Self-assembly and steric stabilization at heterogeneous, biological surfaces using adsorbing block copolymers

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Background: We present the synthesis, characterization and initial structure–function analysis of a new class of bioactive agent that allows the application of techniques from colloid science to biological surfaces. Stable colloidal suspensions can be generated by immobilizing a dense brush of soluble polymer at the colloidal surface, creating a zone protected against the adhesion of approaching particles, a phenomenon termed polymeric steric stabilization. This is often accomplished for aqueous colloidal dispersions using adsorbing block copolymers. We demonstrate that water-soluble block copolymers can be designed to adsorb onto heterogeneous biological surfaces and block cell–cell and cell–surface adhesion, using polymer compositions and architectures that are quite different from surfactants used for stabilizing nonbiological colloidal dispersions.

Results: Comb copolymers were synthesized having polycationic backbones (poly-L-lysine, PLL), serving as the anchor for binding to the net negatively charged biological surfaces, grafted with water-soluble polynonionic chains (polyethylene glycol, PEG), to block biological recognition, producing PLL-*graft*-PEG copolymers. Specific copolymers were found to sterically stabilize red blood cells from lectin-induced hemagglutination and fibroblasts from adhesion to fibronectin-coated surfaces. The polymer design principles, which appear to be unique for adsorption to heterogeneous biological surfaces, require the use of very high molecular weight comb copolymers, perhaps because anionic sites are non-uniformly distributed on biological surfaces, and the ability of larger copolymers to span between highly anionic sites.

Conclusions: Water-soluble copolymers were produced that can block recognition at biological surfaces, on the basis of nonspecific physicochemical phenomena rather than specific biochemical interactions.

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Introduction

Although not extensively exploited for therapeutic use, colloidal phenomena are important in many biological interactions [1]. For example, one function of the glycocalyx (the carbohydrate-rich protein coat on the surfaces of cells) is to sterically stabilize cells against nonspecific interactions, allowing specific biological recognition to predominate [2]. Just as the glycocalyx limits nonspecific interactions, we sought to design copolymers that could self-assemble at a cell or tissue surface and prevent both nonspecific interactions and specific recognition at the biological surface. The use of self-assembly to prevent interactions at biological surfaces is analogous to stabilizing aqueous colloidal dispersions by the adsorption of hydrophilic/hydrophobic block copolymers [3,4]; however, different design principles are required, particularly for the anchor block. Biological surfaces are hydrophilic, and hydrophobic molecules are not expected to adsorb with high affinity; rather, extracellular biological surfaces are characterized by a net negative charge, principally due to protein glycosylation, and these anionic sites are suitable targets for binding by using

polycationic molecules as anchoring segments [5,6]. Biological surfaces are also more heterogeneous than typical non-biological colloidal surfaces, and a high molecular weight (MW) anchoring segment may be necessary to bridge across domains that lack anionic sites [7]. With these design principles in mind, we used a comb copolymer configuration, allowing a very high molecular weight poly-L-lysine (PLL) backbone to be used as the anchoring segment [8]. For the teeth of the comb copolymer, we used polyethylene glycol (PEG), a hydrophilic nonionic polymer that has been employed in numerous applications to limit biological interactions, such as preventing protein adsorption on surfaces, and prolonging the circulation of therapeutic proteins *in vivo* [9]. If the copolymers are capable of immobilizing a high concentration of PEG to the biological surface, then PEG should be capable of preventing biological recognition at that surface [10,11].

Results and discussion

Monomethoxy PEG (MW 5000) was chemically activated so as to produce a single reactive terminus per chain, and a

controlled number of PEG chains were subsequently coupled to a PLL backbone, producing a comb copolymer [12,13]. The number of grafted PEG chains per PLL backbone was varied under stoichiometric control and is expressed here as the 'graft ratio', the number of lysine residues per attached PEG or the average number of lysine residues −1 between attached PEG chains. Aqueous size exclusion chromatography indicated between 85–99% coupling for reaction of activated PEG with PLL, and a monotonic increase in average molecular weight of the PLL-*graft*-PEG (PLL-*g*-PEG) with increasing amounts of attached PEG. Two series of PLL-*g*-PEG comb copolymers were synthesized from PLL of MW 20,000 Da or PLL of 375,000 Da, to produce copolymers of MW ranging from 54,000 to 4,700,000 Da, to determine the effect of the size of the PLL anchor and the effect of the graft ratio on the ability to sterically stabilize biological surfaces.

Lectin-induced hemagglutination was employed as a model for the manipulation of cell–cell recognition [14]. Rat red blood cell suspensions were treated by addition of 0.1% aqueous solutions containing the water-soluble PLL-*g*-PEG copolymers. After allowing 5 min for adsorption of the copolymers onto the red blood cell surface, serially diluted solutions of wheat germ agglutinin (WGA), a lectin protein that rapidly agglutinates rat red blood cells, were added. The vehicle control was phosphate-buffered saline (PBS); following treatment of the red blood cells with PBS alone, hemagglutination occurred for WGA concentrations as low as 2 μg/ml (see Table 1 and Figure 1). Hemagglutination was not affected by pretreating the cells with PEG, nor with a nonionic surfactant (Pluronic F-68), a copolymer consisting of one hydrophobic poly(propylene glycol) block and two PEG blocks. Hemagglutination was actually induced by the addition of PLL homopolymer of either molecular weight, even in the absence of WGA, because the polycation can induce bridging flocculation of the cells [1,15]. PLL-*g*-PEG copolymers containing a PLL backbone of 20,000 Da and high amounts of grafted PEG per PLL chain (graft ratios of 1.75:1 to 7:1) did not affect WGA-induced agglutination, and copolymers with low amounts of grafted PEG (graft ratios of 10.5:1 and 14:1) induced hemagglutination without the addition of WGA, thus behaving like the PLL homopolymer. By contrast, comb copolymers employing a very high molecular weight anchor (PLL 375,000 Da) at moderate graft ratios (5.6:1 and 7:1) completely inhibited WGA-induced hemagglutination, even at WGA concentrations as high as 125 μg/ml.

To study the effects of PLL-*g*-PEG copolymers on cell–surface interactions, an anionic polystyrene surface with and without pre-adsorbed serum proteins was used. The surfaces were treated with copolymers by incubation of the surfaces with 0.1% aqueous solutions of copolymers, followed by a wash with buffered saline so as to

Table 1

Agglutination and aggregation of red blood cells treated with polymers and incubated with wheat germ agglutinin.

Polymer or control	Highest WGA conc. without agglutination (μg/ml)	Highest WGA conc. without aggregation (μg/ml)	Comments
PBS (vehicle)	2	1	No effect
mPEG 5000	2	1	No effect
PLL 20,000	0	0	Agglutinates RBC
PLL 418,000	0	0	Agglutinates RBC
Pluronic F-68	2	1	No effect
PLL-*g*-PEG (PLL MW/graft ratio):			
20,000/1.75:1	2	1	No effect
20,000/3.5:1	2	1	No effect
20,000/7:1	2	1	No effect
20,000/10.5:1	1	0.2	Agglutinates RBC
20,000/14:1	0	0	Agglutinates RBC
375,000/1.75:1	2	1	No effect
375,000/3.5:1	2	2	Slight effect
375,000/5.6:1	125	125	Prevents agglut. by WGA
375,000/7:1	125	125	Prevents agglut. by WGA
375,000/10.5:1	125	2	Complex behavior
375,000/14:1	0	0	Agglutinates RBC

Red blood cells were treated with polymer-containing solutions, followed by incubation with varying concentrations of wheat germ agglutinin. The highest concentration of WGA that could be added to the red blood cells without causing agglutination, as judged by observing settling of the red blood cells, or aggregation, as judged by microscopy, were assessed. The ability of polymers to protect the red blood cells from lectin-induced agglutination indicated a steric stabilization phenomenon at the red blood cell surface. PBS, phosphate buffered saline; RBC, red blood cells; WGA, wheat germ agglutinin.

observe only effects of adsorbed PLL-*g*-PEG. PLL-*g*-PEG copolymers with optimal graft ratios were able to eliminate fibroblast cell spreading on the anionic polystyrene surface not containing pre-adsorbed serum proteins, regardless of the molecular weight of the PLL backbone, indicating that self-assembly of the copolymers occurred readily on this relatively homogenous anionic surface (Figure 2). In contrast, PLL-*g*-PEG copolymers with a low molecular weight PLL backbone had no effect on fibroblast spreading when used to treat the protein-adsorbed surface, but PLL-*g*-PEG copolymers with a PLL backbone of 375,000 Da and optimal graft ratios were able to completely sterically stabilize cell spreading on the protein-adsorbed surface.

Adsorption of copolymers to protein-adsorbed surfaces and subsequent cell spreading was further studied using another model that was designed to mimic the serum protein-adsorbed surface (Table 2). This surface was

Figure 1
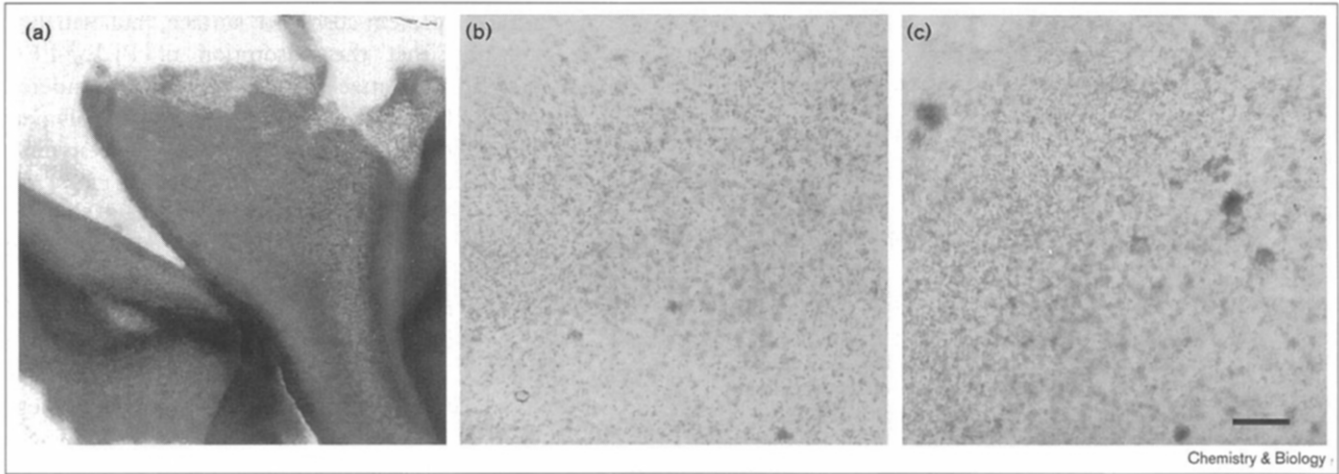
Adsorption of some forms of PLL-*g*-PEG sterically stabilized red blood cells from hemagglutination induced by wheat germ agglutinin. Photomicrographs of red blood cells after stirring are shown: **(a)** PBS treatment (vehicle control), WGA at 5 μg/ml; **(b)** PBS treatment, no WGA added; **(c)** PLL-*g*-PEG (PLL MW 375,000, graft ratio 5.6:1) treatment, WGA at 125 μg/ml; Scale bar = 100 μm.

generated by adsorbing two serum proteins onto anionic polystyrene, fibronectin, the serum protein often responsible for cell adhesion to artificial surfaces, and albumin, the most abundant serum protein, which also adsorbs in high concentrations to artificial surfaces but alone does not support cell spreading onto a surface. PLL-*g*-PEG

Figure 2
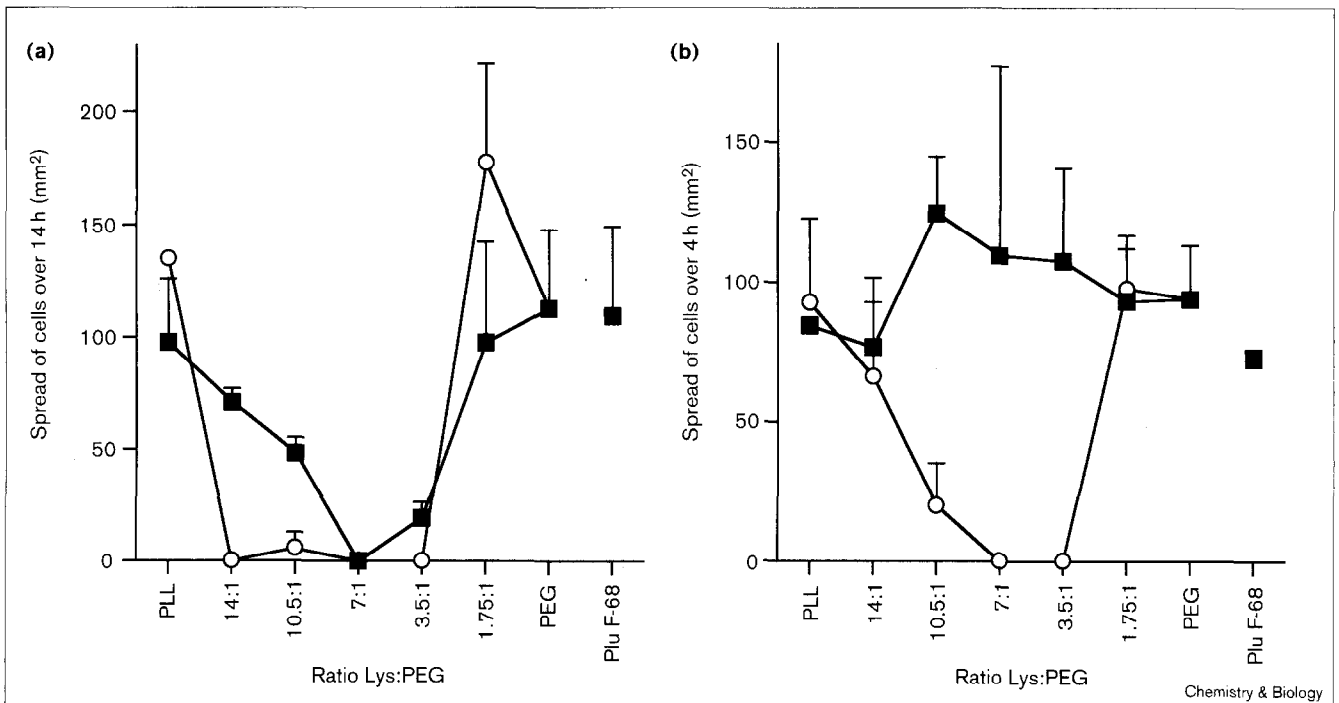
The prevention of fibroblast spreading by adsorption of a series of PLL-*g*-PEG copolymers synthesized from mPEG 5000 and either PLL 20,000 (■) or PLL 375,000 (O) in different graft ratios (ratio of lysine residues to attached PEGs). Plu F-68 is Pluronic F-68, a PEG-based nonionic surfactant. The surfaces were **(a)** anionic polystyrene 14 h post-seeding and **(b)** adsorbed serum proteins on anionic polystyrene 4 h post-seeding. Mean ± standard deviation (s.d.), n = 3.

Table 2

Effect on cell spreading of PLL-*g*-PEG adsorption to fibronectin and albumin-coated surfaces.

Treatment (PLL MW/ graft ratio)	Cell spreading on surfaces (cells/mm²)			
	Fn	Alb	99% Alb, 1% Fn	90% Alb, 10% Fn
PLL-*g*-PEG (375,000/5.6:1)	221 ± 50	0 ± 1*	8 ± 8*	59 ± 11*
PLL-*g*-PEG (20,000/7:1)	251 ± 17	0 ± 0*	265 ± 54	173 ± 82
PBS	249 ± 19	372 ± 44	255 ± 61	208 ± 18

The polymers that were most effective in preventing cell spreading in other models were used to treat surfaces containing albumin and fibronectin (designed to mimic surfaces treated with serum). Fibroblast cells were seeded onto the surfaces, and spreading was measured at 4 h. For surfaces treated with both albumin and fibronectin, the proteins were mixed in PBS, for a total protein concentration of 1 mg/ml, and the percentages indicate the concentration of each protein in solution. Fn, fibronectin; Alb, albumin; PBS, phosphate buffered saline. Mean ± s.d., n = 3. *Significantly different from the appropriate PBS treatment, by an ANOVA, with Scheffe post-hoc test at 5%.

copolymers with optimal graft ratios were adsorbed onto surfaces containing either adsorbed fibronectin, albumin, or both. On the fibronectin-adsorbed surface, no copolymer could prevent cell spreading. On the albumin-adsorbed surface, the molecular weight of the PLL backbone was unimportant; copolymers with optimal graft ratios could prevent cell spreading on the albumin-adsorbed surface, with either low or high molecular weight PLL backbones. If albumin and fibronectin were mixed in solution before adsorption upon the anionic polystyrene surface, the high molecular weight PLL backbone was necessary to prevent cell spreading, as was observed with the serum protein-adsorbed surface. The treatment using a 1% concentration of fibronectin was assumed to result in a much higher surface concentration of fibronectin than treatment of the surface with media containing 10% serum, as the percentage by weight of fibronectin and other adhesive glycoproteins in serum is about 0.04–0.05% of total protein mass in the media containing 10% serum media (assuming 200 µg/ml of fibronectin in serum [16]).

Radiolabeling indicated that the amount of copolymer with a low molecular weight PLL backbone that adsorbed to the serum protein-adsorbed surface was only ~25% lower than the adsorption of the copolymer to the simple anionic surface. This is surprising, because this copolymer was completely effective in preventing cell spreading upon the simple anionic surface, and completely ineffective in preventing cell spreading upon the protein-adsorbed surface, indicating that the overall concentration of polymer on a surface may be unimportant compared with the local concentration of polymer near the cell adhesive sites on the surface.

Optimal copolymer graft ratios were observed for adsorption and efficacy at all model surfaces. On anionic polystyrene and the protein-adsorbed surface, radiolabeling studies indicated that the adsorption of PLL-*g*-PEG copolymers with many attached PEG chains was hindered by the many PEG 'teeth' (for adsorption of copolymers with 20,000 Da PLL backbones onto anionic polystyrene, the surface concentration of PLL-*g*-PEG with a graft ratio of 7:1 was 322 ± 14 ng/cm² and the surface concentration of PLL-*g*-PEG with a graft ratio of 1.75:1 was 84 ± 5 ng/cm²; adsorption of PLL (20,000 Da) was 327 ± 23 ng/cm²). PLL-*g*-PEG with very few attached PEG chains adsorbed more mass to the surfaces than even PLL (PLL-*g*-PEG with a MW 20,000 PLL backbone and a graft ratio of 14:1 was adsorbed at concentrations of 450 ± 16 ng/cm²), but these copolymers presumably did not immobilize sufficient amounts of PEG to provide steric stabilization.

The interruption of biological recognition described here is based on nonspecific physicochemical interactions and not on cytotoxicity or specific biological activity. Hemagglutination induced by WGA occurs by recognition of specific, uncharged carbohydrate residues on the surfaces of red blood cells, which does not require metabolic activity, and occurs even with glutaraldehyde-fixed red blood cells. The protection from WGA-induced hemagglutination by PLL-*g*-PEG was observed with fixed red blood cells and with red blood cells that were centrifuged twice following treatment with PLL-*g*-PEG (with resuspension in PBS containing no dissolved polymer), indicating that the observed effect was physical, rather than toxicological, and that the effect was due to polymer that was associated with the red blood cell surface, and not due to polymer free in solution. Cell toxicity studies, using fluorescein diacetate, indicated that the PLL-*g*-PEG copolymers did not lead to enhanced cell death of fibroblast cells, even after a 75 minute incubation of cells with a 0.2% solution of the copolymer. Moreover, the effect of the copolymer was restricted to the precise site of its application. In Figure 3, a photomicrograph shows the ability of the copolymer to locally eliminate cell spreading on the protein-adsorbed surface. Only a portion of the surface was treated with a solution of copolymer, with the aid of a rubber stamp. The ability to pattern cell spreading on the surface illustrates that the effect is due to a surface phenomenon, rather than a solution or toxicity effect, because the surfaces were washed extensively with buffered saline alone before cell seeding. Additionally, the cells were able to spread up to the interface between the treated and untreated regions, indicating that the observed effect is not the result of a diffusible toxic agent. To generate patterns on the surface, much higher concentrations of cells were seeded on the surfaces than in the previous cell spreading study. The copolymers were still able to eliminate cell spreading on the simple anionic surface, although some cell spreading was observed on the copolymer-treated protein-containing surface.

Figure 3

Steric stabilization of cell adhesion was a local phenomenon and regions of the surface lacking the adsorbed PLL-*g*-PEG copolymer supported cell adhesion and spreading, with patterns formed on surfaces by excluding contact with the polymer solution using a rubber stamp. This demonstrates a surface physicochemical effect, rather than a solution or toxicity effect. **(a)** Fibroblasts form the shape of 'XYZ' on anionic polystyrene selectively treated with PLL-*g*-PEG (PLL MW 20,000; graft ratio 7). **(b)** Fibroblasts form the numbers '123', on a surface containing adsorbed serum proteins selectively treated with PLL-*g*-PEG (PLL MW 375,000; graft ratio 5.6:1). Scale bar = 500 μm.
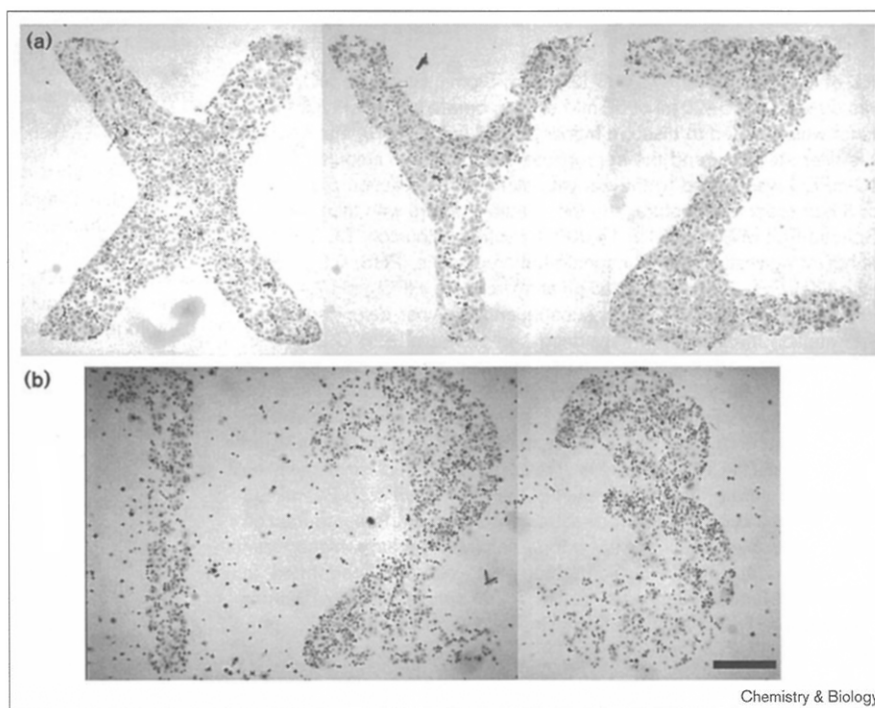
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A consistent response emerged from the structure–function studies performed on the anionic polystyrene substrate with and without adsorbed serum proteins, as well as with red blood cell agglutination. On more homogeneous, nonbiological surfaces (i.e. anionic polystyrene), copolymers with low molecular weight anchors were able to induce steric stabilization (it is typically observed that smaller anchor blocks are adequate to provide effective steric stabilization of nonbiological colloidal particles [17]). On the more heterogeneous, biological surfaces (adsorbed serum proteins, red blood cells), however, only copolymers with high molecular weight anchors were effective, presumably because such large anchors can bridge between the sites on the heterogeneous surface that bear net anionic charge. The graft ratios of PLL-*g*-PEG with high molecular weight backbones that led to effective steric stabilization on the heterogeneous biological surfaces (i.e. the adsorbed serum protein surface and the red blood cell surface) were intermediate values. Only copolymers with a polycationic anchor were effective; copolymer with a hydrophobic block that is effective in producing stable dispersions of aqueous colloidal particles (Pluronic F-68) was ineffective in steric stabilization on biological surfaces.

Significance

Nonspecific colloidal interactions have been used extensively in industrial processes. Despite the important roles they play in natural biological processes, such interactions have not been extensively explored for pharmacological use (although the ability to sterically stabilize virus

particles and prevent binding to cells has been demonstrated using polymers containing saccharide residues that specifically bind to viral surface receptors [18]). Our goal was to explore whether phenomena such as polymeric steric stabilization could be applied to a biological surface (even to a living surface, such as the red blood cell surface), and if these phenomena might be useful in blocking biological recognition that leads to cell–cell or cell–surface adhesion. The discovery of polymers that effect steric stabilization at a biological surface via self-assembly provides the tools to apply the vast body of knowledge in colloid chemistry to problems in biological systems, such as the manipulation of the wound-healing response by blocking local cell-adhesive interactions. Previous work from our laboratory has used microscopic hydrogel barriers to block such interactions, for example, in the manipulation of arterial healing [19,20]. Here, we have employed the red blood cell and protein-coated surfaces to explore the possibility of preventing cell–cell and cell–tissue recognition in such situations using nanoscopic barriers, via block copolymers that adsorb and sterically stabilize such biological interfaces.

Materials and methods

Monomethoxy PEG of MW 5000 (Shearwater Polymers, Huntsville, AL, USA) was dried by azeotropic distillation in benzene. The succinimidyl carbonate of PEG was produced by reaction of 1 equivalent of dry PEG with 5 equivalents of disuccinimidyl carbonate (Fluka, Ronkonkoma, NY, USA) and 5 equivalents dimethylaminopyridine (Fluka) in anhydrous DMF (Aldrich, Milwaukee, WI, USA) for 24 h with stirring. The reaction mixture was precipitated in cold diethyl ether. The product was purified

by recrystallization in ethyl acetate, three times. SC-mPEG–^{1}H-NMR (CDCl$_3$): ppm–2.81 (s, succinimide, 4 H); 3.6 (PEG, 452 H); 4.4 (t, CH$_2$–O-CO$_2$–, 2 H).

PLL of MW 20,000 or 375,000 (200 mg; Sigma, St. Louis, MO, USA) was dissolved in 5–20 ml of 50 mM sodium borate buffer, pH 8.5 (more buffer was required to dissolve higher MW PLL and PEG). The solution was filter sterilized, and the appropriate stoichiometric amount of solid SC-mPEG was added to the vial with stirring. The reaction proceeded for 6 h at room temperature, and the reaction mixture was then dialyzed (Spectra-Por, MW cutoff 12–14,000; Spectrum, Houston, TX, USA) for 24 h, first against 10 mM phosphate buffered saline (PBS; 0.2 g/l KCl, 0.2 g/l KH$_2$PO$_4$, 8 g/l NaCl, 1.15 g/l anhydrous Na$_2$HPO$_4$, pH 7.4 ± 0.1, 285 mOsm/kg H$_2$O ± 5%), and subsequently against deionized water. The reaction mixture was freeze-dried and stored at –20°C under Ar. PLL-g-PEG–^{1}H-NMR (D$_2$O): ppm–1.35, 1.60, 1.68 (–CH$_2$–); 2.88 (–CH$_2$-N–); 3.55 (PEG); 4.20 (–N–CHR–COO–). PLL-g-PEG copolymers with high molecular weight PLL backbones contained a small fraction that was poorly soluble in water that was removed using a 5 μm syringe filter. This small insoluble fraction consisted of small, swollen gels, which were probably the result of the addition of solid SC-mPEG to the PLL, as opposed to predissolved PEG, leading to some fraction of poorly soluble, ultrahigh MW copolymer (MW perhaps higher than 10,000,000 Da). Size exclusion chromatography was performed in sodium carbonate/bicarbonate buffer, 0.2 M, pH 10, with a Shodex OHpak column SB-804HQ (Alltech, Deerfield, IL, USA).

Red blood cells were obtained by collecting blood from rats by cardiac puncture, withdrawing into citrate-phosphate-dextrose solution with adenine (Sigma), with approval from the Institutional Animal Care and Use Committee at the California Institute of Technology. Red blood cells were collected by centrifugation at 200 g for 20 min, three times, with resuspension in Alsever's solution (Sigma). The red blood cells were diluted to a 1% (v/v) concentration with PBS, and 100 μl of the red blood cell suspension was added to 50 μl of a 0.1% solution of the polymer treatment in PBS in a 96-well plate. After a 5 min incubation at room temperature, 50 μl of a wheat germ agglutinin (Sigma) solution was added to the cells (final concentrations of WGA: 125, 42, 14, 5, 2, 1, 0.2 or 0 μg/mL in PBS). The highest concentration of WGA that could be present with the red blood cells without causing hemagglutination is reported, with hemagglutination determined by two methods: the formation of a compact pellet after settling by brief centrifugation of the multiwell plates (compact pellet formation is only possible if the cells are not agglutinated), or microscopic observation of large aggregates while stirring the solution with a pipet tip. Similar studies were performed in which the red blood cells were treated with copolymer solutions, then centrifuged for a 1 s pulse using an Eppendorf centrifuge twice, with resuspension in PBS lacking dissolved polymer. No red cell lysis was observed following any polymer treatment.

Serum proteins were adsorbed to anionic polystyrene (Falcon Tissue Culture Polystyrene; Becton Dickinson, Lincoln Park, NJ, USA) from fibroblast cell culture medium (containing 10% serum; see below for composition), for 30 min at 37°C, followed by a wash with PBS for 30 min at 37°C. Test surfaces were treated with solutions of polymers prior to the seeding of cells onto the surfaces, by incubating the surfaces with 0.1% solutions of the polymers in PBS at pH 7.4 for 5 min, followed by a 5 min wash with PBS alone to ensure that the surface, and not the cells subsequently seeded on the surface, were treated with the polymers. For studies with albumin and fibronectin adsorbed surfaces, anionic polystyrene was treated as above with 1 mg/ml bovine serum albumin (Sigma) in PBS, 50 μg/ml fibronectin (Sigma) in PBS, or mixtures in PBS with 1% or 10% fibronectin plus albumin for a total protein concentration of 1 mg/ml. For cell patterning on surfaces, a rubber stamp was pressed onto the tissue culture surface, and a 0.1% solution of copolymer was added to the well for 2 min. The copolymer solution was aspirated, and the surface was washed three times with PBS before removing the stamp from the surface.

Copolymer adsorption on anionic polystyrene was measured by reacting amino groups on the polymers with ^{125}I Bolton-Hunter reagent (ICN, Costa Mesa, CA, USA; final specific activity typically about 15,000 cpm/μg of polymer) in 50 mM borate buffer, pH 8.5. Excess Bolton-Hunter reagent was removed using Sephadex PD-10 size exclusion columns (Pharmacia Biotech, Piscataway, NJ, USA) equilibrated with PBS.

Human fibroblast cells (neonatal normal human dermal fibroblast; Clonetics, San Diego, CA, USA) were grown in fibroblast cell culture medium (Dulbecco's Modified Eagle's Medium, with 10% fetal bovine serum and 1% antibiotic-antimycotic, GIBCO BRL, Life Technologies, Grand Island, NY, USA) at 37°C and 5% CO$_2$. Fibroblast cells were removed from culture substrates using trypsin/EDTA (GIBCO BRL), centrifuged at 500g for 5 min, and resuspended in culture media (all experiments were done in culture media containing 10% serum). Cells were seeded onto test substrates at a density of 25,000 cells/cm^2. After 4 h or 14 h of culture, the cells were fixed with 10% formalin and counted. Spread cells were considered to be cells extending any pseudopodial processes. For cell patterning, cells were seeded at 250,000 cells/cm^2. As a viability assay, cells were incubated with 5 μl/ml of a solution of 5 mg fluorescein diacetate (Sigma) in 1 ml acetone. After 5 min at 37°C, the percentage of cells exhibiting fluorescence was measured by viewing cells on a microscope in both fluorescence and bright field modes, counting at least 100 cells.

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