buffer solution may lead to the increase of the LDH release after isotonic recovery because the corresponding LDH release in control experiment was small ($12 \pm 0.6\%$). We sought to evaluate the protective ability of adding polymers throughout the experiment, except during the lysis with Triton X-100. With this cellular assay, the effect of block copolymer architectures on cell membrane protection was systematically investigated.

3.2. Polymer Synthesis and Characterization

Parameters used in this study were categorized as follows: (1) number of blocks (i.e., triblock or diblock), (2) end group on PPO block (i.e., hydroxyl, methyl, or *tert*-butyl), (3)

PPO block length, (4) PEO block length, and (5) molecular weight. Block copolymers are listed in Table 1 with chemical structures shown in Figure 2.

3.3. Copolymer Architecture

First, we compare the membrane protection efficacy between a triblock P188 ($E_{75}P_{30}E_{75}$) and a diblock analog ($E_{75}P_{16}\underline{m}$) that has the half length of the P188 and methyl end group on PPO block. $E_{75}P_{30}E_{75}$ and $E_{75}P_{16}\underline{m}$ are comparably effective in protecting against hypo-osmotic stress at both 134 and 74 mOsm (Figure 3). The $E_{75}P_{16}\underline{m}$, at high concentration (150 μ M), also protects during isotonic recovery as robustly as $E_{75}P_{30}E_{75}$ ($20 \pm 4\%$ vs. $12 \pm 3\%$ LDH release relative to non-treated samples, respectively, after 134 mOsm hypo-osmotic stress). Unlike the triblock, a reduced concentration (14 μ M) of $E_{75}P_{16}\underline{m}$ is unable to protect membrane during isotonic recovery. Nevertheless, the protective efficacy of the diblock architecture, albeit at higher concentrations, enables its use for efficient evaluation of the impact of copolymer design on membrane protection.

Here, we chose to maintain molar concentration for comparisons. The $E_{75}P_{16}\underline{m}$ provides comparable protection to the triblock at 150 μ M despite reduced mass concentration (Figure 3). Reduced efficacy at 14 μ M may be partially attributable to reduced mass concentration.

3.4. End Group

Based on Haman⁵³ and Houang *et al.*⁵⁴ we hypothesized that diblock architecture could benefit from increased hydrophobicity to favor interaction in the core of the membrane. Thus, we compared protection efficacy of block copolymers having hydroxyl, methyl, or *tert*-butyl end group on PPO block. All five molecules effectively protect against enzyme release during hypo-osmotic stress (Figure 4). After isotonic recovery, LDH release decreased with a *tert*-butyl end group ($E_{48}P_{16}\underline{t}$ and $E_{78}P_{16}\underline{t}$) versus hydroxyl or methyl end group on PPO block ($\underline{m}E_{46}P_{16}$, $\underline{m}E_{75}P_{15}$, and $E_{75}P_{16}\underline{m}$). Most notably, at reduced copolymer concentrations (14 μ M), *tert*-butyl end groups enable protective function, which is lacking in the alternatively terminated counterparts, indicating that the hydrophobic *tert*-butyl end group on the PPO block plays a prominent role to aid the cell membrane protection.

3.5. PPO Length

To complement hydrophobic end group modification, PPO segment length was also varied across a series of PEO lengths. When the PPO end group and PEO length are maintained, longer PPO blocks improve protection during hypo-osmotic stress as well as isotonic recovery for $\underline{m}E_{120}P_{32}$ vs. $\underline{m}E_{120}P_9$ and $\underline{m}E_{46}P_{16}$ vs. $\underline{m}E_{46}P_5$ (Figure 5). Moreover, PEO polymers lacking any PPO are non-functional (Figure 6). Thus, the polymers exhibiting inferior protection to both membrane injuries are those with the shortest PPO segments and without a *tert*-butyl end. Strong protection is observed for both PPO lengths for $E_{43}P_{21}\underline{t}$ vs. $E_{48}P_{16}\underline{t}$ and $E_{87}P_{49}\underline{t}$ vs. $E_{78}P_{16}\underline{t}$ (Figure 5). One anomaly is that $E_{87}P_{49}\underline{t}$ was much less protective than $E_{78}P_{16}\underline{t}$ at 150 μ M after isotonic recovery whereas the difference of protection efficacy was not distinct at 14 μ M concentration.

To assess conformational state, dynamic light scattering was performed on block copolymer solutions at 37 °C (Figure S3). Only E₈₇P₄₉t showed evidence for micelle formation among the diblock copolymers used in this study. Hydrodynamic radius (R_H) of E₈₇P₄₉t micelle at 150 μ M is 9.1 ± 0.5 nm, which is significantly larger than the estimated R_H of Gaussian conformation (ca. 1.9 nm).

As extensive hydrophobicity can also drive permeabilization^{45,46,63}, copolymers were evaluated for LDH release upon copolymer addition prior to hypo-osmotic stress. No significant enzyme release was observed for any tested polymers (Figure S4).

3.6. PEO Length

The diblock architecture hypothetically enables simultaneous interaction of the PPO segment (and *tert*-butyl end group when present) with the hydrophobic core of the membrane and the PEO segment with the hydrophilic head groups. We hypothesize that extension of the PEO segment would enable increased interaction but would also increase conformational freedom of the unbound state thereby elevating the entropic cost of binding. Thus, the optimal PEO length is not immediately evident. To investigate the PEO length effect, diblock copolymers were synthesized with a consistent 16 PPO units and a *tert*-butyl end group along with 48, 78, or 182 PEO units (corresponding to 70, 80, or 90 wt% PEO, respectively). All three diblock copolymers effectively protect against enzyme release after hypo-osmotic stress and isotonic recovery at 150 μ M (Figure 6). Differences of protection efficacy among the diblock copolymers become evident at 14 μ M: as PEO length increases, protection efficacy of the diblock copolymers increases. In contrast to diblock copolymers, PEO homopolymers are unable to protect the cell membrane at all after isotonic recovery. Note here that the mass concentrations are appreciably different and all show substantial protection at the higher concentration (150 μ M), thus it is not possible to identify the relative protective efficacies at comparable mass concentrations.

3.7. Molecular Weight

Impact of molecular weight effect was assessed by comparing two sets of triblock copolymers (Figure 7). One set of the polymers has ca. 80 wt% PEO: 8400 g/mol (E₇₅P₃₀E₇₅) vs. 14600 g/mol (E₁₄₀P₄₄E₁₄₀); the other set has ca. 70 wt% PEO: 7800 g/mol (E₆₄P₃₇E₆₄) vs. 12100 g/mol (E₁₀₁P₅₆E₁₀₁). Protection efficacy of E₁₄₀P₄₄E₁₄₀ and E₁₀₁P₅₆E₁₀₁ are less than the efficacy of their counterparts at 150 μ M after isotonic recovery. DLS confirmed that E₁₄₀P₄₄E₁₄₀ and E₁₀₁P₅₆E₁₀₁ form micelles at 150 μ M whereas the other two do not. R_H of the micelles are 7.7 ± 0.5 nm for E₁₄₀P₄₄E₁₄₀ and 10.7 ± 0.4 nm for E₁₀₁P₅₆E₁₀₁. All triblocks effectively reduce enzyme release with 14 μ M concentration under hypo-osmotic stress and isotonic recovery. Based on the results, increasing molecular weight may not be always beneficial because of micelle formation.

3.8. Overall

Overall membrane protection efficacies of the diblock copolymers are summarized in a composition map in terms of PPO and PEO molecular weight (Figure 8). Significant membrane protection to isotonic recovery is only observed with a hydrophobic segment of at least 900 g/mol (16 PPO monomer units), which requires a *tert*-butyl group. Without *tert*-

butyl, greater than 900 g/mol of PPO was needed with around 1800 g/mol (32 PPO monomer units) being sufficient. Molecules were capable of protective efficacy over the entire range of hydrophilic segment length tested (around 2000 to 12000 g/mol which correspond to 43 to 280 PEO monomer units) provided that sufficient hydrophobicity was present, with an observed functional range of 57% to 90% hydrophilic. The greatest membrane protection was observed for E₁₈₂P_{16t}, a 8900 g/mol, 90% hydrophilic diblock copolymer.

3.9. Concentration Titration

The sensitivity of protection was evaluated by titrating several copolymers from 0–90 μ M concentrations. For P188, P338, and E₇₅P_{16t}, 14 μ M concentration is needed to reduce LDH release to half compared to non-treated control in isotonic recovery. However, almost 7-fold increase of concentration (90 μ M) is required for E₇₅P_{16m} to reduce LDH release to half (Figure 9).

4. Discussion

The *in vitro* cellular assay implemented in this study is able to efficiently report on the functional outcome for a collection of copolymer variants. This method enables insight into copolymer-membrane interaction and provides a guide for improved molecular design. The ability of PEO-PPO diblock copolymers to effectively protect damaged membranes from enzyme leakage *in vitro* demonstrates that the second hydrophilic PEO segment – present in triblock P188 – is not required for efficacy, in agreement with Houang, *et al.*⁵⁴ This result, and the impacts of composition and size, are consistent with a model in which the hydrophobic segment anchors the copolymer to the membrane while the hydrophilic segment interacts with the lipid head groups and the aqueous extracellular environment.

The strong benefit of a highly hydrophobic *tert*-butyl terminus on the PPO segment (Figure 4) supports the notion that strong membrane anchoring is needed for protection. The necessary PPO length required for potent membrane protection is lowered by incorporating a hydrophobic *tert*-butyl end group to the PPO block (Figures 4 and 8). The minimum required length of PPO with hydroxyl end group is 20–32 monomer units, corresponding to 1200–1900 g/mol. In contrast, with a *tert*-butyl end group, the minimum PPO length drops to below 16 monomer units, which is 928 g/mol.

The membrane interaction hypothesis is further supported by the lack of efficacy observed for diblock copolymers with short PPO segments (mE₄₆P₅ and mE₁₂₀P₉; Figure 5) in both low and high concentrations. Thus, there is a lower limit for the hydrophobic segment to provide membrane anchoring at sensitive μ M concentrations: PPO₉ is insufficient (with PEO₁₂₀) while PPO_{16-*tert*-butyl} or PPO₃₂ is sufficient. Above the lower limit, the polymers have improved protection efficacy with increasing PPO length at low concentration (Figure 5). Yet with sufficient PPO length (i.e., 16 PPO monomer units with *tert*-butyl end group), superiority of copolymers with longer PPO blocks is diminished, if not inverted, at high concentrations (150 μ M). For example, although both polymers can protect the membrane, fractional LDH release drops significantly with concentration for E₇₈P_{16t}, while the efficacy does not improve substantially for E₈₇P_{49t} (Figure 5). Notably, E₈₇P_{49t} forms micelles at

high concentration at physiological temperature (37 °C), which still enable moderate protective efficacy. Moreover, triblock copolymer predominant in PPO causes membrane permeability^{45,46,63} (i.e., PEO₂-PPO₁₆-PEO₂ or PEO₂₅-PPO₄₀-PEO₂₅) because of extensive hydrophobicity. Collectively, these data suggest upper and lower limits on PPO length for protective efficacy. Provided the hydrophobic segment satisfies the lower limit, protection efficacy increases with PEO length as evidenced from comparison of E₄₈P_{16t} vs. E₇₈P_{16t} vs. E₁₈₂P_{16t} (Figure 6). The enhanced protection efficacy might be attributed to the increased interaction between PEO chain and lipid head groups. Previous studies report interaction between liposomes and E₁₉₅ in that E₁₉₅ could prevent liposome degradation from peroxidation stress but requires 5–10 fold higher polymer/lipid ratio than that of E₇₅P₃₀E₇₅⁴¹. Another study, probing local hydration dynamics on liposome surface, observed weak interaction of E₁₉₅ with liposomes at high polymer concentration⁴². Our results show that E₁₉₅ did not protect myoblast against hypo-osmotic stress and isotonic recovery at the same concentration as E₇₅P₃₀E₇₅. Overall, the hydrophobic block may function to increase the local concentration of the hydrophilic block, which stabilizes the membrane. Based on the comparison of homopolymer to diblock, we hypothesize that the hydrophobic PPO block is partially inserted into the lipid bilayer whereas the hydrophilic PEO block interacts with the lipid head groups, cellular surface, and surrounding aqueous environment. There is a possibility that such physical contact can affect biological function related to membrane protection. We also cannot exclude the possibility that the polymers interact with other elements of the cell membrane such as proteins. Further study would be needed to validate the hypothesis.

Notably, at highly elevated concentration (3 mM) PEO with high molecular weight (15000–20000 g/mol) were able to protect cardiac myocyte membrane from hypoxia- and reoxygenation injury⁶⁴. The hydrophilic block may also provide direct stabilization but requires a substantial hydrophilic component to properly position the block at the membrane interface to avoid permeabilizing the membrane. The assay implemented herein is not designed to explicitly parse these important fundamental differences. Rather it is able to efficiently report on the functional outcome for a collection of copolymer variants, which provides insight on the copolymer-membrane interaction and guides improved molecular design.

Three pairs of molecules provided molecular weight comparisons: E₇₅P₃₀E₇₅ vs. E₁₄₀P₄₄E₁₄₀ triblocks, E₆₄P₃₇E₆₄ vs. E₁₀₁P₅₆E₁₀₁ triblocks, and E₄₃P_{21t} vs. E₈₇P_{49t} diblocks. The low and high molecular weight molecules exhibited comparable protection at low concentration whereas the smaller molecules were superior to their larger counterparts at high concentrations, although the trend is not prominent in triblocks. Notably, the inferior molecules – E₁₄₀P₄₄E₁₄₀, E₁₀₁P₅₆E₁₀₁, and E₈₇P_{49t} – have the longest PPO length among the polymers used in this study and are the only three molecules to form micelles at 37 °C. We hypothesize that single polymer chains provide membrane protection efficacy by interacting with the cell membrane. Micelle formation would reduce the net amount of free polymer chains that would otherwise interact with the cell membrane, leading to the decreased efficacy.

The current study was performed on C2C12 mouse muscle myoblasts. The generalizability of these results across other cell types will need to be tested. As the polymers bind to model lipid membrane such as lipid bilayers and liposomes^{41,49}, the polymers are likely to interact significantly with lipids in the cell membrane. To the extent that lipid compositions across mammalian cell types are reasonably consistent^{65,66} and specific protein interactions are not expected, we hypothesize that these results will extend to additional cell types.

The membrane protection effects of varying copolymer size, composition, and architecture can be compared across the current myoblast LDH release assay and concurrent studies on liposomal integrity under free radical challenge⁵³ and maintenance of muscle torque in dystrophin-deficient (*Dmd^{mdx}*) mice under mechanical stress⁵⁴. A slight protective efficacy of diblock E₇₅P₁₆ was also observed in *mdx* mice upon intraperitoneal injection though not upon subcutaneous injection, inverse of the result for triblock E₇₅P₃₀E₇₅.

Pharmacokinetic and pharmacodynamic considerations could also account for this difference. Diblock E₇₅P₁₆ also effectively protected liposomes from challenge by free radical-generating 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH). Yet, unlike the current myoblast isotonic renormalization assay, diblock was superior to triblock E₇₅P₃₀E₇₅ in the liposomal assay. As in the current *in vitro* myoblast protection assay, the addition of a *tert*-butyl group to the end of the PPO segment was also beneficial in the *mdx* mouse assay. Conversely, *tert*-butyl addition was insufficient to provide protection in the liposomal/AAPH assay. Also, short PPO lengths hindered protection in the current myoblast isotonic recovery assay, as well as the liposomal/AAPH assay. Both low and high molecular weight 80% hydrophilic triblock copolymers were functional in protecting *mdx* mice albeit with different optimal delivery routes (subcutaneous and intraperitoneal, respectively), as in the triblock/diblock architecture comparison.

In summary, there is reasonable agreement between the abilities of the polymers to protect myoblasts from isotonic recovery and to maintain muscle torque in dystrophin-deficient mice upon mechanical stress⁵⁴. Yet considerations must be taken for pharmacodynamics and pharmacokinetics regarding differential modes of delivery. Concordance was less robust between the myoblast assay and protection of liposomes from free radical challenge. Differences could result from variations in membrane composition (e.g. lipid distribution, cholesterol, proteins), mode of stress (isotonic recovery vs. free radical-driven lipid peroxidation), and protection metric (LDH release vs. liposome reshaping). Reduction of radicals reaching liposomal fatty acyl chains may be mechanistically different from protection of cellular membrane stability as measured by enzyme release or survival. Future studies will further validate and extend this *in vitro* platform to guide *in vivo* membrane protection.

The efficiency of chemical synthesis of diblocks and cellular analysis enable further broadening of chemical space. Future experiments could include additional hydrophilic monomers, hydrophobic monomers (note that 60% vinylpyrrolidone – 40% vinylacetate copolymer VA64 effectively resealed cells after controlled cortical impact²⁴), termini, hydrophilicity, molecular weights, and architectures.

5. Conclusion

We have systematically investigated the effect of block copolymer designs on cell membrane protection with an *in vitro* cellular assay, in which cultured murine myoblasts are exposed to hypotonic stress and isotonic recovery followed by evaluation of membrane integrity via LDH release. Comparison of PEO-PPO diblock and PEO-PPO-PEO triblock copolymers reveals that the diblock copolymer can also protect the cell membrane. Significant membrane protection to isotonic recovery is only observed with a hydrophobic segment of at least 16 PPO monomer units as well as a *tert*-butyl end group. Without *tert*-butyl, greater than 16 units of PPO were needed with around 32 PPO monomer units being sufficient. With sufficient hydrophobic segment, increasing protection efficacy was observed with increasing PEO length. Overall, we have found that hydrophobicity controlled by PPO length and end group is a critical factor to determine membrane protection efficacy, in addition to the PEO length. The result is consistent with a model in which the hydrophobic segment anchors the copolymer in the membrane while the hydrophilic segment interacts with the lipid head groups and the aqueous extracellular environment. The assay implemented herein is able to efficiently report on the functional outcome for a collection of copolymer variants, which provides insight on the copolymer-membrane interaction and guides improved molecular design.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

EO	ethylene oxide
PO	propylene oxide
PEO	poly(ethylene oxide)
PPO	poly(propylene oxide)
P188/P338/P237/P407	Pluronic 188/338/237/407 triblock copolymers
E₇₅P₁₆m	PEO ₇₅ -PPO ₁₆ -methyl diblock copolymer
E_xP_yt	PEO _x -PPO _y -(<i>tert</i> -butyl) diblock copolymers
mE_xP_y	methyl-PEO _x -PPO _y diblock copolymers
C2C12	mouse muscle myoblast
LDH	lactate dehydrogenase
DLS	dynamic light scattering

R_H	hydrodynamic radius
AAPH	2,2'-azobis(2-amidinopropane) dihydrochloride
SEC	size exclusion chromatography
THF	tetrahydrofuran
MALDI-TOF	matrix-assisted laser desorption/ionization time-of flight

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Figure 1.

In vitro cellular assay with C2C12 myoblast: (A) Scheme of cellular assay with hypo-osmotic stress and isotonic recovery. Cells were cultured in 96-well plates and exposed to isoosmotic solution, hypotonic buffer, and isotonic recovery prior to lysis. Solutions were collected after each step (①–④). (B–D) Fractional LDH release at each step without the polymers. Buffer condition was only varied in the second step: (B) 330 mOsm for control experiment, (C) 134 mOsm and (D) 74 mOsm hypo-osmotic buffer. Average $\pm$ standard error are shown for three independent experiments with 2–6 wells per experiment.

Figure 2.

Chemical structure of polymers: (a) PEO-PPO-PEO triblock copolymer; (b–d) PEO-PPO diblock copolymers with (b) methyl, (c) *tert*-butyl, or (d) hydroxyl end group on PPO block; (e) PEO homopolymer. *m* and *n* are degrees of polymerization of PEO and PPO block, respectively.

Figure 3.

Impact of copolymer architecture on membrane protection. 14 or 150 μM triblock copolymer $E_{75}P_{30}E_{75}$ and diblock copolymer $E_{75}P_{16}M$, which comprises approximately one half of the symmetric triblock, were added to myoblast cultures throughout hypo-osmotic stress and isotonic recovery. LDH release was measured and normalized to non-treated control (buffer-only). Average $\pm$ standard error are shown for 6–8 independent experiments with 3–6 wells per experiment. * indicates $p < 0.05$. ** indicates $p < 0.001$.

Figure 4.

Impact of PPO end group on membrane protection. 14 or 150 μM diblock copolymers – in sets with varying PPO end groups – were added to myoblast cultures throughout hypo-osmotic stress and isotonic recovery. LDH release was measured and normalized to non-treated control (buffer-only). Average $\pm$ standard error are shown for 3–6 independent experiments with 3–6 wells per experiment. * indicates $p < 0.05$. ** indicates $p < 0.001$.

Figure 5.

Impact of PPO length on membrane protection. 14 or 150 μM diblock copolymer pairs with near-matched PEO lengths and varying PPO lengths were added to myoblast cultures throughout hypo-osmotic stress and isotonic recovery. LDH release was measured and normalized to non-treated control (buffer-only). Average $\pm$ standard error are shown for 3–6 independent experiments with 3–6 wells per experiment. * indicates $p < 0.05$. ** indicates $p < 0.001$.

Figure 6.

Impact of PEO length on membrane protection. Diblock copolymers with 16 PPO monomers, a *tert*-butyl end group, and 48, 78, or 182 PEO monomers, as well as PEO homopolymers with 92 or 195 monomer units, were added to myoblasts throughout hypo-osmotic stress and isotonic recovery. Polymer concentrations of 14 or 150 μM were used. LDH release was measured and normalized to non-treated control (buffer-only). Average $\pm$ standard error are shown for 2–5 independent experiments with 3–6 wells per experiment. * indicates $p < 0.05$. ** indicates $p < 0.001$.

Figure 7.

Impact of triblock molecular weight on membrane protection. 14 or 150 μ M triblock copolymers with 80% ($E_{75}P_{30}E_{75}$, $E_{140}P_{44}E_{140}$) or 70% ($E_{64}P_{37}E_{64}$, or $E_{101}P_{56}E_{101}$) PEO content but varying molecular weight were added to myoblast cultures throughout hypo-osmotic stress and isotonic recovery. LDH release was measured and normalized to non-treated control (buffer-only). Average $\pm$ standard error are shown for 2–7 independent experiments with 3–6 wells per experiment. * indicates $p < 0.05$.

Figure 8.

Impact of copolymer architecture, block length, and composition on membrane protection. Plotted are average LDH release (% non-treated) values during isotonic recovery after 134 mOsm hypo-osmotic stress with 14 μ M polymer concentration. Circle diameter is proportional to the average LDH release for 6–21 measurements. Colors of circles are the same as the colors used for corresponding block copolymers in Figures 3–7. End group on the PPO block of diblock copolymers are denoted by borderline: thick solid line (*tert*-butyl group), double line (hydroxyl group), or dashed line (methyl group). Circles without borderline denote the triblock copolymers and the semicircles are PEO homopolymers. Dashed diagonal lines indicate the EO wt% of the polymers.

Figure 9.

Copolymer titration to quantify sensitivity. Copolymers (P188, P338, $E_{75}P_{16m}$, and $E_{78}P_{16t}$) with varying concentrations were added to myoblast cultures throughout 134 mOsm hypo-osmotic stress and isotonic recovery. LDH release was measured and normalized to non treated control (buffer-only). Average $\pm$ standard error are shown for three independent experiments with three wells per experiment.

Table 1

Block copolymers used in this study.

	End group on PEO	EO units ^a	PO units ^a	End group on PPO	Total MW ^b	EO wt% ^c	<i>d</i>
Triblock copolymer	P188	150	30	-	8400	79	1.07
	P338	280	44	-	14600	84	1.17
	P237	128	37	-	7800	72	1.09
	P407	202	56	-	12100	73	1.27
Diblock copolymer	E ₇₅ P ₁₆ <u><i>m</i></u>	75	16	-CH ₃	4300	78	1.10
	E ₄₈ P ₁₆ <i>l</i>	48	16	-C(CH ₃) ₃	3000	70	1.06
	E ₇₈ P ₁₆ <i>l</i>	78	16	-C(CH ₃) ₃	4450	77	1.05
	E ₁₈₂ P ₁₆ <i>l</i>	182	16	-C(CH ₃) ₃	8900	90	1.07
	E ₄₃ P ₂₁ <i>l</i>	43	21	-C(CH ₃) ₃	3100	60	1.06
	E ₈₉ P ₄₀ <i>l</i>	87	49	-C(CH ₃) ₃	6700	57	1.10
	<u><i>m</i></u> E ₄₆ P ₅	46	5	-OH	2300	88	1.04
	<u><i>m</i></u> E ₁₂₀ P ₉	120	9	-OH	5800	91	1.04
	<u><i>m</i></u> E ₄₆ P ₁₆	46	16	-OH	2900	70	1.04
	<u><i>m</i></u> E ₇₅ P ₁₅	75	15	-OH	4200	79	1.08
Homopolymer	<u><i>m</i></u> E ₁₂₀ P ₃₂	120	32	-OH	7200	73	1.04
	PEO 8K (E195)	195	-	-	8581	100	1.01 ^e
	PEO 4K (E92)	92	-	-	4101	100	1.01 ^e

^aTotal number of EO or PO monomer units in a polymer^bNumber average molecular weight (*M_n*) in g/mol^cPEO weight percent to total molecular weight^dDispersity *M_w*/*M_n* from SEC^eObtained from MALDI-TOF