Blocking Adhesion to Cell and Tissue Surfaces via Steric Stabilization with Graft Copolymers containing Poly(Ethylene Glycol) and Phenylboronic Acid

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Abstract

Graft copolymers were designed to coat biological surfaces and thereby block subsequent adhesion and recognition events with other proteins, cells, and tissues. Inspired by polymeric steric stabilization of colloidal dispersions, the copolymers contain a backbone with affinity for biological surfaces and grafted side-chains that are resistant to adhesion of biological elements. Phenylboronic acid (PBA) moieties in the polymer backbone provided for binding, by forming reversible covalent complexes with cis-diols in oligosaccharides that are ubiquitous on cell surfaces and secreted macromolecules. The PBA moieties were conjugated to a poly-L-lysine (PLL) backbone via a secondary amine linker. Grafted poly(ethylene glycol) (PEG) side-chains provided for resistance of adhesion of proteins and cells. It was hypothesized that these PLL-g-(PEG;PBA) copolymers would spontaneously assemble on biological (e.g., cell or tissue) surfaces, where the PBA-containing backbone would bind to the surfaces and thereby anchor a dense PEG brush on the surface.

PLL-g-(PEG;PBA) copolymers were synthesized with varying degrees of PEG and PBA grafting. The pKa of the PBA groups was found to be circa 6, allowing for stronger binding at physiological pH than most PBA groups, which have pKas circa 8.8. The PLL-g-(PEG;PBA) copolymers were found to bind specifically to a mannan resin, where the PEG grafting ratio sterically controlled the binding. The copolymers coated red blood cells and blocked their agglutination by lectins and by blood group antibodies. The copolymers were also found to coat tissue culture polystyrene, adsorbed serum proteins, and extracellular matrix and to prevent the adhesion, spreading, and/or migration of rabbit lens epithelial cells on those surfaces. The copolymers displayed evidence of toxicity in $vitro$ but no toxicity was seen when administered in $vivo$ in models of posterior capsule opacification or peritoneal adhesion formation. PLL-g-(PEG;PBA) was found to interfere with the worsening of peritoneal adhesions following adhesiolysis. The efficacy of the

copolymers was a function of the degree of PEG and PBA grafting, and PLL-g-(PEG;PBA) copolymers were found to be more effective than electrostatically-binding PLL-g-PEG copolymers. PLL-g-(PEG;PBA) copolymers have many possible clinical applications where blocking protein or cell interactions with cell, tissue, or biomaterial surfaces via a simple aqueous lavage is desired.

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Abbreviations

Abbreviations used in this text:

ECM	extracellular matrix
Graft ratio	# of PEG chains grafted per # of Lys mers in PLL backbone
HBS	HEPES-buffered saline, pH 7.4
IOL	intraocular lens
mAb	monoclonal antibody
mPEG-CDI	α-monomethoxy, ω-oxycarbonylimidazole PEG
MW	molecular weight
PBA	phenylboronic acid
PBS	phosphate-buffered saline, pH 7.4 unless otherwise noted
PCO	posterior capsule opacification
PEG	poly(ethylene glycol)
PLL	poly-L-lysine
PLL-g-PEG	PEG grafted to PLL backbone
PLL-g-(PEG;PBA)	PEG and PBA grafted to PLL backbone
RBC	red blood cell
RI	refractive index
rLEC	rabbit lens epithelial cell
rt	room temperature
TBS	tris-buffered saline, pH 7.4
TCPS	tissue culture polystyrene
WGA	wheat germ agglutinin

1. Chapter 1

Introduction

1.1 Motivation for this Work

Many undesirable biological responses begin with adhesion and recognition at cell or extracellular matrix surfaces within the body. For example, patients with sickle cell anemia or thalassemia receive chronic blood transfusions and can begin to develop antibodies to red blood cell antigens other than the ABO blood group antigens (Vichinsky, E. P. et al. 1990). This is called alloimmunization, and binding of these antibodies to transfused red blood cells can cause delayed transfusion reactions and life threatening events. This complication arises in up to 40% of sickle cell anemia cases. In another example, after transplantation of porcine organs into humans (xenotransplantation), host antibody binding to the α-galactosyl epitope on endothelial cells lining the organ vasculature quickly leads to hyperacute rejection of the transplant (Rother, R. P. and Squinto, S. P. 1996). This is a major obstacle to the development of xenotransplantation as a solution to organ donation shortages. Following cataract surgery, lens epithelial cells that remain after the surgical procedure can migrate onto the posterior lens capsule, proliferate, deposit fibers, and wrinkle the capsule (Apple, D. et al. 1992). This happens in up to 50% of the surgeries and leads to obstruction of vision termed a secondary cataract or posterior capsule opacification. Reperfusion injury is a common occurrence following tissue ischemia induced by myocardial infarction, stroke, or organ transplantation (Carden, D. L. and Granger, D. N. 2000). The generation of superoxide radicals upon re-introduction of oxygenated fluid creates a cascade of events that includes neutrophil adhesion to the vascular endothelial cells. This can lead to tissue injury including microvascular dysfunction, multiple organ dysfunction syndrome, and systemic

inflammatory response syndrome. As a final example, manipulations involved in laparoscopic tumor resection can produce free intraperitoneal tumor cells (Muller, J. M. et al. 1999). The cells can then adhere to surfaces in the wound site and thereby metastasize.

All of these events involve proteins and/or cells binding to cell and/or extracellular matrix surfaces within the body. By blocking these binding events, the pathological cascades could be blocked. Such interactions are often inhibited pharmacologically through the use of competitive inhibitors, which employ biological recognition to bind to the receptors or ligands involved. Placing physical biomaterial barriers over surfaces to block adhesion to those surfaces has also been used to block binding events. However, the former approach requires knowledge of the ligands and receptors involved in the cascade and synthesis and administration of effective inhibitors. Besides these technical difficulties, immune responses to many protein-based inhibitors and side-effects of inhibitors at distal locations are frequent complications. The latter approach of application of physical barriers can involve difficult surgical manipulations and frequently does not provide complete protection from adhesion to all the surfaces involved due to lack of intimate contact with the surface.

The work described in the present dissertation involves using a different approach to blocking deleterious adhesion and recognition events. A family of therapeutics designed to block these recognition events using physicochemical interactions was envisioned, where the principles of colloid science would be harnessed. Polymers were designed that would spontaneously bind to cell and tissue surfaces and sterically stabilize the underlying surface from interactions with other components in the surroundings. The principles of polymeric stabilization of colloidal dispersions were used, where the polymeric backbone would bind to groups that are ubiquitous on biological surfaces, and grafted pendant side chains that are highly resistant to protein and cell interactions would then form a dense polymer brush on the treated surface. Thus a water-soluble polymer was designed that would spontaneously protect all lavaged surfaces from biological interactions in a

nonspecific fashion. Such a less specific approach might be particularly useful, especially when multiple receptor-ligand interactions are involved in the overall response. In addition, this approach would allow application by a simple lavage that could be applied following conventional or laparoscopic surgery. The application method would be much more facile than placing biomaterial barriers or photopolymerizing gels *in situ* on all the surfaces to be treated. This approach potentially could be immediately applicable to a large number of undesirable biological responses, while enabling further tailoring to specific pathologies if necessary. Naturally, due to the inherent lack of specificity of the binding interactions, the approach would be limited to situations where the copolymer could be directly applied to the tissue of interest. These would include, for example, surgical, endoscopic or catheter-based interventional procedures, as opposed to systemic injection into the vasculature.

This work was designed to investigate such a copolymer, building on previous work by Elbert and Hubbell (Elbert, D. L. 1997). Surfactant copolymers have frequently been used to sterically stabilize biomaterial surfaces, but rarely living biological surfaces such as the surfaces of cells and tissues. The remainder of this chapter will describe the nature of biological adhesion interactions, the design considerations inherent to this approach, the properties of the binding and adhesion-resisting chemical moieties chosen, and finally the design of the copolymer and an outline of the experiments conducted to test the binding and stabilizing properties of these copolymers.

1.2 Biological Adhesion Interactions

Biological adhesion events usually result from non-covalent recognition interactions that are governed by electrostatic, hydrogen bonding, hydrophobic and van der Waals forces (Alberts, B. et al. 1994; Stryer, L. 1995). The sum of these interactions can result in strong, specific binding interactions. For example, antibodies have a variable Fab region

that fits specifically with its antigen, binding together with incredible specificity. The antigens can be proteins, polysaccharides, or nucleic acids. Binding of antibodies to their antigens can initiate the cascade of events in immune response to a substance. Also, cells possess myriad receptors with low or high binding affinity on their surfaces, which recognize ligands including secreted signaling molecules and adhesion molecules on surfaces. Binding of the receptors can then induce specific responses in the cells, including migration, secretion of other molecules, proliferation, or adhesion. In contrast, proteins can interact nonspecifically with biomaterial surfaces, adsorbing irreversibly in a monolayer (Ratner, B. D. et al. 1996).

Cell adhesion to surfaces such as extracellular matrix (ECM) involves a number of steps. The integrin family forms the primary class of cell surface receptors that bind ECM proteins such as laminin, collagen, and fibronectin. Cells initially attach in a rounded form to proteins on the surface, then form focal contacts and spread on the surface. The focal contacts include clusters of integrin receptors linked to intracellular actin filaments that allow the cell to exert tension and to migrate. Most cells must be attached and spread upon surfaces in order to survive. Cells can then secrete ECM - a dense organized meshwork of macromolecules that holds the cells within tissue together in a hierarchically organized and temporally coordinated manner. The major components of ECM are proteoglycans, containing many glycosaminoglycan chains linked to a core protein, and glycoproteins that are mostly structural (i.e., collagen) or adhesive (i.e., fibronectin and laminin). A specialized ECM is the basal lamina (a.k.a. basement membrane), a 40 - 120 nm thick sheet that is secreted underneath all epithelial cell sheets. Basal lamina are comprised mostly of type IV collagen, the heparan sulfate proteoglycan perlecan, laminin, and entactin. While integrins recognize specific matrix molecules, surfaces containing small peptide fragments from the matrix molecules such as the peptide RGD can induce many of the same effects on cells as the entire protein does.

A common method employed to block biological binding events is to add a soluble competitor (an antagonist). For instance, soluble RGD has been added to lens capsules following cataract surgery in order to bind to integrins on the lens epithelial cells, so the integrins would then be unavailable to bind to matrix molecules on the capsule surface and migrate (Nishi, O. et al. 1997). The limited efficacy seen by these authors may have been in part because RGD-integrin binding is not the only pathway of cell adhesion and spreading at play in this situation. This approach of RGD administration has also been proposed to inhibit tumor metastasis (Dechantsreiter, M. A. et al. 1999). Similarly, the addition of soluble carbohydrate antigens or soluble complement receptor type 1 have been proposed to block immune responses to organ transplants (Cooper, D. K. et al. 1993; Nagasaka, T. et al. 1997; Pruitt, S. K. et al. 1994). However, this would require constant infusion to keep binding sites saturated, and would still only block a fraction of the recognition and rejection pathways.

Another approach to blocking biological adhesion events is to block binding via a physical barrier. For example, expanded poly(tetrafluoroethylene) and oxidized regenerated cellulose have been placed on peritoneal surfaces following peritoneal surgery to block the formation of adhesions between organ surfaces (Risberg, B. 1997). Hydrogels containing poly(ethylene glycol) have been coated on the peritoneal cavity to block adhesion formation (Hill-West, J. et al. 1994), coated on islets of Langerhans to block antibody binding to the islets (Cruise, G. M. et al. 1999), and coated on artery surfaces following balloon angioplasty to block intimal thickening (Hill-West, J. L. et al. 1994). Attempts have also been made to place intraocular lenses in such as way as to physically block lens epithelial cell migration onto the visual axis following cataract surgery (Apple, D. et al. 1992). In all the above approaches, a treatment in which long-lasting, conformal, intimate contact between the barrier and the surface is necessary for efficacy.

The approach in this work is to block adhesion via a molecular layer that coats the involved surfaces and sterically blocks the interaction of other substances with the treated

surface. Thus it will be necessary to keep the adhesive moieties (i.e., the receptor and protein ligand) far enough apart so that forces involved in biological adhesion do not overcome the repulsive steric forces offered by the polymer brush on the surface. A discussion of the repulsive properties of polymeric steric stabilization and of the basic principles of the design of these polymers is presented in the following section.

1.3 Polymeric Steric Stabilization of Colloids

The concept of preventing attractive interactions at surfaces has long been studied in the field of colloid stabilization (Leckband, D. et al. 1999; Napper, D. H. 1983; Overbeek, J. T. G. 1977; Russel, W. B. et al. 1989). Identical colloidal particles, typically ranging in size from 1 nm to 10 μm, in a chemically different liquid will always be thermodynamically driven to flocculate due to attractive van der Waals forces. These attractive forces work at ranges of approximately 5 nm and stabilization techniques must prevent the particles from approaching close enough for these attractive forces to become significant. The most common methods to prevent flocculation (and thus to stabilize the colloidal suspension) are based on electrostatic or polymeric stabilization. In electrostatic stabilization, like charges are introduced on the surfaces of all the particles, either by chemical modification or by binding of charged polymers to the surfaces. The approach of particles is then inhibited by the electrostatic repulsion between like charges and is dependent on the Debye length. The Debye length in a 100 mM aqueous solution of a 1:1 electrolyte is typically 1 nm. Thus for physiological fluids with electrolyte concentrations of approximately 150 mM, the Debye length is typically not adequate to prevent the particles from approaching close enough such that van der Waals attractive forces can start to dominate the interactions. By contrast, polymeric stabilization is usually independent of the ionic strength of the media and may be more suitable to provide for strong reliable stabilization in physiological fluids.

In polymeric stabilization, polymers are adsorbed or chemically grafted to the surfaces of the colloidal particles. When two particles come in close proximity to each other, the local concentration of surface-bound polymer increases and the number of configurations available to the polymers decreases, causing repulsive osmotic and entropic forces that drive the particles apart again. This is also termed steric stabilization. The particles may be stabilized by adsorbing homopolymers or by block or graft copolymers (Figure 1.1).
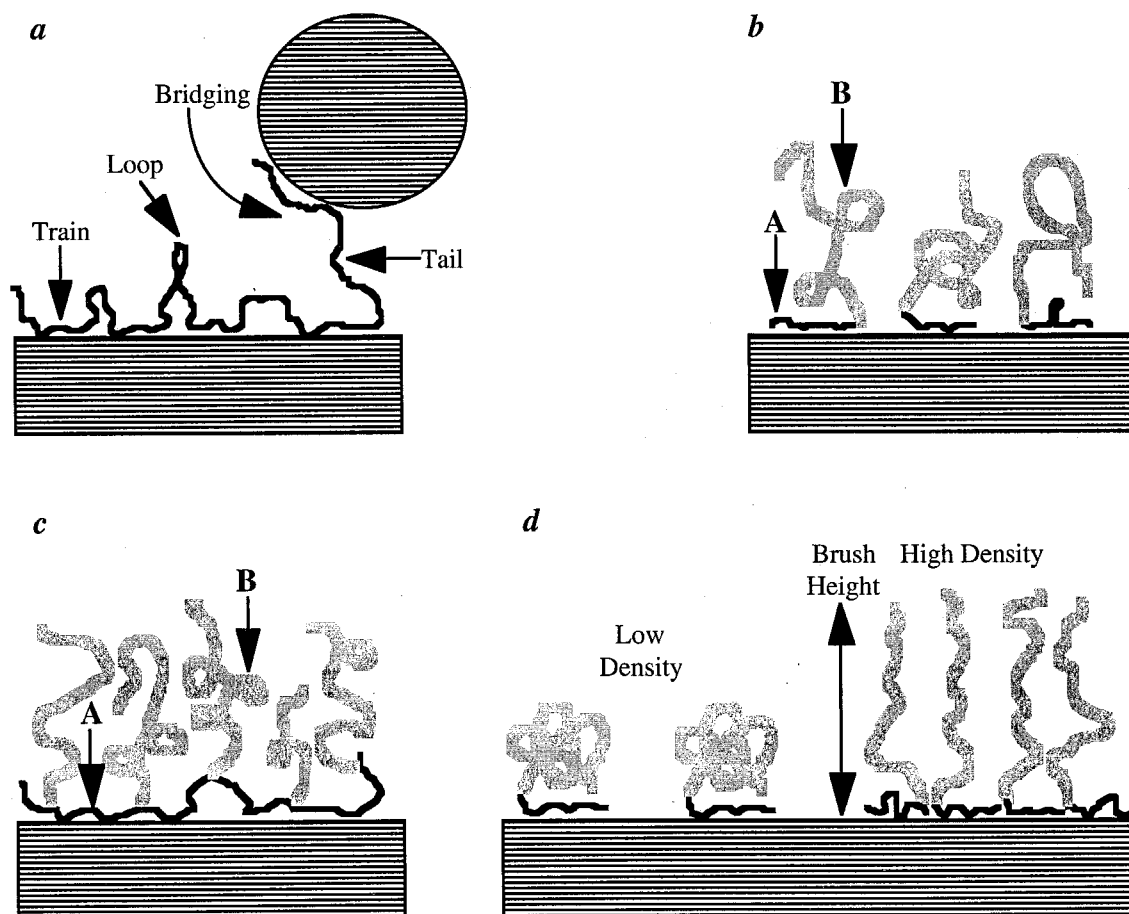
Figure 1.1. **Modes of polymeric stabilization of surfaces.**

a: An adsorbing homopolymer can form loops, trains, and tails on a surface. Tails typically extend far into solution and can cause bridging between two surfaces. b: An AB block copolymer has an anchor block that binds to the surface with high affinity (A) and a buoy block that is highly soluble in the solvent (B). c: A graft copolymer has an anchor backbone (A) with many buoy blocks (B) grafted to one backbone. d: Buoy blocks present at low and high densities. Buoys at low densities form mushrooms where the

height of the polymer is proportional to $N^{3/5}$. Buoys at high densities are forced to extend normal to the surface and the height of the brush thus formed is proportional to N.

Adsorbing homopolymers can bind to the surface at multiple points and have some affinity for the solvent as well as for the surface. Thus these polymers are in a dynamic equilibrium and usually form loops, trains, and tails. The tails can extend far into the bulk solution and can also lead to bridging, which is undesirable in the light of particle stabilization. The adsorption energy must compensate for the loss of configurational freedom of the polymer in solution and the forced additional segment-segment interactions, thus polymers with higher molecular weights (MWs) adsorb more easily because they sacrifice fewer configurational states. Polymers with higher MWs also desorb more slowly, due to the lower probability of all segments desorbing from the surface at the same time. If the homopolymers have strong adsorption interactions and full coverage on the surfaces, the interaction between particles is always repulsive. However, if the homopolymers have strong adsorption interactions and partial coverage on the surfaces or if the homopolymers have only weak adsorption, the particles may experience attractive interactions due to the potential for bridging. For this reason, block or graft copolymers are considered the most effective at stabilization of colloidal dispersions.

With block or graft copolymers, the anchor block or backbone is designed to bind strongly to the particle surfaces, while the buoy block(s) are highly soluble in the solvent and non-interacting with the substrate. Thus the polymer spontaneously assembles on the particle surfaces, with the anchor bound to the surface and the buoy block(s) extending into solution. This approach can also be used by chemically grafting the end of a polymer to the particle surface. The conformation of the buoy blocks is dependent on the surface density of the polymer. If there is a low surface density, the buoy blocks are in a similar conformation as to when they are in solution. They form "mushrooms" on the surface with a Flory radius of approximately $aN^{3/5}$ where a is the monomer size, N is the degree of polymerization, and when a good solvent for the buoy block is used. When the surface

density increases such that overlap of polymer chains can occur, the buoy blocks extend into solution and form a brush when the area per chain is small compared to the cross section of the free coil. At high surface coverage, the height of the polymer brush is directly proportional to N, instead of just $N^{3/5}$ as for low coverage. The interaction between two polymerically stabilized colloidal surfaces can either be no interaction, interpenetration of polymer chains from the two surfaces, or compression of the polymer chains. With at least moderate coverage in a good solvent for the buoy, approach of the surfaces leads only to compression and thus strong repulsion. A minimum layer thickness is necessary to mask van der Waals forces, and thus optimum stabilization occurs with a strong anchoring block, full surface coverage, and a sufficiently high buoy MW to maintain the minimum layer thickness. The binding strength of the anchor block depends on the affinity of the block for the surface and the solvent and on the MW of the block as discussed in the homopolymer section. While Flory-Huggins, Derjaguin-Landau-Verwey-Overbeek, and self-consistent field theories have all been used to model these polymer interactions, no adequate theory yet exists to describe thermodynamics of aqueous polymer solutions. Theoretical models and experimental tests of steric stabilization of surfaces will be further discussed in reference to poly(ethylene glycol) in section 1.6.

The effect of non-adsorbing polymers present in solution can also be important. Because fewer conformations are available for the polymer when it is close to the surface of a particle, a zone is depleted of polymer segments near each particle surface. Because these depletion zones have a lower concentration of polymer than the bulk solution, an osmotic force can drive together two particles if their zones overlap. Thus non-adsorbing polymer in solution can cause colloids to flocculate. This phenomenon has been shown to be the mechanism for high solution concentrations of poly(ethylene glycol) causing cells to fuse (Kuhl, T. et al. 1996).

1.4 Steric Stabilization of Biological Surfaces

1.4.1 Stabilization Used by Nature

The concepts of steric (polymeric) and electrostatic stabilization are utilized by living organisms. Cell and tissue surfaces are densely coated with oligosaccharides and with anionic charges, and these factors help prevent non-specific adhesion interactions between surfaces so that only specific interactions such as between receptors and ligand will occur (Foa, C. et al. 1996). The thick coating of oligosaccharides and charged residues that surrounds cell membranes is called the glycocalyx (Lodish, H. et al. 1995) and can be 20 - 30 nm or more thick. An example of a component of the glycocalyx is glycophorin A, the major glycoprotein in red blood cell (RBC) membranes. This glycoprotein is 60% carbohydrate and extremely rich in anionic sialic acids residues (Jackson, R. L. et al. 1973; Marchesi, V. T. et al. 1972). These sialic acid residues comprise 25% of the carbohydrate, being most concentrated at the most exterior region of the glycoprotein, and are responsible for the majority of the net anionic charge on RBC surfaces. The anionic charge of the sialic acids is important for electrostatic stabilization of the RBCs, helping prevent adhesion of the RBCs to the vasculature as well as to other cells. If these sialic acid residues are removed by enzymatic methods, macrophages show increased uptake of the RBCs and a decreased gap size in the contact area (Foa, C. et al. 1996). Removal of sialic acid residues from monocytes also increases adhesion of antibody-coated particles to the monocytes under flow conditions (Sabri, S. et al. 1995). Cell surface proteoglycans can also modulate cell adhesion via steric exclusion (Morris, J. 1993). The polysaccharides on the surfaces of bacteria have been shown to cause repulsive steric interactions that inhibit adhesion of the bacteria to surfaces (Rijnaarts, H. H. M. et al. 1995). Adhesion of bacteria to surfaces is modulated by electrostatic, steric, and van der Waals interactions. However, at an ionic strength of 0.1 M, steric interactions control adhesion to surfaces for many combinations of bacteria and surfaces (Rijnaarts, H. et al.

1995). These cell surface macromolecules can extend from the cell surface up to 100 nm into the surroundings and thus can modulate interactions at long distances. Steric stabilization of cell surfaces by surface polysaccharides can generate sufficient repulsive forces to overcome the thermal energy on the order of kT of a cell (modeled as a rigid body) (Foa, C. et al. 1996). A major determinant of the steric stabilization effects is the relative lengths of specific adhesion molecules and of adhesion resisting molecules on the surface, which is complicated by the folds, protrusions, and microvilli structures on the surfaces of cells (feature sizes on the order of 100 nm). Clearly, organisms have evolved a complex interplay of nonspecific electrostatic, steric, and van der Waals interactions as well as specific receptors and ligands that modulate interactions between surfaces.

1.4.2 Design of Stabilizers for Biological Surfaces

It is interactions with these complex surfaces that we now wish to control with polymers designed to block both nonspecific and specific adhesion interactions. The high concentration of oligosaccharides and acidic groups in the glycocalyx of cells renders it highly hydrophilic. The extracellular matrix is also dominated by highly hydrophilic and charged proteoglycans and by hydrophilic oligosaccharides on glycoproteins and glycolipids (Alberts, B. et al. 1994; Teti, A. 1992). Thus the traditional design of a surfactant with a hydrophobic backbone and hydrophilic side chains may not be the optimal design for coating biological surfaces with stabilizing polymers. However, it has been shown that poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) ABA copolymers, known as poloxamers or Pluronics, can adsorb to cell surfaces (Melik-Nubarov, N. S. et al. 1999). The limiting factor in the effectiveness of Pluronics on surfaces has often been shown to be the length of the hydrophobic backbone (and thus the strength of backbone binding) (Amiji, M. and Park, K. 1992; Malmsten, M. and Muller, D. 1999; McGurk, S. L. et al. 1999). Given the limiting strength of hydrophobic binding

and the hydrophilic nature of cell and tissue surfaces, an affinity interaction other than hydrophobicity may be the best choice for this polymer design.

Elbert and Hubbell first used the approach of polymeric steric stabilization of biological surfaces, and harnessed electrostatic attraction for their binding interaction (Elbert, D. L. and Hubbell, J. A. 1998; Elbert, D. L. and Hubbell, J. A. 1998). Because most cell and extracellular matrix surfaces and many secreted proteins are net anionic, a cationic poly-L-lysine (PLL) backbone was used. They constructed a graft copolymer, with a PLL backbone and pendant poly(ethylene glycol) (PEG) side chains (PLL-g-PEG), as PEG is well known for its protein- and cell- adhesion resisting properties (Harris, J. M. 1992; Leckband, D. et al. 1999). The size of the PLL backbone and the ratio of grafted PEGs were varied, while the binding to and steric stabilization of cell and protein surfaces was studied. A polymer with a 20 kDa PLL backbone and 1 grafted PEG chain (MW 5 kDa) for every 6-7 lysine mers was found to coat anionic tissue culture polystyrene or gelatin and to block cell spreading to those surfaces. This polymer also prevented adhesion of macrophages to confluent mesothelial cells in vitro. However, this PLL-g-PEG polymer was ineffective at blocking cell spreading on adsorbed serum proteins, extracellular matrix, and fibronectin mixtures. The mechanism of these effects was unclear, as radiolabelling studies showed approximately monolayer adsorption of the polymer to serum protein and extracellular matrix surfaces, and much lower polymer adsorption to a gelatin surface. The polymer was also shown to reduce the formation of peritoneal adhesions when applied as a lavage in vivo, but the effect may have been due to modulation of the structure of fibrin formed in the presence of the polymer. This PLL-g-PEG polymer was also ineffective at coating RBCs and blocking their agglutination by a lectin. When a similar polymer with the same PEG grafting ratio but a very large PLL backbone (375 kDa) was used, it was found to be more effective at steric stabilization. This polymer blocked cell spreading on tissue-culture polystyrene at many PEG grafting ratios and blocked cell spreading on adsorbed serum proteins and fibronectin mixtures at the optimal PEG grafting ratio. This

polymer also coated RBC surfaces and blocked their agglutination by a lectin, although it was ineffective at blocking agglutination induced by antibodies to blood group specific antigens (Elbert and Hubbell, unpublished data). Thus it appeared that the high molecular PLL backbone produced a more effective, albeit not completely effective, polymer. Considering the arguments presented earlier in section 1.3, it is likely that the longer backbone had significantly stronger binding to the surfaces due to greater multivalency of the binding (cationic lysine) groups and smaller losses in entropic freedom when adsorbing. Given the high ionic strength and low Debye lengths in physiological media, electrostatic interactions are rather effectively shielded and a high multivalency interaction may be required. In addition, the charge distribution on biological surfaces may be quite heterogeneous in many cases, and a longer backbone provides for "bridging" over uncharged sites. As an interesting side note, Elbert and Hubbell also found that a Pluronic was unable to block RBC agglutination by a lectin.

Given these results, it was sought to explore other polymer designs, to determine if the efficacy of these polymer therapeutics could be improved and if lower MW polymers (which generally would exhibit more favorable pharmacology and toxicology) could be used. Given the hydrophilic nature of biological surfaces and milieu, the need for strong binding to prevent desorption and bridging, the heterogeneity of biological surfaces, and the desire for a nonspecific interaction that would have a general affinity for most all biological surfaces, we turned to phenylboronic acid-containing blocks as moieties that can form reversible covalent bonds with *cis*-diols present in saccharides residues.

1.5 Phenylboronic Acid

The objective in this work was to design a polymer that could nonspecifically coat biological surfaces. This requires a binding interaction to groups that are ubiquitous on cell, extracellular matrix, and secreted protein surfaces. In addition, an affinity interaction

stronger than electrostatic was desired. Phenylboronic acid (PBA) is a moiety that can form reversible covalent bonds with *cis*-diols and other hydroxyls. Given that most cell surface and extracellular matrix proteins are heavily glycosylated with sugars that can contain *cis*-diols, PBA seemed an appropriate moiety to fit the design criteria.

1.5.1 Affinity of PBA for Biological Surfaces

There are many *cis*-diols present in saccharide residues in the body. Glycoproteins and proteoglycans are ubiquitous on cell surfaces, in serum, and in the extracellular matrix (Stryer, L. 1995). Cellular glycocalyces are dominated by saccharides, most secreted mammalian proteins are glycosylated, and the extracellular matrix is also rich in modified polysaccharides in the form of proteoglycans. The reducing ends of carbohydrates are attached to proteins either by O-glycosidic linkages to serine or threonine or by N-glycosidic linkages to asparagine (Kobata, A. 1992). N-linked sugar chains have a common pentasaccharide core containing 3 mannose and 2 N-acetylglucosamine residues. High-mannose type sugar chains then contain only further α-mannosyl residues. Complex type sugar chains contain no further mannose residues but contain N-acetylglucosamine, galactose, fucose, sialic acids, N-acetylgalactosamine, and sulfate groups. Hybrid type sugar chains contain features of both the high-mannose and complex types. O-linked sugar chains follow fewer structural rules. Sterically, a polymer is most likely to interact with the sugar residues at the terminal position. Most frequently, sialic acid residues occupy the terminal position on carbohydrates of glycoconjugates, and these usually contain hydroxyls at C7, C8, and C9 to which PBA can bind (Schauer, R. 1982). Other common terminal sugar residues which contain *cis*-diols include galactose, fucose, N-acetylgalactosamine, and mannose (Kobata, A. 1992).

The diversity in glycosylation of proteins is extremely high, and the greatest diversity is in the terminal sugar residues (Gagneux, P. and Varki, A. 1999). This allows

them to perform important functions in cell adhesion and recognition, including forming the sialyl-Le[a] antigen present on some cancer cells, the Galα1->3Gal epitope that causes hyperacute rejection of porcine xenotransplants, and the A and B blood group antigens. The ability of sugar residues to link by any of their hydroxyl groups, to take on both furanose and pyranose forms, and to be further modified by acetylation, sulfation, etc., contributes to their great diversity in structure. Common glycsoaminoglycans on proteoglycans include chondroitin sulfate, keratan sulfate, heparin, dermatan sulfate, and hyaluronate (Stryer, L. 1995). These are made of disaccharide repeating units and are modified by carboxylate, sulfate, or acetyl groups which also add to diversity. Glycosylation patterns of glycoproteins, proteoglycans, and glycolipids also vary for different species, tissues, ages, transformed states, and even within the same cell types depending on the local environment (Dennis, J. W. et al. 1999; Hardingham, T. E. and Fosang, A. J. 1992; Hughes, R. C. 1992; Kobata, A. 1992; Paulsson, M. 1992). Thus it is difficult to predict the exact saccharides that will be presented on surfaces tested in this work, but there is in general a pervasive presence of terminal sugar residues on biological surfaces to which we can anticipate that the polymer's PBA groups may bind.

It has been demonstrated that molecules containing PBA can bind to cell surfaces. Burnett et al. synthesized a fluorescent boronic acid which could bind to bacterial cell walls and a diboronic acid that could bind to RBC surfaces and agglutinate them (Burnett, T. J. et al. 1980). Aoki et al. found that endothelial cells adhered to and differentiated into capillary structures when cultured on a surface coated with a PBA-containing polymer (Aoki, T. et al. 1995). Miyazaki et al. synthesized a PBA-containing polymer which acted as a mitogen for lymphocytes, presumably cross-linking cell surface glycoproteins as mitogenic lectins do (Miyazaki, H. et al. 1993). Ikeya et al. coated a surface with a PBA-containing polymer and demonstrated selective B cell adhesion to the surface, with little binding of T cells (Ikeya, T. et al. 1998).

1.5.2 PBA interaction with *cis*-diols

The interaction between PBA and *cis*-diols has been known for some time (Figure 1.2). It has been exploited mostly for chromatographic applications (Singhal, R. P. and DeSilva, S. S. M. 1992), but recent applications have expanded to include sugar sensing (James, T. D. et al. 1995; James, T. D. et al. 1996; Kimura, T. et al. 1995; Kitano, H. et al. 1998; Niwa, M. et al. 1998), glucose and glycation product detection (Eggert, H. et al. 1999; Frantzen, F. et al. 1995; Kataoka, K. et al. 1995; Kikuchi, A. et al. 1996), glucose responsive insulin delivery (Kataoka, K. et al. 1998; Kitano, S. et al. 1992; Shiino, D. et al. 1994), controlling enzyme activity (Hamachi, I. et al. 1997; Snow, R. J. et al. 1994), and transport of hydroxyl-containing species through membranes (Gallop, P. M. et al. 1982; Mohler, L. K. and Czarnik, A. W. 1993; Takeuchi, M. et al. 1996; Westmark, P. R. et al. 1996).

PBA can form reversible covalent complexes with 1,2 diols, 1,3 diols, 1,3,4 triols, dicarboxylic acids, α-hydroxy carboxylic acids, 1,2 dihydroxybenzenes and polyalcohols such as poly(vinyl alcohol) (Kitano, S. et al. 1991; Norrild, J. C. and Eggert, H. 1995; Pizer, R. D. and Tihal, C. A. 1996; Singhal, R. P. and DeSilva, S. S. M. 1992). However, binding with 1,2 and 1,3 diols is the most common and this interaction is shown in Figure 1.2. The structure of boronic acid complexes in aqueous solution is still under dispute, but considerable evidence exists that the furanose form of sugars is preferred over pyranose forms (Cooper, C. R. and James, T. D. 1998; Norrild, J. C. and Eggert, H. 1995). PBA shows preferential affinity among sugars, and the binding affinity among common sugars decreases in the following order: fructose > galactose > mannose > glucose (James, T. D. et al. 1996).
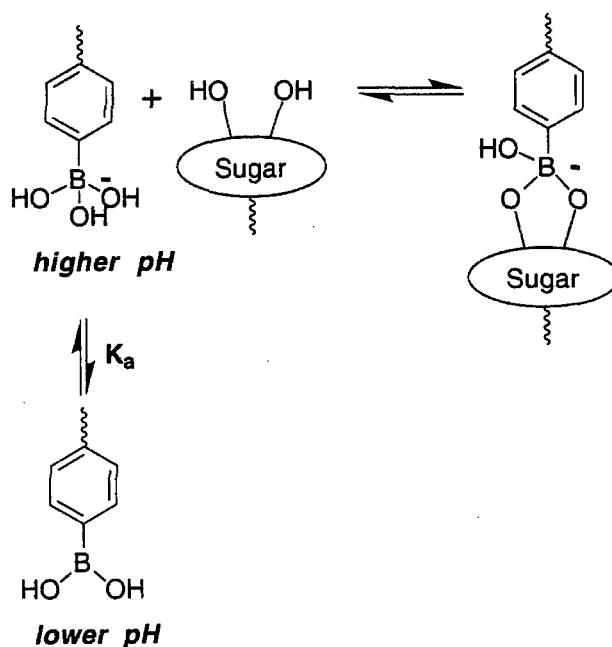
Figure 1.2. Schematic of PBA interaction with hydroxyls in aqueous solution.

PBA acts as a Lewis acid and accept a hydroxyl at high pHs. When in the higher pH tetrahedral form, PBA can form a reversible covalent complex with *cis*-diols as shown here for a sugar residue. The complex formed is quite stable, especially because the pKa of the PBA is lowered once it has bound a sugar. The pKa of free PBA is usually around 8.8.

As can be seen in Figure 1.2, in aqueous solutions, PBA complexation with diols occurs predominantly when the boron is in the tetrahedral form (which occurs at higher pHs). Because the pKa of typical PBA moieties is around 8.8, this significantly limits complexation of PBA at physiological pH as well as requires harsher conditions for chromatography applications (Kitano, S. et al. 1991; Singhal, R. P. et al. 1991). Thus, significant effort has been made to synthesize PBA moieties with lower pKas, by stabilizing the anionic tetrahedral form (Singhal, R. P. and DeSilva, S. S. M. 1992). Techniques include adding electron withdrawing groups such as nitro, carboxyl, or fluorocarbons to the phenyl ring (Shiino, D. et al. 1993; Singhal, R. P. et al. 1991; Soundararajan, S. et al. 1989), adding internal coordinate bonds between the boron and a carbonyl oxygen (Liu, X. C. and Scouten, W. H. 1994), or forming a ferrocene derivative

of the PBA with oxidation of the iron atom (Moore, A. N. J. and Wayner, D. D. M.

1999). A great deal of work has shown that placing cationic amines in proximity to the

PBA can also lower the effective pKa of the moiety. Yurkevich et al. found that quaternary

amines in proximity to PBAs on a resin allowed binding over a wide pH range (Yurkevich,

A. M. et al. 1975), Wulff et al. lowered the pKa by linking a tertiary amine on the phenyl

ring ortho to the boron such that an intramolecular B-N bond could form (Wulff, G. 1982),

and Fischer and Havinga found that the boron in 3 and 4-pyridineboronic acids had a pKa

≤ 4 (Fischer, F. C. and Havinga, E. 1974). A number of researchers have since

demonstrated PBA pKa lowering by tertiary amines linked to the ortho position allowing

B-N interactions (James, T. D. et al. 1995; James, T. D. et al. 1994; Takeuchi, M. et al.

1996), a tertiary amine substituent in the meta carbon position of the phenyl ring (Eggert,

H. et al. 1999), and copolymers containing PBA groups and tertiary or quaternary amine

groups (Kitano, H. et al. 1998; Kitano, S. et al. 1991; Niwa, M. et al. 1998; Shiino, D. et

al. 1996), all hypothetically working via electron withdrawing effects or by electrostatic

stabilization of the anionic boron (Takeuchi, M. et al. 1996). Ludwig et al. even found that

adding a soluble quaternized polyamine or quaternized amphiphilic amine to a monolayer of

amphiphilic PBAs at an air-water interface increased sugar binding by the monolayer at

neutral pH (Ludwig, R. et al. 1993). Thus covalent bonding to amines is not necessary to

lower the effective pKa of PBA, only proximity in solution which can provide a cationic

environment. Nevertheless, only a handful of PBA moieties cited in the literature have

measured pKas lower than 7, and most involve complicated syntheses or have limited

water solubility (Eggert, H. et al. 1999; Fischer, F. C. and Havinga, E. 1974; James, T.

D. et al. 1994; Moore, A. N. J. and Wayner, D. D. M. 1999; Shiino, D. et al. 1993;

Wulff, G. 1982).

1.5.3 PBA Toxicology

PBA itself is not likely to cause problems when administered *in vivo*. Most of the toxicology and metabolic studies of borates have focused on boric acid, which can form the same types of complexes with hydroxyls and amines as PBA can (Hunt, C. D. 1998). Borates can interact with the *cis*-diols on ribose moieties such as found in NAD^+, NADP, and FAD nucleotides, and thus can affect the activity of enzymes that require these as cofactors. In fact, boron is known to influence the activities of at least 26 enzymes directly, by binding cofactors, or by unknown mechanisms. These include oxidoreductases, transferases, hydrolases, and isomerases; serine proteases have received special attention for their importance in regulatory roles. Borates can act as reversible competitive inhibitors, and this has been postulated as one of boron's roles as an essential nutrient. Borates have also been postulated to influence ion transport across the cell membrane. These biological roles may lead to boron's effects on plasma estradiol, testosterone, HDL cholesterol, and vitamin D levels (Samman, S. et al. 1998). Borates are known to be an essential nutrient for plants and are believed to also be so for humans. Borates are freely adsorbed orally and are distributed throughout the body water and also concentrate in bones (Murray, F. J. 1998). However, borates are not metabolized and are excreted renally with an elimination half life less than 24 h. This may explain why, in spite of their ability to interact with many enzymatic pathways, only in cases of very high doses do borates cause toxic effects (Hubbard, S. A. 1998). With chronic exposure to boric acid, no-observed-adverse-effect-levels of 9 - 43 mg boron/kg/day have been found, with higher doses resulting in testicular atrophy and reduced male fertility, and developmental effects to the fetus and mother, especially reduced fetal weight. No evidence of mutagenicity or carcinogenicity has been found in boric acid, borax, or disodium octaborate tetrahydrate.

In the applications considered in this work, the boronic acid-containing species would be attached to a water-soluble polymer. This immediately limits the metabolic

pathways that might be influenced. The polymer component would largely prevent entry of the phenylboronate compound into the intracellular compartment except via endocytosis from membrane-bound materials, with shuttling to the lysosomal pathway of material destruction.

1.6 Poly(ethylene glycol)

Besides the PBA binding groups, the other component of the polymer designed in this work must be capable of blocking the undesired adhesion interactions. Hydrophilic polymers have often been employed to block biological interactions with surfaces (Elbert, D. L. and Hubbell, J. A. 1996; Monfardini, C. and Veronese, F. M. 1998). Examples of these include dextran (Frazier, R. A. et al. 2000; Holland, N. B. et al. 1998; Osterberg, E. et al. 1995), polymers with phospholipid polar groups (Ishihara, K. et al. 1999), hyaluronic acid (Morra, M. and Cassineli, C. 1999), alginic acid (Morra, M. and Cassineli, C. 1999), heparin (Amiji, M. and Park, K. 1993), albumin (Amiji, M. and Park, K. 1993), and poly(ethylene glycol). Associating these polymers with surfaces has been shown to reduce protein, platelet, and cell adhesion to the surfaces as well as thrombus formation. However, of all the hydrophilic polymers, poly(ethylene glycol), or PEG, has received by far the most attention and seems to be the most effective at sterically stabilizing underlying surfaces.

1.6.1 PEGylating Surfaces

PEG has received much attention ever since it was shown to block immunological recognition of proteins to which it was covalently bound (Abuchowski, A. et al. 1977) and to suppress the binding of blood elements to surfaces onto which it was immobilized (Mori, Y. et al. 1982). PEG has been found to be extremely effective at "masking" underlying surfaces, and a full review of its properties and uses can be found in Harris's

books (Harris, J. M. 1992; Harris, J. M. and Zalipsky, S. 1997). PEG has very unique properties, including high solubility in both water and organic solvents, both an upper and lower critical solution temperature in water, hydrogen bonding and metal complexing, high flexibility and high mobility, non-immunogenicity and lack of toxicity, not being harmful to cells even though it interacts with cell membranes, and being rather chemically inert except for reactions at the end groups (Harris, J. M. 1992; Leckband, D. et al. 1999). The effectiveness of PEG at steric stabilization in aqueous systems has been attributed to its high degree of water bonding, low interfacial free energy with water, lack of binding sites for proteins, high surface mobility and highly extended conformation, and consequent large excluded volume. Steric stabilization forces from PEG on surfaces have been measured (Claesson, P. and Golander, C. 1987) and PEG's effect on surfaces has been shown to be due to more than just lowering the contact angle of water (Prime, K. and Whitesides, G. 1991). PEG is also sometimes referred to as poly(ethylene oxide), though this term is usually used for molecules with MWs above 20 kDa that have only one terminal hydroxyl due to a different catalytic method used in synthesis, whereas PEG MWs between 1 and 20 kDa are most frequently used in biological steric stabilization applications.

The literature is replete with examples of PEG rendering surfaces more resistant to protein adsorption and cell adhesion. PEG has been covalently grafted to poly(ethylene terephthalate) (Gombotz, W. R. et al. 1991), poly(styrene) (Delden, C. J. V. et al. 1996; Osterberg, E. et al. 1995), poly(tetrafluoroethylene) (Wang, P. et al. 2000), silicon (Zhang, M. et al. 1998), glass (Jenney, C. R. and Anderson, J. M. 1999), and liposomes (Needham, D. et al. 1992). PEG has been included in copolymers or interpenetrating networks with poly(styrene) (Delden, C. J. V. et al. 1996; Dunn, S. et al. 1994), poly(ethylene terephthalate) (Desai, N. and Hubbell, J. 1992), trimethylolpropane triacrylate (Drumheller, P. and Hubbell, J. 1995), and poly(urethane) (Kim, J. H. et al. 2000). Surfactants with PEG as the buoy segments have also been extensively used. The most widely used are Pluronics, which contain two PEG buoy blocks separated by a

poly(propylene oxide) anchor block. They have been used on poly(styrene) (Green, R. J. et al. 1998; Harper, G. et al. 1991; Harper, G. et al. 1995; McGurk, S. L. et al. 1999), poly(ethylene) (Amiji, M. and Park, K. 1992; Lee, J. H. et al. 1989), and hydrophobic-treated glass (Amiji, M. and Park, K. 1992; Gingell, D. and Owens, N. 1994). Other PEG surfactants include PEG-poly(butylene oxide) on hydrophobic glass or poly(ethylene) (Gingell, D. et al. 1994; Lee, J. H. et al. 1989; Malmsten, M. and Muller, D. 1999), PEG-poly(styrene) on poly(ethylene terephthalate) (Nakayama, Y. et al. 1999), PEG-poly(lactide) on hydrophobic glass (Malmsten, M. and Muller, D. 1999), and PEG and hexanal grafted to poly(vinyl amine) adsorbed onto graphite and poly(ethylene) (Vacheethasanee, K. and Marchant, R. E. 2000). These surfaces have been shown to resist the adhesion of proteins, platelets, cells, and bacteria, to resist uptake by macrophages and Kupffer cells, to fail to stimulate the formation of foreign body giant cells, and to exhibit reduced inflammatory and fibrotic responses when implanted *in vivo*.

The goal in this work was to associate PEG with living biological surfaces, rather than non-living material substrates. This can present a challenge to preserving viability and function but has been previously explored with some success. Interfacial photopolymerization of acrylated PEG macromers has been utilized to form gels on surfaces within blood vessels (Hill-West, J. L. et al. 1994), surfaces within the abdominopelvic cavity (Hill-West, J. et al. 1994), and islets of Langerhans (Cruise, G. M. et al. 1999; Cruise, G. M. et al. 1998). Thermally gelling Pluronics have been gelled on surfaces within the abdominopelvic cavity (Leach, R. E. and Henry, R. L. 1990) and around blood vessels (Simons, M. et al. 1992). Chemical grafting of activated PEG molecules has also been employed to PEGylate and block biological recognition of red blood cell surfaces (Armstrong, J. K. et al. 1997; Jeong, S. T. and Byun, S. M. 1996; Murad, K. L. et al. 1999; Scott, M. D. et al. 1997), blood vessel surfaces (Deible, C. R. et al. 1998), and islets of Langerhans (Panza, J. L. et al. 2000).

1.6.2 Modeling PEGylated Surfaces

An overall conclusion from most studies of steric stabilization by PEG in biological applications is that higher densities and larger MWs of PEG on the surface are generally more effective (Brindley, A. et al. 1995; Delden, C. J. V. et al. 1996; Drumheller, P. and Hubbell, J. 1995; Dunn, S. et al. 1994; Harper, G. et al. 1995; Wang, P. et al. 2000). However, the relative importance of these two factors of grafting density and molecular weight and the absolute values that are necessary for efficacy are less clear. These issues have been addressed theoretically by a number of investigators.

Jeon et al. conducted an extensive investigation of steric stabilization by PEG grafted to surfaces, in terms of blocking protein adhesion to those surfaces (Jeon, S. and Andrade, J. 1991; Jeon, S. et al. 1991). Steric repulsion, van der Waals attraction, and hydrophobic interactions were considered, where the interaction of a hydrophobic domain on a protein with a hydrophobic surface to which a PEG brush had been grafted was modeled. In their first, simpler approach, the protein was given an infinite size. They then found that a high surface density and large MW of the PEG was optimal, with the surface density being the more important parameter. In a second approach, the protein was given a finite spherical size and hydrophobic interactions between PEG and the protein were considered, although the interaction parameters were approximated. In this case, an optimum surface density for PEG occurred, at which point steric repulsion developed more rapidly than hydrophobic attractions. This optimum density was a function of the size of the protein, with the optimum distance between grafted PEGs being 9 - 11 Å for a protein with a radius of 20 Å, 11 - 15 Å for a radius of 40 Å, and 13 - 17 Å for a radius of 60 - 80 Å. However, maximal MWs of the PEG were still optimal. Martin and Wang recently showed that this model does not fully predict behavior when the degree of polymerization is less than 1000 (Martin, J. I. and Wang, Z. G. 1995). However, the general conclusions and trends that can be drawn from this model continue to hold.

Szleifer modeled the interactions of lysozyme with a PEG-grafted surface, using Single-Chain Mean-Field theory (Szleifer, I. 1997). Inhomogeneity in the z direction was allowed, a spherical protein was considered, and protein-polymer, protein-solvent, and polymer-solvent interactions were all considered to be equal. When no polymer attraction to the underlying surface was allowed, Szleifer found that the equilibrium protein adsorption was independent of the degree of polymerization of the PEG when the value was above 50. A distance less than 16 Å between PEGs was sufficient to completely block protein adsorption. Interestingly, that number correlates quite well with the results of Jeon et al. When attraction between the polymer and the surface was approximated, larger degrees of polymerization were more effective at blocking protein adsorption. Szleifer also noted that the rate of protein adsorption also slows with increasing polymer MW, by orders of magnitude. Depending on the application intended for a PEGylated surface, it may be possible to slow the rate enough such that the surface is adequately kinetically protected, if not thermodynamically at equilibrium.

In an effort to understand why PEG seems so much more effective at sterically stabilizing surfaces than other similarly hydrophilic polymers, Torchilin et al. compared a flexible and a rigid polymer (Torchilin, V. P. et al. 1994). The density of the polymer as a function of distance from the surface was modeled for a flexible polymer and a more rigid one (which had 5 times greater distance between mobile segments than for the flexible polymer). They showed that a flexible polymer was much more concentrated near its grafting point on the surface, and thus more effectively sterically blocked the surface, when grafted at a high enough density. While the model did not consider interactions between nearby polymers, it did effectively illustrate their result that PEG MW 5 kDa is much more effective at blocking protein adsorption to a liposomal surface than dextran MW 6 kDa, at the same molar grafting densities.

Leckband et al. focused on the design criteria to create non-fouling (protein and cell resistant) surfaces with PEG (Leckband, D. et al. 1999). In blocking binding of proteins

to surfaces, these systems differ from colloidal steric stabilization in that the density of the PEG brush will interact differently with proteins of different size, and that the PEG brush will increase the local viscosity and thus provide a diffusional as well as steric barrier to the protein adsorbing to the underlying surface. In addition, hydrogen bonding, the heterogeneous hydrophobicity and non-rigidity of proteins, the non-simplistic nature of PEG (such as its ability to form helices in water), the polydispersity of PEG, and non-smooth surfaces are not accounted for in theoretical models. Also, kinetic stability may be sufficient for given applications, even if the eventual thermodynamic equilibrium would result in unacceptable adhesion. The rate of protein adsorption is directly proportional to the inverse of the degree of polymerization of the grafted PEG. Their recent work showed that PEG-containing bilayers can be <u>attractive</u> to streptavidin-containing bilayers, when two bilayer surfaces are pushed together with sufficient force (Sheth, S. R. and Leckband, D. 1997). However, at lower compressive loads, the bilayers showed repulsive forces with ranges and magnitudes as a function of grafting density that did fit the theories of Jeon et al. This work again pointed to the complex nature of PEG and the inability of current models to fully predict its interactions.

1.6.3 Structure-Function Studies with PEG

A number of experiments have also been conducted in an effort to elucidate the greater efficacy of PEG over other hydrophilic polymers in sterically stabilizing surfaces. Prime et al. formed self-assembled monolayers on gold, and attached various end groups to the alkane thiols in mixed monolayers (Pale-Grosdemange, C. et al. 1991; Prime, K. and Whitesides, G. 1991). They found that monolayers containing oligo(ethylene glycol) headgroups ($N = 6$) repelled the adsorption of five different proteins more effectively than glucose or hydroxyl terminated surfaces, even when the surfaces had the same advancing water contact angle. Osterberg et al. showed that end-grafting PEG to poly(styrene)

rendered it more protein-resistant than end-grafted or side-grafted dextran (Osterberg, E. et al. 1995). End-grafted dextran was the least effective, which may likely be due to dextran's rigid helical structure, whereas the PEG chains were highly extended even though packed at a lower molar density on the surface, implying high water content and mobility of the PEG chains. Marchant et al. designed surfactant copolymers where hexanal side chains assembled on graphite or poly(ethylene), and hydrophilic PEG or dextran side chains were then ordered on the surface (Holland, N. B. et al. 1998; Vacheethasanee, K. and Marchant, R. E. 2000). PEG side chains repelled bacterial adhesion more effectively than dextran chains, even when present at the same mol% in the copolymer.

A major difficulty in studying the steric stabilization of surfaces by PEG is that the density of PEG chains on the surface is usually not known, and when the MW is varied, the density of PEG chains on the surface also varies (because PEG chains bound to the surface repel the binding of more PEG chains to the surface, with this steric hindrance being a function of MW). Marchant et al. did show that surfaces containing 1 PEG of MW 2 kDa every 55 Å were not as effective at blocking cell adhesion as surfaces that presumably had denser degrees of PEGylation. Gombotz et al. found that increasing PEG MW increased protein resistance but plateaued at 3.5 kDa; however, the molar density of PEG chains on the surface varied at the same time (Gombotz, W. R. et al. 1991). Jenney et al. found that their highest MW PEG (18.5 kDa) was most effective at blocking long term macrophage density and foreign body giant cell formation (Jenney, C. R. and Anderson, J. M. 1999). While very approximate, their data implied that a similar PEG mer density was present on surfaces grafted with PEG 18.5 kDa and 4.6 kDa, yet the 18.5 kDa surface was the most resistant. Irvine et al. compared surfaces grafted with linear PEG MW 10 or 20 kDa or grafted with star PEGs MW 233 and 360 kDa (PEG stars have a central core and many pendant PEG arms) (Irvine, D. J. et al. 1998). The distances between PEG molecules on the surfaces were 33, 39, 139, and 132 Å, respectively. All surfaces were resistant to the adsorption of proteins after 4 h of incubation, except that a

small amount of the smallest protein adsorbed to the surface with the 360 kDa star PEG. They were able to show that this binding was due to the star's geometry, which concentrated the PEG arms at 80 Å from the surface and allowed proteins to be present at $z < 80$ Å, whereas the other PEGylated surfaces had the PEG concentrated within 50 Å of the surface, with the peak density near $z = 0$.

When PEGylating surfaces via surfactants, the relative affinity of the PEG and anchor chains for the surface and solvent is also important. Many studies have shown that stabilization by Pluronics is dependent on having a long enough anchor backbone (Amiji, M. and Park, K. 1992; Green, R. J. et al. 1998). This is balanced by the PEG buoy chains, as more/longer PEG chains will tend to attract the entire surfactant copolymer into solution. Thus high MW PEG chains are desirable, but only when balanced by a long enough anchor (Lee, J. H. et al. 1989; McGurk, S. L. et al. 1999). Longer PEG chains have been shown to repel particles at longer distances from the surface (Gingell, D. and Owens, N. 1994). Malmstem and Muller found that, for a given number of anchor and buoy mers, surfactants with an AB geometry bound more to surfaces than those with an ABA or BAB geometry, as the AB geometry allowed the buoy chains to stretch out more in the z direction (Malmsten, M. and Muller, D. 1999). Kenausis et al. studied the binding of PLL-g-PEGs to metal oxide surfaces (Kenausis, G. L. et al. 2000). They found that similar amounts of polymer adsorbed to the surfaces whether the PLL MW was 20 or 375 kDa and whether the PEG was 2 or 5 kDa, and all surfaces were quite resistant to protein binding.

Due to PEG's great affinity for water, solutions containing linear PEG can have a depletion zone of PEG near any surface (Kuhl, T. et al. 1996). If the center of the PEG is less than half the solution radius of gyration away from the surface, its conformations are limited, thus fewer molecules are present near the surface. This has been shown to cause attractive forces between phospholipid vesicles in PEG solutions and is the proposed mechanism for cell fusion induced by PEG solutions. A common biochemical protocol is

to fuse cells by incubating them in a PEG solution for a short period of time. However, this requires very high concentrations of PEG (i.e., 50 vol%) and can be quite toxic to the cells (Freshney, R. I. 1994).

1.6.4 PEG Toxicology

In spite of PEG's toxicity at very high concentrations, PEG is in general very nontoxic (Harris, J. M. 1992; Wade, A. and Weller, P. J. 1994; Working, P. K. et al. 1997). PEG is FDA approved for internal consumption and injection and is used as a pharmaceutical excipient, as well as in cosmetics and personal care items. Low MW PEGs (i.e., less than 400 Da) can be oxidized to toxic molecules *in vivo*, but this toxicity decreases rapidly with MW increase. Oral LD_{50} values of 50 g/kg have been found for PEG 4 or 6 kDa (Wade, A. and Weller, P. J. 1994). An intravenous LD_{50} value of 16 g/kg was found for PEG 4 kDa in mice (Wade, A. and Weller, P. J. 1994). Chronic intravenous infusion of 90 mg/kg/day of PEG 3350 Da for 178 days showed no adverse effects in dogs (Working, P. K. et al. 1997). The no-observed-adverse-effect-levels of subcutaneous injections of PEG MW 1450 were determined to be 600 - 1500 mg/kg/day for chronic doses up to 28 days in dogs and rats (Working, P. K. et al. 1997). PEG of MW 1 kDa or greater is minimally absorbed from the gastrointestinal tract. PEG is renally excreted and the terminal elimination half life of PEG MW 1450 in plasma was found to be 3 - 4 days (Working, P. K. et al. 1997). PEG has also been found safe in mutagenicity, reproductive effect, teratogenicity, and carcinogenicity assays, and PEG is poorly immunogenic. Solutions of PEG are hospitable to living cells, and are even used in organ preservation.

1.7 Outline of Experiments

The work reported in this dissertation investigated the steric stabilization of biological surfaces by a copolymer containing segments designed to anchor the polymer on surfaces and other segments designed to resist binding of biological elements. PBA groups will form the anchoring segments, binding to saccharide groups on proteoglycans and other sugar-modified species. The buoy segments will be comprised of PEG, a highly hydrophilic and mobile polymer.

Preliminary experiments with polymer architectures determined a graft copolymer to show the most promise. In this configuration, a long backbone containing PBA groups will have a number of PEG side chains grafted to it. This architecture has a larger MW backbone than an AB architecture, which may promote binding of the polymer to the surface as discussed in section 1.3. Many researchers have shown that placing many binding moieties on a polymer backbone can produce much stronger interactions with the ligand than the same concentration of monovalent binders (Reuter, J. D. et al. 1999; Tagawa, K. et al. 1999). Thus the avidity effect of multivalent binding on a long backbone may provide for stronger, longer lasting binding to surfaces. This may also reduce the likelihood of bridging events, if binding of the anchor to surfaces is strong enough, and there is adequate PEG present to prevent other surfaces from approaching close enough to bind to any unbound PBA residues.

It was chosen to build the copolymer on a PLL backbone. The amine functionalities would be conducive to chemical grafting reactions with PBA and PEG groups. In addition, the amine functionalities could be harnessed to lower the effective pKa of the PBA moieties. PLL is water soluble, commercially available in a number of molecular weights, and will undergo slow hydrolysis to yield the naturally occurring amino acid lysine.

Thus a family of PLL-g-(PEG;PBA)s was synthesized, where the numbers of PEG chains and PBA moieties grafted per PLL backbone were varied. The hypothesized

interaction of this polymer with biological surfaces is shown in Figure 1.3. The PBA backbone could spontaneously assemble on cell or tissue surfaces and surround them with a dense PEG brush that would then sterically inhibit other proteins and cells from interacting with the surfaces. The following chapters describe the synthesis of these polymers, testing the binding of the polymers to model surfaces, testing the ability of the polymers to sterically stabilize model surfaces, and applying the polymers to two clinically-relevant models *in vivo*.

In Chapter 2, the synthesis, purification and characterization of the copolymers will be discussed. Grafting PBA to the PLL backbone by reductive amination produced a secondary amine linker. This construction of the polymer produced PBA moieties with a pKa around 6, which provides for efficient binding at physiological pH and represents a simple route to low PBA pKas from commercially available reagents. The PBA and PEG grafting ratios were varied, and the following chapters investigate the effect of the grafting ratios on the binding and steric stabilization of these polymers on surfaces.

In Chapter 3, the binding of the polymers to a model carbohydrate surface and the blocking of protein binding by steric stabilization of RBC surfaces will be explored. The PLL-g-(PEG;PBA) polymers bound to a mannan-conjugated resin via the PBA moieties. It was found that the PEG grafting ratio sterically controlled how much of the polymer bound to surface, with higher degrees of PEG grafting leading to less surface binding. Polymers with optimal graft ratios spontaneously chemisorbed to RBCs and prevented their agglutination by lectins and antibodies. Moreover, the PLL-g-(PEG;PBA) copolymers sterically stabilized cell surfaces more effectively than the electrostatically-binding PLL-g-PEG analogs.
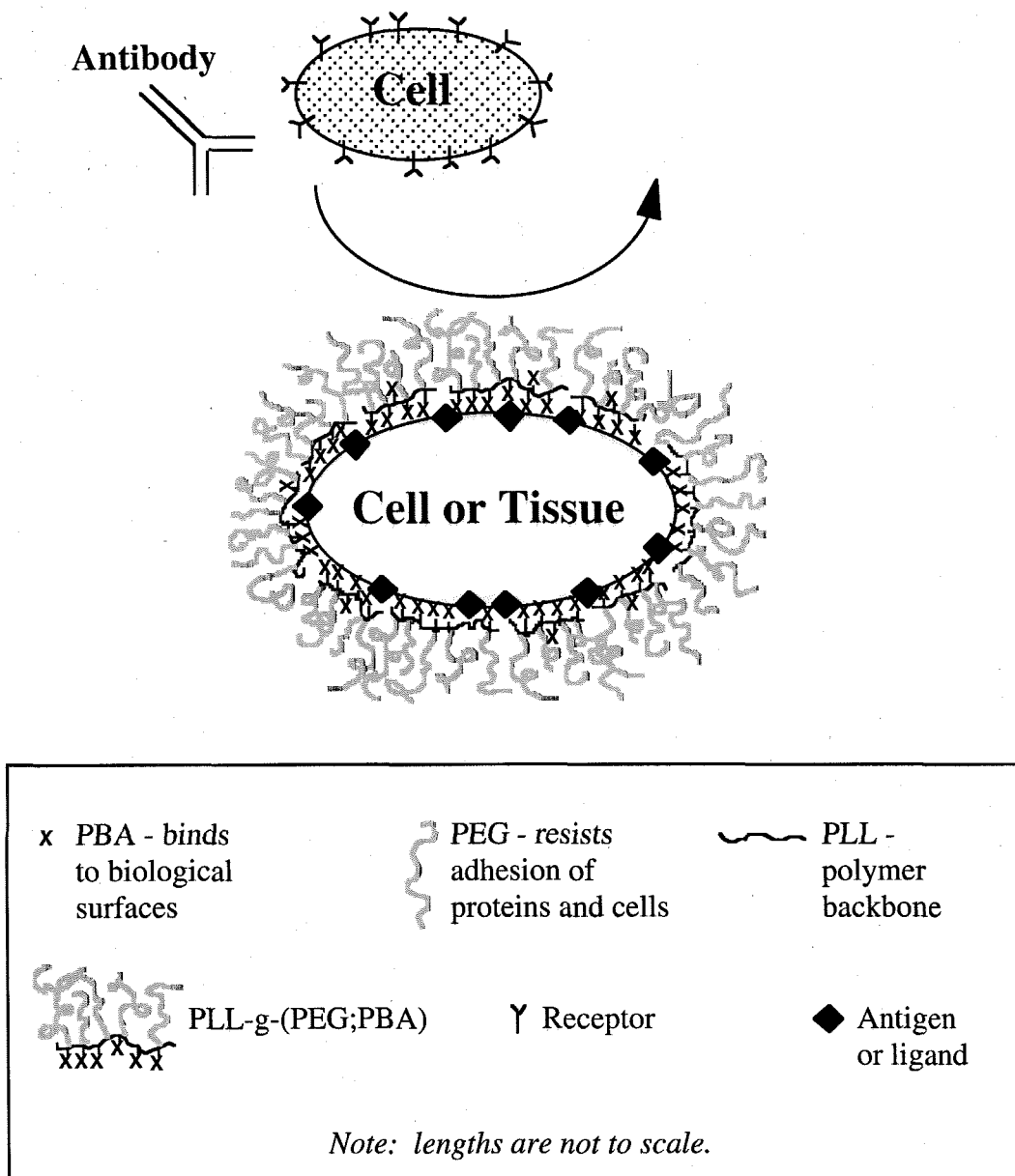
Figure 1.3.   Interaction of PLL-g-(PEG;PBA) with biological surfaces.

PBA moieties on the polymer backbone spontaneously assemble on cell or tissue surfaces. The surface is then surrounded by a dense PEG brush which sterically blocks other proteins and cells from interacting with the treated surface.

Blocking cell adhesion to surfaces *in vitro* via steric stabilization with PLL-g-(PEG;PBA) will be discussed in Chapter 4. PLL-g-(PEG;PBA) polymers with optimal grafting ratios blocked adhesion and/or spreading of cells onto tissue culture polystyrene, adsorbed serum proteins, or ECM. On tissue culture polystyrene, the polymer effect was shown to be local, long lasting, and easy to pattern. The polymers were less effective on the proteinaceous surfaces, and not effective on a surface of Matrigel proteins, but were still more effective than their PLL-g-PEG analogs. The polymers may be more effective at binding to cell surfaces than to ECM surfaces due to the differing glycosylation patterns of these surfaces. Toxicity testing *in vitro* indicated significant dose-dependent toxicity of some of the polymers. However, the response of the cells was anomalous and the polymers did not exhibit apparent toxicity *in vivo* as discussed in the following two chapters.

Chapters 5 and 6 examine the application of the polymers to two clinically relevant *in vivo* models. The models were chosen in part because polymer binding to ECM (more so than living cells) would be necessary, and because ECM has only slow turnover, the polymer would be expected to remain for quite some time. Chapter 5 discusses application of the polymer to lens capsules following cataract surgery to prevent posterior capsule opacification. While preliminary results showed some promise for the polymer, difficulties with achieving the opacification response in control animals prevented a true test of the polymer. Chapter 6 discusses application of the polymer to surfaces within the peritoneal cavity following lysis of peritoneal adhesions, to prevent peritoneal adhesion reformation. Application of the polymer was shown to prevent the animals' conditions from worsening, whereas control animals' adhesions became significantly worse. Because the polymers may be more efficient at binding to cell surfaces than ECM surfaces, they may have more efficacy in models that require polymer binding to RBC surfaces or to other cell surfaces where surface clearance by endocytosis is not limiting. In both surgical models, no toxic responses to the high doses of polymers applied were noted.

This thesis concludes with Chapter 7, where general conclusions and suggestions for future work are made. The PLL-g-(PEG;PBA) polymers were shown to be capable of sterically stabilizing biological surfaces, both from protein adhesion and from cell adhesion. Their construction can be further varied for tailoring to specific applications as well as to avoid cytotoxicity if indeed they can be toxic. The polymers also show versatility for applications not investigated in this work, including drug delivery and treatment of biomaterial implants.

2. Chapter 2

Synthesis and Characterization of PLL-g-PEG and PLL-g-(PEG;PBA)

2.1 Introduction

The goal of this work was to design a copolymer that would spontaneously bind to biological surfaces and protect those surfaces from undesirable adhesion and recognition interactions. This would provide a physicochemical method of blocking these interactions, similar to the stabilization of colloids by polymeric steric stabilization. Thus it was desired to design a water-soluble graft copolymer that contained backbone groups that would strongly bind ubiquitous molecules on cell and extracellular matrix surfaces, consequently anchoring the grafted side chain non-binding groups to the surface and rendering the surface highly resistant to protein binding and cell adhesion. Such a polymer treatment would provide a molecular barrier to adhesion that could be applied by simply exposing the desired surfaces to an aqueous polymer solution.

Poly(ethylene glycol) (PEG) was chosen for the non-binding groups, as side chains of a graft copolymer. PEG is well known for its protein-adsorption- and cell-adhesion-resisting properties (Harris, J. M. 1992). Phenylboronic acid (PBA) was chosen as the binding domain moiety in the backbone of the graft copolymer. PBA forms reversible covalent complexes with cis-diols (Figure 2.1), such as those found in sugar residues in glycoproteins that are found on the cell surface and in the extracellular matrix, e.g., sialic acid, galactose, mannose, and fucose residues. These complexes only form when the boron is tetrahedral and thus are most prevalent above the PBA pKa, which is usually around 8.8. Kataoka and colleagues have demonstrated that incorporation of amines into PBA-containing polymers can lower the effective pKa of the PBA to around 7, and that

these polymers can bind strongly to cell surfaces via interaction with plasma membrane glycoconjugates (Aoki, T. et al. 1995; Shiino, D. et al. 1996). Thus, we chose to construct a polymer using PBA binding domains on an amine-containing polymer backbone and PEG non-binding domains grafted as pendant side chains. In such a graft copolymer, the PBA-bearing backbone would be expected to complex with biological surfaces, while the attached pendant PEGs would form a polymer brush that could sterically protect the underlying surface from adhesive interactions.
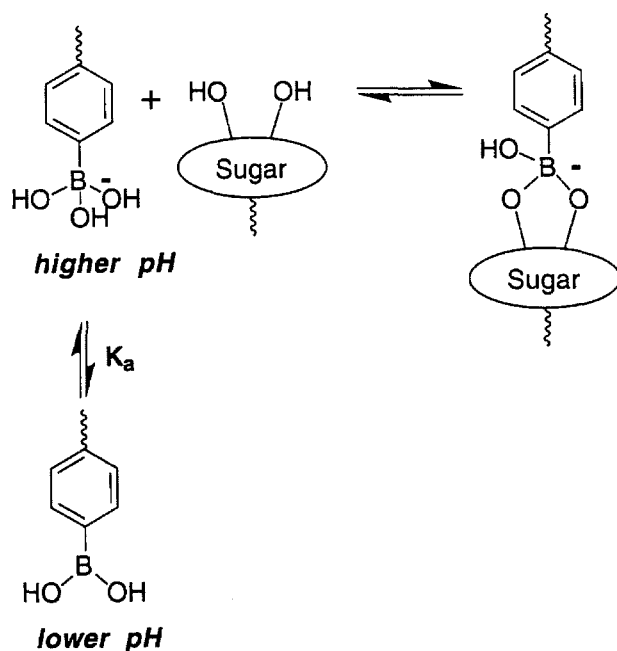
Figure 2.1. PBA complexation with a *cis*-diol-containing moiety.
Shown here complexing with a residue of an oligosaccharide. PBA is in the trigonal, non-binding state below its pKa and in the anionic tetrahedral binding state above its pKa. The pKa of the PBA moiety is usually approximately 8.8, but the PBA moieties synthesized in this work have a pKa around 6.

It was hypothesized that the ability of the PBA-containing copolymers to sterically stabilize biological surfaces would be governed by a balance of binding (via PBA) and non-binding (via PEG) interactions. Increasing the number of grafted PEGs per polymer backbone would increase the ability of a surface-bound polymer to sterically protect the

surface, but would also sterically inhibit the polymer backbone from binding to the surfaces to be treated. Thus it was expected that there would be an optimum PEG grafting ratio to provide the most effective steric stabilization of an otherwise adhesion-promoting surface. It was also hypothesized that a maximum number of PBA moieties per polymer backbone would provide for the strongest binding of the polymer to surfaces.

To evaluate such a construction, poly-L-lysine-*graft*-(polyethylene glycol; phenylboronic acid) (abbreviated PLL-g-(PEG;PBA)) was synthesized, where the numbers of PEG chains and PBA moieties per PLL backbone were varied. PLL was chosen as the polymer backbone because amines on the backbone provide a facile handle for reaction with PEG and PBA, with easy variance of grafting ratios. The PLL also allows for placing amines in proximity to the PBA groups, which may lower the pKa of the PBA moieties. Further, the PLL backbone is derived from naturally-occurring amino acids and will eventually degrade along the amide backbone.

PEG was first grafted to the PLL, to enhance solubility of the PLL for further reaction with PBA as well as to avoid any possible side reactions between the activated PEG and PBA. By first synthesizing PLL-g-PEG, this also provided control polymers with the exact same PEG grafting ratio but without the function of the PBA groups, allowing further investigations of the PBA function in binding and stabilization studies. The well-known reaction between carbonyldiimidazole-activated PEG and lysine amino groups was used to conjugate the PEG to the PLL (Beauchamp, C. O. et al. 1983).

PBA was then grafted to the PLL-g-PEG via reductive amination. 4-formyl-PBA is commercially available and can form a stable, secondary amine linker following reductive amination to a primary amine. The reductive amination protocol was adopted from Abdel-Magid et al., using sodium triacetoxyborohydride as a reducing agent in a one-step synthesis (Abdel-Magid, A. F. et al. 1996). This reducing agent is mild, selective, and often superior to other reducing agents such as sodium cyanoborohydride, while avoiding the well-known contamination with the toxic by-products HCN and NaCN that can occur

when using sodium cyanoborohydride. Because PLL (and thus PLL-g-PEG) is highly charged and hydrophilic, whereas 4-formyl-PBA is hydrophobic, it is difficult to find a solvent common for all components of the reaction system. Dichloromethane was found to be appropriate, allowing at least slight solubility of all the components involved. This approach would not have been possible had not PLL-g-PEG been first synthesized, to bring the PLL into organic solvent by conjugation with the organic-soluble PEG.

The PLL-g-PEG and PLL-g-(PEG;PBA) polymers were synthesized using the methods described above, with varying graft ratios. Covalent conjugation was confirmed and grafting ratios were calculated by ^{1}H NMR and HPLC analysis. The pKa of the PBA moieties in the polymers was analyzed by base titration, and found to be approximately 6, which should lead to strong binding of these groups at physiological pH.

2.2 Materials and Methods

Copolymer synthesis

PEG was grafted to PLL via carbonyldiimidazole activation. 500 mg PLL•HBr, MW 24 kDa (Sigma, St. Louis, MO) was dissolved in 12.5 mL of sodium borate buffer (50 mM, pH 9) and sterilized by passing through a 0.2 µm filter. Various stoichiometric ratios of solid α-monomethoxy, ω-oxycarbonylimidazole PEG, MW 5 kDa (mPEG-CDI, donated by Shearwater Polymers, Huntsville, AL) were added. Each reaction mixture was stirred for 24 h at room temperature (rt). The reaction mixtures were then dialyzed (10 kDa MWCO) against phosphate-buffered saline (PBS: 137 mM NaCl, 10 mM phosphate, 3 mM KCl, pH 7.4). The mixtures were subsequently dialyzed against 0.14 M saline, then deionized distilled water. The purified PLL-g-PEG product was lyophilized.

PBA was conjugated to the backbone of PLL-g-PEG via reductive amination. PLL-g-PEG was combined with 2 equivalents of 4-formyl-PBA (Aldrich, Milwaukee, WI), where the number of non-PEGylated lysine mers was considered 1 equivalent. The

reaction flasks were then filled with Ar and remaining operations were performed under an Ar atmosphere. ≥2 equivalents of sodium triacetoxyborohydride (Aldrich) were added. Anhydrous dichloromethane was added so the lysine mer concentration was 0.05 M, following which 2 equivalents of triethylamine were added. The reaction mixtures dissolved into a slurry within 30 min, as expected due to limited solubility of various components in the solvent, and the reaction mixtures were stirred for 24 h, rt. Following this, ≥1 equivalent of sodium triacetoxyborohydride and 2.5 equivalents of glacial acetic acid were added, and the reaction mixtures were stirred for another 24 h, rt. The reaction mixtures were then removed from the Ar atmosphere. Dichloromethane was removed by rotary distillation, and the reaction products were neutralized with 1 M NaOH, with additional rotary distillation as necessary. The solutions were lyophilized, dissolved in 0.1 - 1 M HCl such that the pH was 1, and then desalted on a polyacrylamide column (exclusion limit 6 kDa, Econo-Pac 10DG, BioRad, Hercules, CA), using 0.01 M HCl as the mobile phase (the acidity was necessary to prevent precipitation of products in the column). The reaction products were dissolved at 75 mg/mL and up to 3 mL of this solution was applied to the desalting column. The UV absorbance of the eluent at 265 nm was monitored, and baseline separation of the polymer peak and small molecule peak was obtained. The polymer-containing aliquots (mL 3-7) were combined, and this PLL-g-(PEG;PBA) solution was lyophilized.

Analysis of copolymer composition

The number of PEG chains grafted per PLL backbone was determined by size exclusion HPLC of the PLL-g-PEG polymers (the PLL-g-PEG polymers were analyzed because the polymers containing PBA would be retained on most size exclusion resins). The polymers were dissolved in sodium bicarbonate buffer (0.2 M, pH 10), and applied at 0.3 mL/min to a size exclusion column (Shodex SB-804 HQ, Alltech, Deerfield, IL). The refractive index (RI) of the eluent was monitored. The RI chromatograms for each PLL-g-

PEG batch showed two clearly separated peaks, one due to the PLL-g-PEG copolymer and one due to unreacted PEG. Each peak in the RI signal was integrated, and the average number of PEG chains grafted per PLL backbone in each batch was calculated using the integral values, the known feed masses of PLL and PEG, and the RI increment for protein (PLL) and PEG (0.18 and 0.134 mL/g, respectively) (Brandrup, J. and Immergut, E. H. 1989).

^{1}H NMR was performed on the PLL-g-PEG and PLL-g-(PEG;PBA) products to confirm their structure and purity. Polymers were dissolved at 10 mg/mL in D_2O and analyzed on a Bruker AM500 spectrometer. The average number of PBA moieties attached per PLL backbone in each batch was calculated by integrating the area of the aromatic PBA protons (located at 7.5 and 7.9 ppm) and of the lysine side chain methylene protons (located between 1.3 and 1.9 ppm).

pKa measurement

The pKas of the PLL-g-(PEG;PBA) polymers were determined by base titration. 75 mg of polymer was dissolved in 3 - 3.5 mL of deionized, distilled H_2O. The solution was degassed and placed under a N_2 atmosphere. 1 M HCl was added so the pH was approximately 2. 0.03 M NaOH (degassed) was injected at 1.98 mL/h with vigorous stirring, and the pH was recorded by computer every 1 s.

2.3 Results

PLL-g-(PEG;PBA) synthesis

PLL-g-(PEG;PBA) copolymers were synthesized by first grafting mPEG-CDI, MW 5 kDa, to the PLL backbone, MW 24 kDa, followed by conjugating 4-formyl-PBA to the PLL-g-PEG via reductive amination (Figure 2.2). A sample of each PLL-g-PEG compound was reserved before PBA conjugation, allowing comparison of polymers that

Figure 2.2. Synthesis scheme for PLL-g-PEG and PLL-g-(PEG;PBA).

PLL is reacted with mPEG-CDI to form PLL-g-PEG. PBA groups are then conjugated to remaining free lysinyl amines to form PLL-g-(PEG;PBA), via reductive amination.

m is the degree of polymerization of the PEG chains (here 113 mers). x is the number of free lysine mers in the polymer, y is the number of PBA groups conjugated to the polymer backbone, z is the number of PEGs conjugated to the polymer backbone, and x+y+z is the number of total lysine mers in the backbone. For PLL-g-(PEG;PBA) 1:2, 1:3, and 1:6, x = 0 (and some lysine mers have 2 PBA moieties conjugated to the epsilon amine, instead of just one as shown in the figure). For PLL-g-(PEG;PBA) 1:9 and 1:21, x > 0. For PLL-g-PEG, y = 0.

did and did not contain the PBA moieties. The copolymers will be referred to by their PEG

graft ratio, which will be defined as the ratio of grafted PEG chains per lysine mers in the

backbone. Thus PLL-g-(PEG;PBA) 1:21 has the lowest number of grafted PEGs on the

backbone (1 grafted PEG per every 21 lysine mers) and PLL-g-(PEG;PBA) 1:2 has

thehighest number of grafted PEGs (1 grafted PEG per every 2 lysine mers).
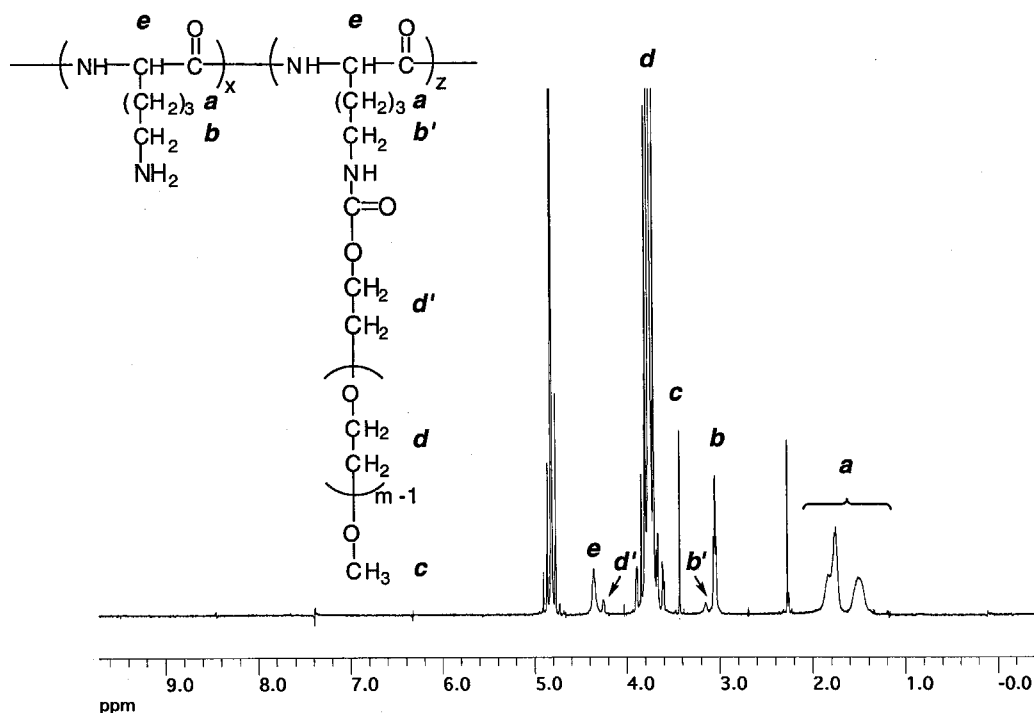
Figure 2.3. ¹H NMR spectrum of PLL-g-PEG 1:9.

Solvent: D_2O. The PLL-g-PEG spectra were equivalent to previously published (Sawhney, A. and Hubbell, J. 1992) spectra (the peak at 2.3 ppm is an impurity in the deuterated solvent). The expected shoulder peaks due to covalent conjugation of PLL to PEG were observed (**b'** and **d'**). These peaks were proportionally larger in polymers with higher graft ratios (i.e., PLL-g-PEG 1:2).

Covalent attachment of PEG to the PLL backbone was confirmed by NMR and by aqueous size exclusion HPLC. Figure 2.3 shows the ^{1}H NMR spectrum for PLL-g-PEG 1:9. The expected urethane linkage peaks (Sawhney, A. and Hubbell, J. 1992) appeared in the PLL-g-PEG NMR spectra, with proportionally larger peaks for copolymers having higher PEG grafting ratios. However, because these peaks are very small and overlap with other peaks, the exact degree of PEG grafting was determined by performing size exclusion HPLC. Figure 2.4 shows the refractive index traces from the HPLC. The traces show two clearly separated peaks, one for the PLL-g-PEG copolymer and one due to unreacted
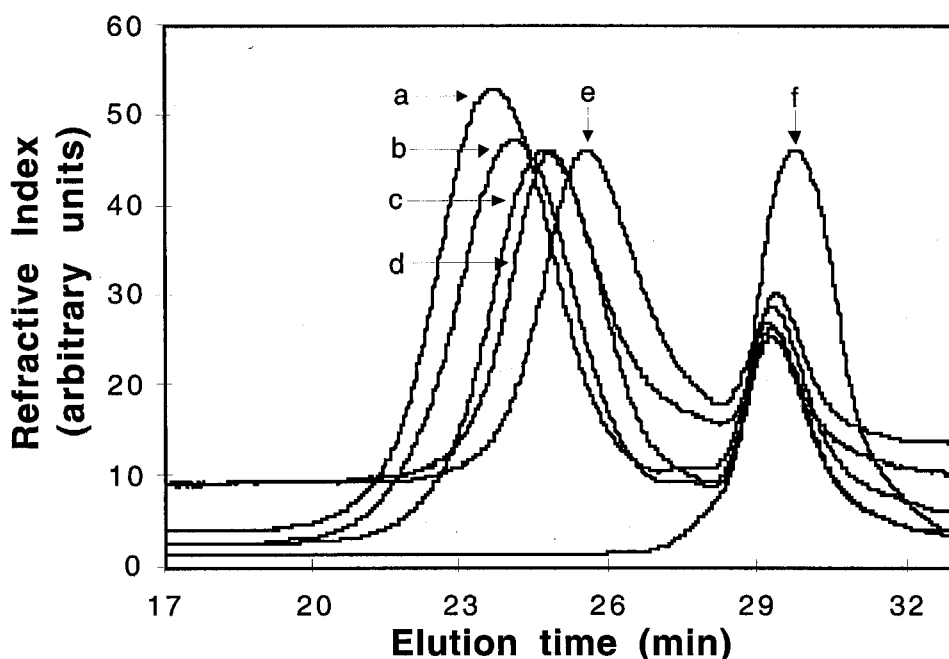
Figure 2.4. Size exclusion HPLC chromatograms of PLL-g-PEG.
PLL-g-PEG 1:2 (a), 1:3 (b), 1:6 (c), 1:9 (d), 1:21 (e), and monomethoxy-PEG MW 5 kDa (f) were analyzed in a pH 10 sodium bicarbonate buffer (the pKa of lysine is approximately 10). The refractive index of the eluent was monitored, and scaled for presentation. Two separate peaks were clearly evident in the traces for each product - one due to the PLL-g-PEG copolymer and one due to unreacted PEG (overlapping with the trace of monomethoxy-PEG MW 5 kDa). These peaks were integrated for calculation of the covalent PEG grafting of each PLL-g-PEG.

PEG. These peaks were integrated and combined with the known feed masses and the RI

increments for PLL and PEG in order to calculate the graft ratios of the polymers.

Covalent attachment of PBA to PLL-g-PEG was confirmed by NMR, gel filtration,

and UV spectroscopy. Following PBA conjugation to PLL-g-PEG, gel filtration of the

reaction mixture provided baseline separation of the PLL-g-(PEG;PBA) polymer from the

unreacted PBA precursor and other small molecules (Figure 2.5). ^{1}H NMR of the purified
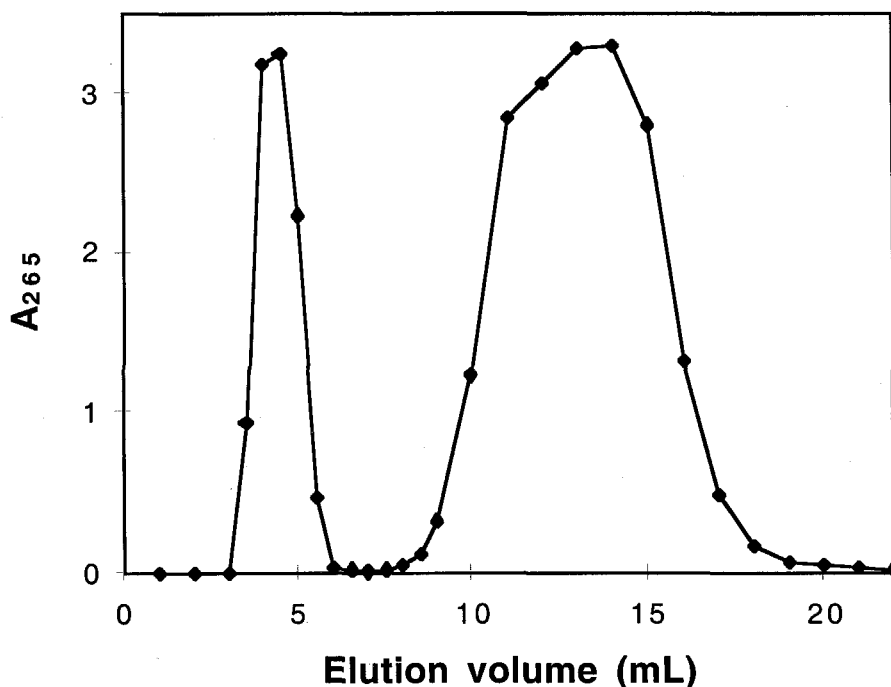
Figure 2.5. Gel filtration of PLL-g-(PEG;PBA) 1:9 reaction mixture.

The PLL-g-(PEG;PBA) reaction mixtures were applied to polyacrylamide gel filtration
columns (exclusion limit 6 kDa) and the UV absorbance of the eluents were monitored.
Baseline separation between the polymer peaks and small molecule peaks was obtained.
Polymer-containing aliquots (mL 3-7) were combined and analyzed.
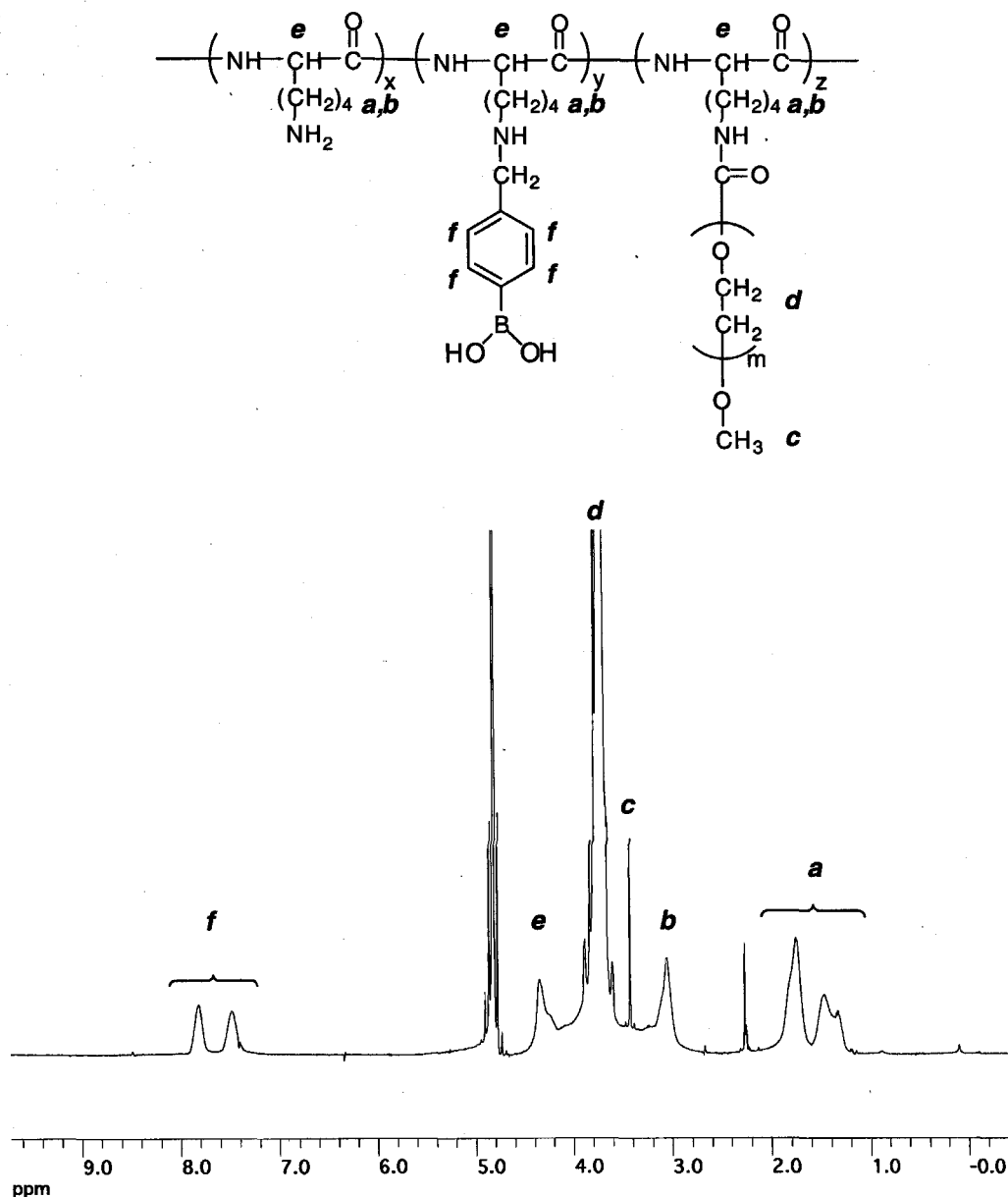
Figure 2.6. ^{1}H NMR of PLL-g-(PEG;PBA) 1:9.

Solvent: D$_2$O. New peaks at **f** confirm addition of the phenyl moiety to PLL-g-(PEG;PBA). The unconjugated PBA in the gel filtration small molecule fraction contained sharp doublets at 7.5 and 7.9 ppm. If gel filtration runs did not result in baseline separation, unconjugated PBA present in polymer fractions was thus easily confirmed in the NMR spectra, which showed sharp doublet peaks overlapping the smooth polymeric PBA peaks.

polymer confirmed conjugation of PBA to the polymer (Figure 2.6). New phenyl peaks were observed at 7.5 and 7.9 ppm. Inadequate separation by gel filtration that did not result in baseline separation of the polymer and small molecules could be confirmed by NMR, as unconjugated PBA peaks appeared as sharp doublets at 7.5 and 7.9 ppm. These sharp doublets could be observed in the gel filtration small molecule fraction, as well as in the polymer fraction of any filtrations that did not result in baseline separation (only polymers purified by gel filtration runs that resulted in complete removal of unconjugated PBA were used in this work). The presence of the PBA moiety in the PLL-g-(PEG;PBA) polymers was also confirmed by their UV spectra, which show an absorbance peak around 265 nm as expected for aromatic-containing molecules (Figure 2.7). The degree of PBA
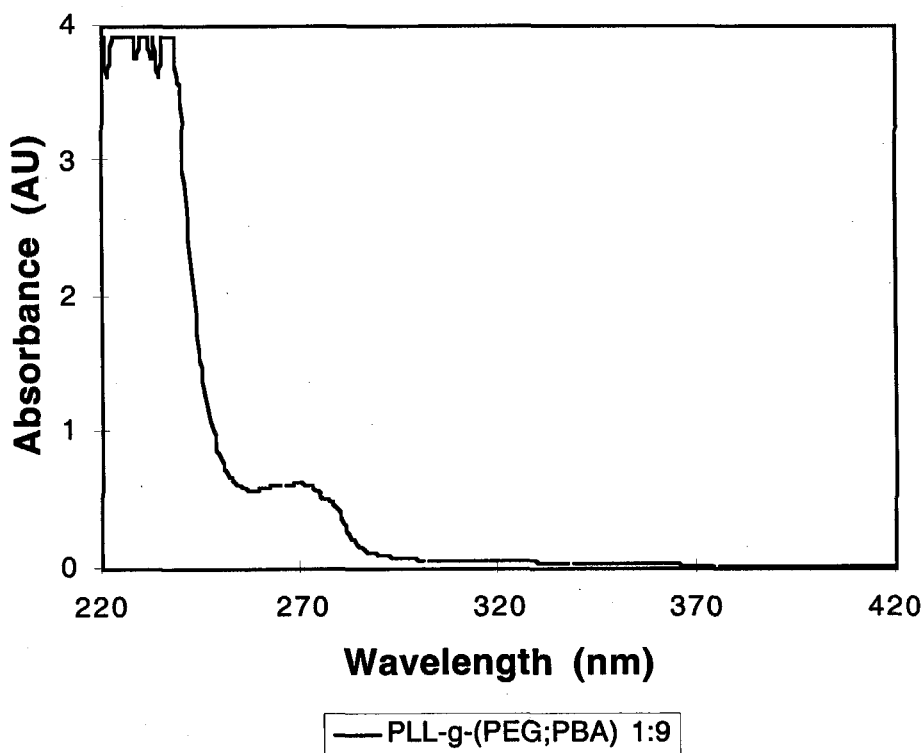
Figure 2.7.   UV spectrum of PLL-g-(PEG;PBA) 1:9.

The PLL-g-(PEG;PBA) polymers were dissolved at 0.2 wt% in PBS, pH 7.4. Strong absorbance peaks at 265 nm correlated with expected absorbance due to a phenyl ring on the polymer (the spectra of the PLL-g-PEGs did not contain any peaks around 265 nm).

conjugation of the PLL-g-(PEG;PBA) polymers was determined from the NMR integrals of the PBA aromatic protons (f) and lysinyl aliphatic protons (a). These values also correlated well with the UV absorbance at 265 nm of PLL-g-(PEG;PBA) solutions of known concentrations. It should be noted that the PLL-g-(PEG;PBA) polymers showed complete solubility in aqueous solutions at the highest concentrations used (2 wt%).

The degree of PEG and PBA grafting in the final PLL-g-(PEG;PBA) compounds is given in Table 2.1. It should be noted that PLL-g-(PEG;PBA) 1:2, 1:3 and 1:6 have an average of ≥ 1 PBA moiety per unPEGylated lysine mer, whereas PLL-g-(PEG;PBA) 1:9 and 1:21 have an average of <1 PBA moiety per unPEGylated lysine mer. It should also be noted that the separations scheme did not remove unreacted PEG (any unreacted mPEG-CDI hydrolyzes to produce monomethoxy-PEG-OH), and this free PEG remains in the final PLL-g-(PEG;PBA) and PLL-g-PEG compounds. As described above, this free monomethoxy-PEG-OH is quantified and is not included in the measurement of the grafting density of the PLL-g-PEG and PLL-g-(PEG;PBA) copolymers.

pKa of PBA moieties

The pKa of the PBA moieties in PLL-g-(PEG;PBA) 1:3 and 1:9 were determined to be 5.8 and 6.4, respectively. The pKas were measured by base titration (Figure 2.8). The precise values of the pKas were then calculated from the titration curves using Gran Titration equations (Figure 2.9). Gran Titration equations are used to determine pKa values of dilute compounds (Pankow, J. F. 1991). These equations use simple equalities such as proton balance equations, coupled with assumptions about dominant species in different areas of the graph (i.e., $[H^+] >>$ [titrand in the basic form] $+ [OH^-]$, in the very low pH region for F1). F1 is plotted in the low pH region, where the strong acid (i.e., HCl) is being neutralized. $F1 = (V_o + V_a + V_t)*10^{-pH}$, where V_o is the initial volume of the solution, V_a is the volume of strong acid added to lower the pH before titration, and V_t is the total volume of basic titrant added (the variable on the x-axis). Based on the

Table 2.1. Analysis results of PLL-g-(PEG;PBA) copolymers.

PEG Graft Ratio	1:21	1:9	1:6	1:3	1:2
# PEGs attached per PLL backbone	6	13	19	36	56
# PBAs attached per PLL backbone	41	65	99	100	123
# PEGs attached per lysine mer in backbone (*graft ratio*)	1:21	1:9	1:6	1:3	1:2
# PBAs attached per unPEGylated lysine mer in backbone	0.4	0.6	1.0	1.2	2.0
MW of polymer (calculated from known PEG and PBA grafting)	49,000	91,000	124,000	210,000	311,000
wt% PEG in polymer (not including free PEG)	65%	82%	86%	92%	95%
wt% unreacted PEG in final product	17%	20%	21%	15%	15%

PLL MW is 24 kDa, PEG MW is 5 kDa. The degree of PEG grafting was determined from the known feed ratios and integration of the PLL-g-PEG and PEG peaks in size exclusion HPLC of the PLL-g-PEG precursors. The degree of PBA conjugation was determined from the NMR integrals of the lysine and PBA peaks. Using the nomenclature in Figure 2.2, row 1 is z, row 2 is y, row 3 is z/(x+y+z), and row 4 is y/(x+y) (except where an average of >1 PBAs are attached per unPEGylated lysine mer, then rows 2 and 4 are adjusted accordingly to account for the greater number of PBAs present per each unPEGylated lysine mer). In the text, the polymers are referred to by their PEG graft ratio.
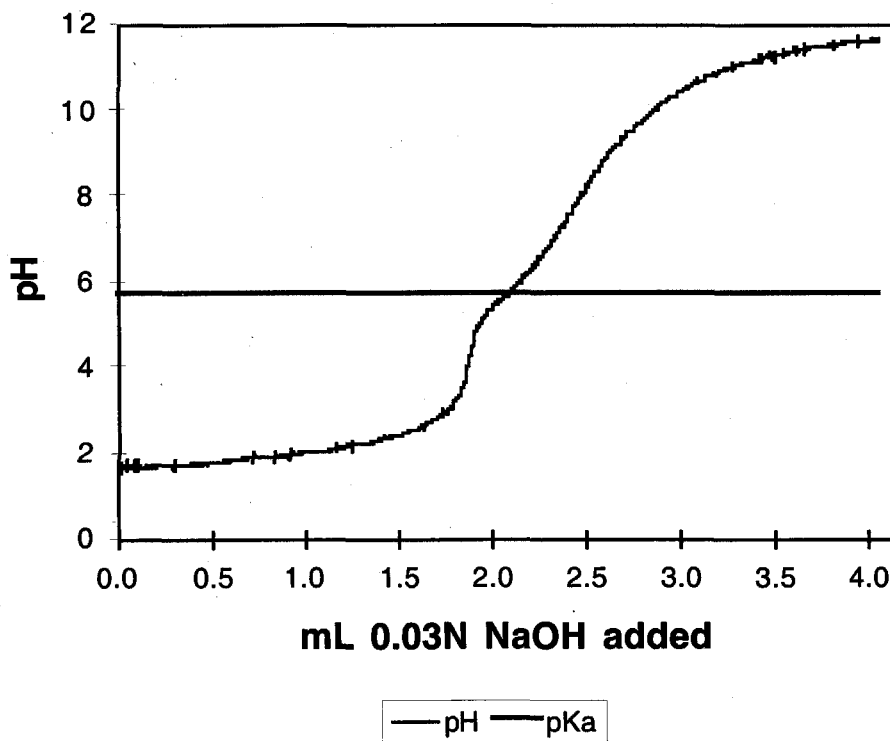
Figure 2.8. Titration curve of PLL-g-(PEG;PBA) 1:3.

The polymer (75 mg) was dissolved in degassed water (3.5 mL) and kept under nitrogen during titration. A distinct hump in the curve is visible near pH 6, and the exact value of the pKa was calculated with Gran Titration equations. The curve for PLL-g-(PEG;PBA) 1:9 looks similar, except it contains additional broadening due to residual free lysinyl amines. A pKa of 5.8 was obtained for the 1:3 copolymer, and 6.4 for the 1:9 copolymer.

assumptions described above, plotting F1 vs. V_t will result in a line with a slope = -[basic titrant], and an x-intercept equal to the volume of basic titrant required to titrate all of the strong acid (V_s). Similarly, F2 can be plotted in the region when the titrand is almost fully deprotonated, and the slope = -Ka and the x-intercept is the volume of basic titrant required to reach complete deprotonation of the titrand. F3 can also be plotted in this region, and the slope is Ka^{-1}, and the x-intercept is V_s. F4 can be plotted in the region after the titrand is fully deprotonated, but was not used here because it is in the region where lysinyl amine deprotonation may be occurring. F1 must be plotted in order to obtain Vs for plotting F2.
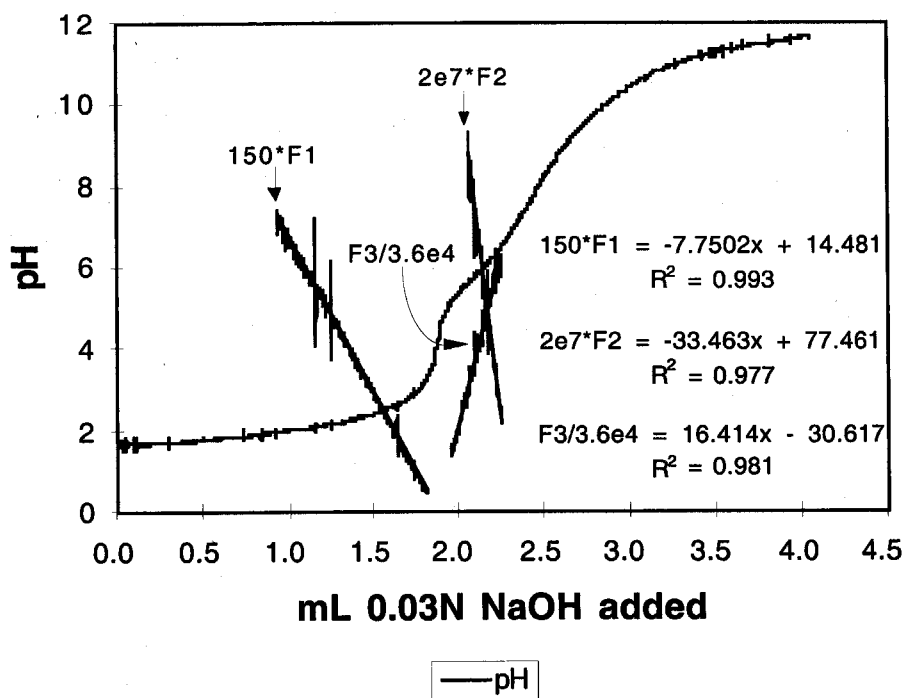
Figure 2.9. Gran Titration equations for PLL-g-(PEG;PBA) 1:3.
The slope of F2 is -Ka (predicting pKa 5.776). The slope of F3 is Ka^{-1} (predicting pKa 5.772). The average of these two values was used for the final pKa (5.8). Equation F4 was not used, as this is used in the region where lysine protonation could also be occurring (as well as being near the final pH of the basic titrant). The Gran Titration equations had similarly good fits (R^2 values) for PLL-g-(PEG;PBA) 1:9, yielding a pKa of 6.4.

The pKa values of the PBA moieties in PLL-g-(PEG;PBA) 1:3 and 1:9 were calculated from the slopes of F2 and F3. Based upon these measurements, at a physiological pH of 7.4, ≥90% of PBA residues in both polymers are expected to be in the tetrahedral form and capable of binding to cis-diols.

2.4 Discussion

PLL-g-(PEG;PBA) polymers were designed containing a backbone with moieties that could bind to biological surfaces (PBA) and grafted side-chains that could resist

adhesion of proteins and cells (PEG). It was hypothesized that the ability of the PBA-containing copolymers to sterically stabilize biological surfaces would be governed by a balance of binding (via PBA) and non-binding (via PEG) interactions. Thus the PEG grafting ratio was varied, and a maximal number of PBA groups on the polymer backbones was sought.

Varying the feed ratio of PEG to PLL resulted in polymers with varying PEG graft ratios, as anticipated. Some of the mPEG-CDI did not react with the PLL, which was expected as the reaction solvent (water) competes with the lysinyl amines for reaction with the CDI (the lysinyl primary amine is much more nucleophilic than water) (Beauchamp, C. O. et al. 1983; Hermanson, G. T. 1996). In addition, ^{1}H NMR of the PEG reactant in $CDCl_3$ only indicated 85% functionalization by CDI. Previous experiments by Elbert and Hubbell (Elbert, D. L. and Hubbell, J. A. 1998) confirmed expectations that free PEG does not block binding of proteins and cells to surfaces, thus it was not anticipated that the presence of the free PEG would effect further experiments with the PLL-g-(PEG;PBA) copolymers.

While reaction conditions were designed to conjugate a PBA to every unPEGylated lysinyl amine in the polymers, the actual degree of PBA conjugation was found to be a function of the PEG graft ratio of the copolymer. Two equivalents of PBA per free lysinyl moiety were added to each PBA conjugation reaction mixture, and much greater reaction times were used compared to the anticipated time for completion. Reaction mixtures first contained triethylamine to deprotonate the PLL-g-PEG amines, but later acetic acid was added as this can accelerate completion of the reaction. Excess reducing agent was present at all times, to drive the reaction as well as to compensate for any reducing agent hydrolysis due to residual water present in the system. However, analysis revealed that the number of PBAs conjugated to each unPEGylated amine varied with the PEG graft ratio, where highly PEGylated polymers contained up to 2 PBAs per available amine, and polymers with little PEG contained less than 1 PBA per available amine. This is not surprising, given the

limited solubility of the reactants in the solvent. As PEG is highly soluble in dichloromethane, PLL-g-PEG polymers containing more PEG were presumably more soluble in this solvent and thus more available for reaction with the PBA reactant. Difunctionalization of amines following reductive amination with aldehydes is common, thus the presence of more than 1 PBA per available amine for PLL-g-PEG 1:2 and 1:3 is also not surprising. While the number of PBA moieties on the backbone of the PLL-g-(PEG;PBA) polymers varied as the PEG graft ratio varied, it will be shown in the next chapter that the PEG grafting ratio controlled how much of the polymer bound to surfaces and sterically stabilized surfaces.

The pKa of the PBA moiety in the PLL-g-(PEG;PBA) polymers is exceptionally low, which means that the great majority of the PBA groups will be able to bind *cis*-diols on biological surfaces at physiological pH. The low percentage of tetravalent PBA groups present at pH 7.4 for a typical PBA moiety (with a pKa of 8.8) has limited the usefulness of potential *in vivo* devices and required harsher conditions for chromatography applications in the laboratory (Kitano, S. et al. 1991; Singhal, R. P. and DeSilva, S. S. M. 1992; Singhal, R. P. et al. 1991). This has motivated many endeavors to synthesize PBA moieties with lower pKas, but very few of these pKas are below 7 (Eggert, H. et al. 1999; James, T. D. et al. 1995; Mohler, L. K. and Czarnik, A. W. 1993; Moore, A. N. J. and Wayner, D. D. M. 1999; Shiino, D. et al. 1993; Wulff, G. 1982). The moiety created herein has a pKa around 6 and was synthesized in one step with commercially available reagents. The presence of secondary (as well as tertiary and/or primary) amines throughout the PLL-g-(PEG;PBA) polymer backbone likely contributed to this exceptionally low pKa by creating a highly cationic local environment. Having cationic amines in the vicinity of PBA has been proposed to facilitate a tetravalent PBA state (James, T. D. et al. 1996; Kitano, S. et al. 1991; Niwa, M. et al. 1998; Yurkevich, A. M. et al. 1975), but few have placed such a high percentage of cationic groups into a polymer backbone with PBA. Indeed, simply placing a quaternized polyamine into a solution containing PBA moieties

can facilitate sp³ hybridization of the boron (Ludwig, R. et al. 1993). Others have conjugated PBA to poly(lysine), but used an amide linkage between the PBA and the PLL, which thus eliminated amine functionalities in the backbone and resulted in a high pKa (Kimura, T. et al. 1995; Nagasaki, T. et al. 1994). The linking scheme employed herein to synthesize PLL-g-(PEG;PBA) resulted in polymers that possess a strong ability to bind *cis*-diol-containing surfaces under physiological conditions.

2.5 Conclusions

PLL-g-(PEG;PBA) polymers were designed and synthesized such that the PBA moieties on the backbone could bind the polymer to biological surfaces, and pendant PEGs grafted to the polymer backbone could then resist binding of other proteins and cells to the treated biological surfaces. The polymers were synthesized by grafting mPEG-CDI to a PLL backbone, followed by conjugating 4-formyl-PBA to the PLL-g-PEG via reductive amination. The PEG grafting ratio was varied by varying the feed ratio of PEG. The number of PBA moieties on the polymer backbone was also varied, where PLL-g-PEGs containing more PEG on the backbone were more reactive and more PBA was conjugated to them. The PBA moiety created by conjugating PBAs to the ε-amines of PLL via a secondary amine linker has a very low pKa, around 6, which is likely due to the high local concentration of amines near the PBA. This was a simple 1-step synthesis resulting in a PBA moiety that can bind *cis*-diols on biological surfaces much more strongly at physiological pH than most PBA moieties currently being used.

2.6 Acknowledgments

3. Chapter 3

PLL-g-(PEG;PBA) Binding to and Sterically Stabilizing Biological Surfaces

3.1 Introduction

Surfactant polymers were designed to spontaneously bind to and sterically stabilize biological surfaces. These could block deleterious adhesion and recognition events, such as antibody binding to transplanted red blood cells (RBCs). For chronically transfused patients, such as those with thalassemia and sickle cell anemia, alloimmunization is a frequent complication of transfusion, resulting in antibody formation and immune reactions (Vichinsky, E. P. et al. 1990). If antigens on the RBCs could be blocked with the polymer before transfusion, these complications potentially could be prevented.

The polymers were designed to contain binding domains with a general affinity for biological surfaces, and also pendant non-binding domains that would sterically prevent biological recognition of the treated surface. These polymers could spontaneously assemble on biological surfaces and block interactions with the surface, similar to polymeric steric stabilization of colloids. PEG was chosen for the non-binding domain, as side chains of a graft copolymer. PEG is well known for its protein-adsorption- and cell-adhesion- resisting properties, and chemical grafting of activated PEG molecules has also been employed to PEGylate and block biological recognition of red blood cell surfaces (Armstrong, J. K. et al. 1997; Jeong, S. T. and Byun, S. M. 1996; Scott, M. D. et al. 1997) and blood vessel surfaces (Deible, C. R. et al. 1998). Elbert and Hubbell have explored sterically stabilizing living surfaces with PEG-containing graft copolymers, using electrostatic interactions for the binding domain (Elbert, D. L. and Hubbell, J. A. 1998; Elbert, D. L. and Hubbell, J. A. 1998). PBA was chosen as the binding domain moiety in

the backbone of a graft copolymer. PBA binds to biological surfaces via *cis*-diols that are components of the oligosaccharides present within glycoproteins and glycoconjugates. Such oligosaccharides are abundant on the cell surface and extracellularly in the eukaryote, and as such they represent a relatively non-specific ubiquitous target for the binding interaction in steric stabilization. Indeed, PBA-containing molecules have been shown to bind to RBC surfaces (Burnett, T. J. et al. 1980). Complexes between PBA and *cis*-diols only form when the boron is tetrahedral and thus are most prevalent above the PBA pKa. In Chapter 2, PEG and PBA were conjugated to a PLL backbone to form PLL-g-(PEG;PBA), and the pKa of the PBA moieties in this polymer was found to be around pH 6.

It was chosen to evaluate PLL-g-(PEG;PBA), with PBA moieties on the PLL polymer backbone, and PEG chains pendant from the backbone (Winblade, N. D. et al. Accepted). The ability of these polymers to bind to *cis*-diol containing surfaces was evaluated using a mannan-conjugated resin as a model surface. The ability of the polymers to sterically stabilize biological surfaces was evaluated using RBCs as a model biological surface, where RBCs were treated with the polymers and their ability to be agglutinated by multivalent proteins was assayed (Figure 3.1). The binding to and stabilizing of surfaces by these polymers was correlated with their PEG and PBA grafting ratios. It was found that PLL-g-(PEG;PBA) polymers with optimal PEG graft ratios efficiently complexed with carbohydrate-containing surfaces. Polymers with optimal graft ratios spontaneously chemisorbed to red blood cells and prevented their agglutination by lectins and by monoclonal antibodies against blood group antigens. Moreover, the PLL-g-(PEG;PBA) copolymers sterically stabilized cell surfaces more effectively than the electrostatically-binding PLL-g-PEG analogs.

RBCs are agglutinated by lectin
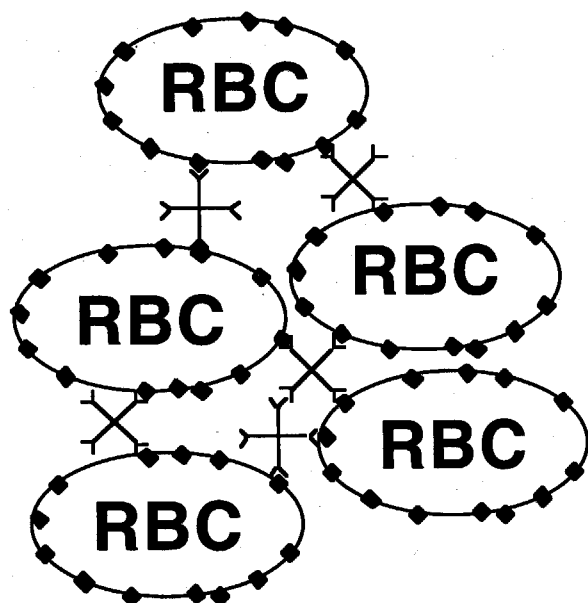
Polymer-treated RBCs cannot be agglutinated
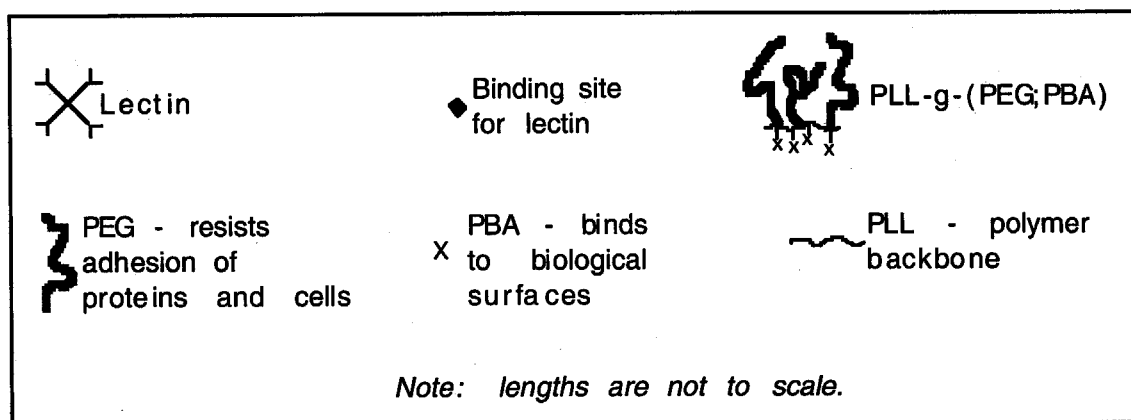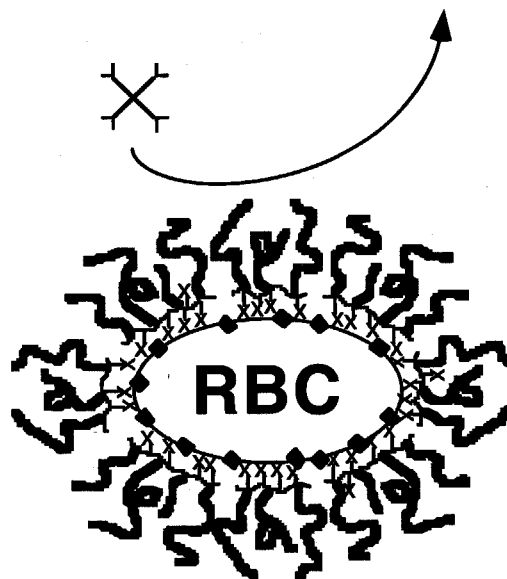
Figure 3.1. Schematic of RBC agglutination assay.

PLL-g-(PEG;PBA) polymers can assemble on RBC surfaces, coating them with a dense PEG brush and blocking the interaction of lectins with the RBC surfaces. Otherwise, the multivalent lectin will bind to RBC surfaces and agglutinate them.

3.2 Materials and Methods

Polymers

PLL-g-(PEG;PBA) and PLL-g-PEG polymers as synthesized and purified as described in Chapter 2 were used in all assays. The graft ratio is defined as the number of PEGs grafted to the PLL backbone divided by the number of lysine mers in the PLL backbone.

Mannan binding assays

Kinetics of polymer binding to *cis*-diol surfaces were assayed using a mannan resin. Mannan resin (mannan bound to 4% cross-linked agarose beads, via cyanogen bromide activation, 7-14 mg mannan/mL resin, Sigma) was suspended at 10 vol% in PBS, shaken gently for 3 min, centrifuged at 200 g for 2 min, and the supernatant and fine particles were aspirated. This washing step was completed ≥ 3 times, until no UV absorbance was seen in the supernatant. Polymers were dissolved at 0.1 wt% in PBS. 100 μL of washed resin was combined with 1 mL of polymer solution and this sample was shaken at rt (this provided a ratio of approximately 10 mannose mers per PBA mer for the PLL-g-(PEG;PBA) polymers). Periodically, the sample was centrifuged, the UV spectrum of the supernatant was recorded, and the supernatant was returned to the sample. Binding of the PLL-g-(PEG;PBA) polymers to the resin was determined from the change in supernatant absorbance at 265 nm, and binding of the PLL-g-PEG polymers was determined from the change in supernatant absorbance at 215 nm. To monitor polymer binding to the resin when the PBA binding groups were blocked, competing soluble mannose was used. D-(+)-mannose was added to the polymer solutions at a final concentration of 0.4 M (this provides for a final ratio of approximately 50 soluble mannose molecules per 1 mannose mer on the resin). These solutions were incubated for ≥ 5 min before the resin was added, then the experiment was continued as described above.

Dissociation kinetics were measured by allowing polymer binding to the resin to reach equilibrium and then replacing the supernatant with polymer-free PBS. Specifically, after equilibrium was reached, the supernatant was removed and the resin was quickly rinsed with 1 mL PBS, twice (the absorbance of the rinses was <10% of the absorbance of the supernatant that was first removed). Then the resin was re-suspended in 1 mL PBS and shaken at 4 °C. Periodically, the sample was centrifuged and the UV spectrum of the supernatant recorded. If the absorbance at 265 nm was greater than approximately 0.01 (4 - 7% of the polymer had dissociated from the resin), the supernatant was discarded and fresh PBS was added to the resin. If the absorbance of the supernatant was <0.01, then the supernatant was added back to the resin. The cumulative amount of polymer dissociated from the resin was then calculated.

Red blood cell agglutination assays

Steric stabilization of polymer-treated surfaces was assayed by testing the ability of proteins with multivalent binding sites for red blood cells (RBCs) to agglutinate polymer-treated RBCs. All agglutination assays took place in PBS (pH 7.4) at rt, using 0.1 wt% polymer solutions, with $n \geq 3$, unless noted otherwise. Statistical significance was assessed using the Student's t-test.

Wheat germ agglutinin assays:

Agglutination by the lectin wheat germ agglutinin was assayed using rat RBCs. Blood was drawn from deeply anesthetized rats before sacrifice, in accordance with the University of Washington Animal Care Committee. The blood was anticoagulated with ethylenediaminetetraacetic acid. Alsever's solution (Sigma) was added so that the RBCs were 10 vol%, the suspension was centrifuged for 20 min at 200g, and the supernatant and buffy coat were removed. This step was performed a total of 3 times. The washed RBCs were suspended at 10 vol% in Alsever's solution, stored at 4 °C, and used within 1 week.

Agglutination by wheat germ agglutinin (*Triticum vulgaris*, Sigma; abbreviated as WGA) was assayed by finding the highest concentration of WGA that could be added to polymer-treated RBCs without the RBCs agglutinating. For assays, the RBCs were diluted to 1 vol% with PBS, centrifuged for 20 min at 200g, and the supernatant removed. This step was repeated a total of 2 times, and then the RBCs were again suspended at 1% in PBS. Wheat germ agglutinin was dissolved at 500 µg/mL in PBS. Serial dilutions were made by diluting this solution 1:3 (e.g., 100 µL WGA solution + 200 µL PBS) to obtain a total of 7 different concentrations. A control with 0 µg/mL WGA was also used during each trial. 50 µL of RBC suspension (1% in PBS) and 25 µL of 0.1 wt% polymer solution were combined in 96-well tissue culture treated polystyrene plates with U-shaped bottoms and incubated for 5 min. 25 µL of a WGA dilution was then added to the well, and the plate was centrifuged for 1.5 min at 200g. Wells were then visually examined for agglutination: agglutinated RBCs formed a diffuse carpet across the bottom of the well, whereas non-agglutinated RBCs could roll over each other and thus formed a distinct pellet at the nadir of the bottom of the well. Each well was then stirred with a pipet tip and microscopically examined to confirm the result and check for microscopic aggregates. The highest final concentration of WGA that could be present without causing RBC agglutination was then recorded (the highest concentration tested was 125 µg/mL).

The mechanism of PLL-g-(PEG;PBA) prevention of RBC agglutination was further investigated by varying the assay conditions. To test the effect of pH, the pH of the polymer solution was adjusted with 1M NaOH so that the pH of the final polymer/RBC/WGA suspension was 8.7. To test the effect of excess free PEG in solution, up to 0.05 wt% free PEG was added to solutions of PLL-g-(PEG;PBA) 1:6 before incubation with RBCs. To test the effect of competing *cis*-diol-containing molecules in solution, various amounts of D-(+)-glucose were added to the polymer solution and incubated for ≥5 min before the polymer solution was added to the RBCs. To test the

effect of removing free polymer from solution, the polymers were incubated with the RBCs

in microcentrifuge tubes. Then the tubes were centrifuged, the supernatant aspirated, and

the RBCs re-suspended at the same concentration in PBS, twice. Then a final

concentration of 125 μg/mL WGA was added to the RBCs, the tubes were centrifuged,

and the cells were examined for agglutination. To test the necessary amount of polymer to

prevent agglutination, the wt% of the 25 μL of polymer solution that was applied to the 50

μL of RBC suspension was varied from 0 - 0.1 wt%. The amount of PLL-g-(PEG;PBA)

1:9 bound to the RBC surfaces (when 25 μL of 0.025 wt% polymer was applied) was

estimated by assuming that all the polymer (excluding free PEG) was bound to the RBC

surfaces and was evenly distributed. The surface area per rat RBC was estimated by a

performing a least squares linear fit of RBC surface area versus volume for humans,

rabbits, and sheep ($R^2 = 0.9991$) and then using this line to calculate the rat RBC surface

area based on the rat RBC volume of 47.5 μm^3 (Bishop, C. and Surgenor, D. M. 1964;

Surgenor, D. M. 1974; Wintrobe, M. M. et al. 1974).

Antibody agglutination assays:

Antibody agglutination of polymer-treated RBCs was assayed by combining type A

human RBCs with a monoclonal antibody to the blood type A antigen. Blood was drawn

from a healthy, blood type A human donor and anticoagulated with

ethylenediaminetetraacetic acid. The RBCs were washed as described for the rat RBCs and

then similarly suspended at 6 vol% in PBS. The human RBCs were always assayed on the

day of collection, to avoid any storage effects. Agglutination was induced using a

commercial blood typing antisera consisting of a monoclonal anti-A IgG antibody

(Carolina Biological Supply, Burlington, NC; abbreviated mAb). Serial dilutions of mAb

were made by diluting the mAb 1:2 (e.g., 100 μL mAb solution + 100 μL PBS). 50 μL

of RBC suspension (6% in PBS) and 25 μL of 0.6 wt% polymer solution were combined

in a microcentrifuge tube and incubated for 5 min, with mixing. 7.5 μL of a mAb dilution

was then added to the tube and incubated 1 min. The tube was centrifuged for 20 s at approximately 1000g. The cell pellet was then gently re-suspended and examined for agglutination, both visually and microscopically. The degree of agglutination of the RBCs was scored as described by Marsh (Walker, R. H. 1990). Briefly, the degree of agglutination is given a score value between 0 and 12, where for example 0 means no agglutination, 2 means only microscopic agglutination, 4 means many macroscopic agglutinates, 8 means large and small agglutinates with no free cells, and 12 means one single agglutinate with no free cells. The final score is taken by adding up the score values for the RBCs for each mAb dilution until the mAb is so dilute that it no longer causes RBC agglutination. For example, if the agglutination is 12 at no dilution of mAb, 6 at the first (1/2) dilution of mAb, 2 at the second (1/4) dilution of mAb, and 0 at the third (1/8) dilution of mAb, the score is 12 + 6 + 2 = 20. Thus higher titration scores mean the RBCs are more easily agglutinated. The titers of the treated RBCs were also determined, where the titer is the reciprocal of the highest dilution of mAb that still causes any agglutination response (in the example, the titer is 4).

3.3 Results

Chemisorption of polymers to mannan resin

The ability of the PLL-g-(PEG;PBA) polymers to chemisorb to a *cis*-diol containing surface was observed to be a function of the degree of PEG grafting and required the presence of available PBA binding moieties in the copolymer (Figure 3.2). Each mannose mer in mannan contains a *cis*-diol, hence tetrahedral PBA moieties would be expected to bind to mannan. 0.1 wt% polymer solutions were incubated with a mannan-conjugated resin and the amount of polymer binding to the resin was measured by depletion, at pH 7.4. The equilibrium amount of polymer bound decreased in the following order:
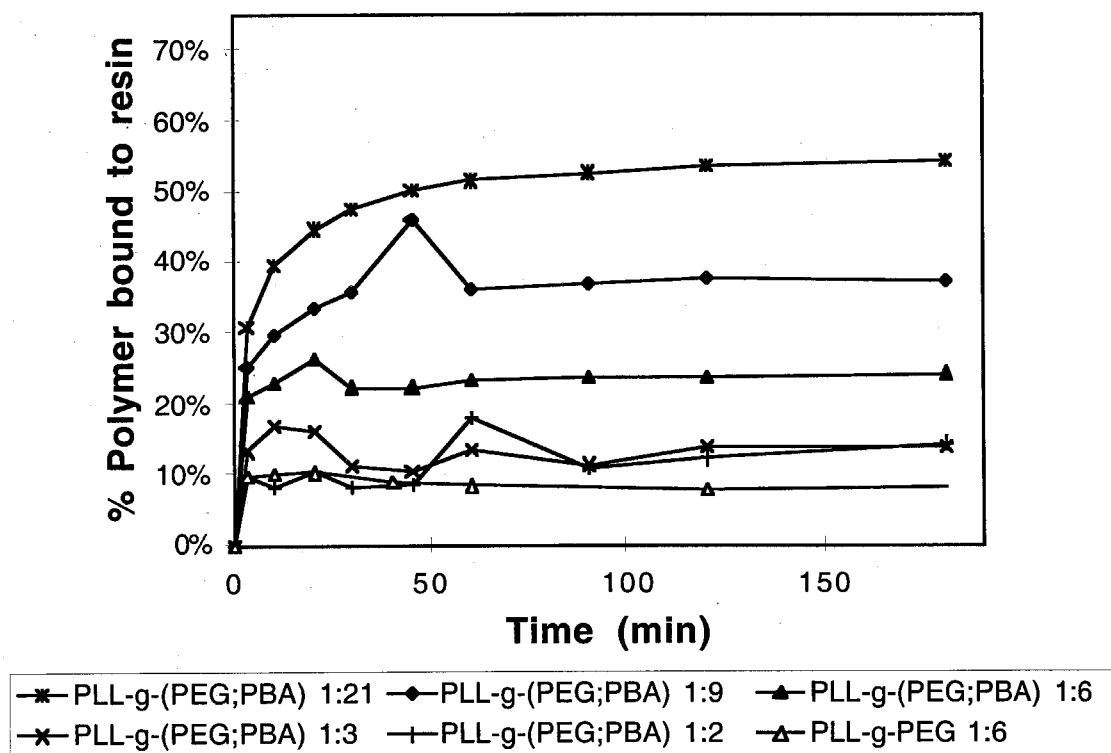
Figure 3.2. Polymer binding to mannan resin.

Polymers were dissolved at 0.1 wt% in PBS (pH 7.4) and incubated with a mannan-containing resin (each mannose mer contains a *cis*-diol to which PBA can bind). PLL-g-(PEG;PBA) binding to the resin was controlled by the PEG graft ratio, where polymers containing less PEG bound in higher amounts to the resin. Less than 15% of PLL-g-PEG polymers adsorbed to the resin at all graft ratios.

PLL-g-(PEG;PBA) 1:21 (58%) > 1:9 (40%) > 1:6 (27%) > 1:3 ≅ 1:2 (15%). The total

amount of PEG side chains immobilized on the resin by these copolymers also followed the

same trend (calculated using the amount bound, and the MW and the PEG graft ratio of the

copolymers). Less than 15% of the PLL-g-PEG polymers adsorbed at any of the grafting

ratios (although PLL-g-PEG polymers containing less PEG also bound to the resin more

than PLL-g-PEG polymers containing more PEG). Pre-incubating the PLL-g-(PEG;PBA)

polymers with competing soluble mannose blocked polymer binding to the resin (Figure

3.3), although a small amount of residual binding for PLL-g-(PEG;PBA) 1:21 was noted.

The dissociation rate of the polymers was also tested, by binding PLL-g-(PEG;PBA) 1:9 or
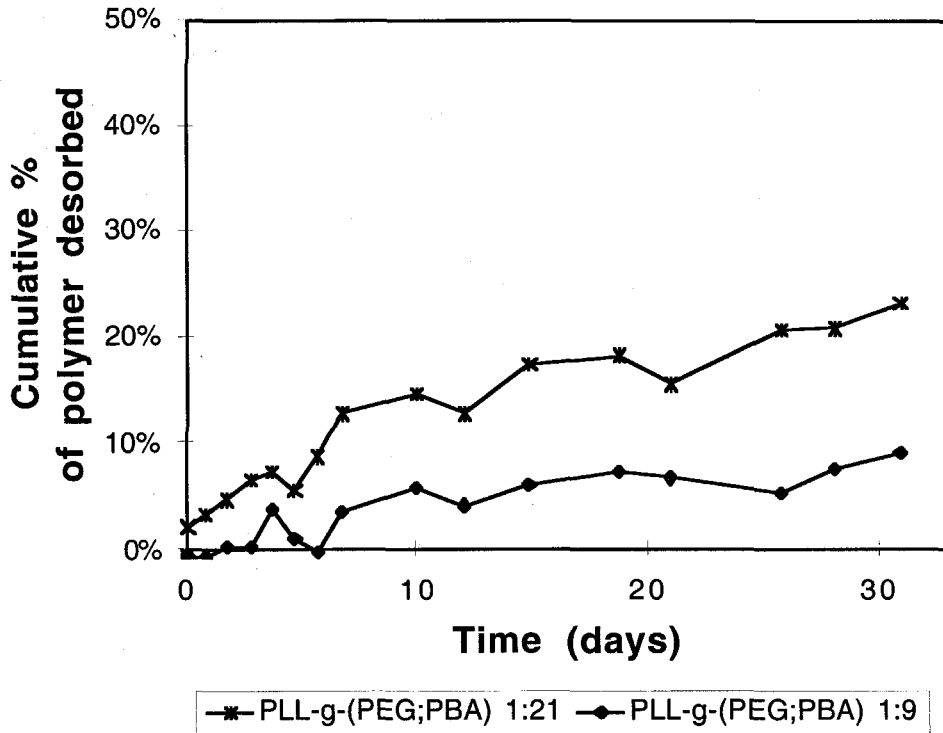
Figure 3.4. Polymer dissociation from mannan resin.

Equilibrium polymer binding to a mannan-containing resin was reached, then the resin was re-suspended in polymer-free PBS (pH 7.4) and polymer dissociation was monitored at 4 °C. Polymer dissociation was very slow, with a half life on the order of months. PLL-g-(PEG;PBA) 1:21 contains less PBA on the backbone, and dissociated faster than PLL-g-(PEG;PBA) 1:9 which contains more PBA.

with four sites that bind N-acetylglucosamine residues and can thus bind and agglutinate

RBCs via sugar residues on the surface of the RBCs (Stryer, L. 1995). RBCs were

incubated with polymer solutions, and the maximum amount of WGA that could be added

without agglutinating the cells was measured (Figure 3.5). PLL-g-(PEG;PBA) 1:9 and

1:21 were able to completely prevent RBC agglutination at the highest concentrations of

WGA tested (statistically different from PBS, $P < 0.001$). PLL-g-(PEG;PBA) 1:6 and

PLL-g-PEG 1:9 had a small effect on WGA agglutination ($P < 0.01$ and $P < 0.001$

respectively, compared to PBS; but each was statistically less effective than PLL-g-
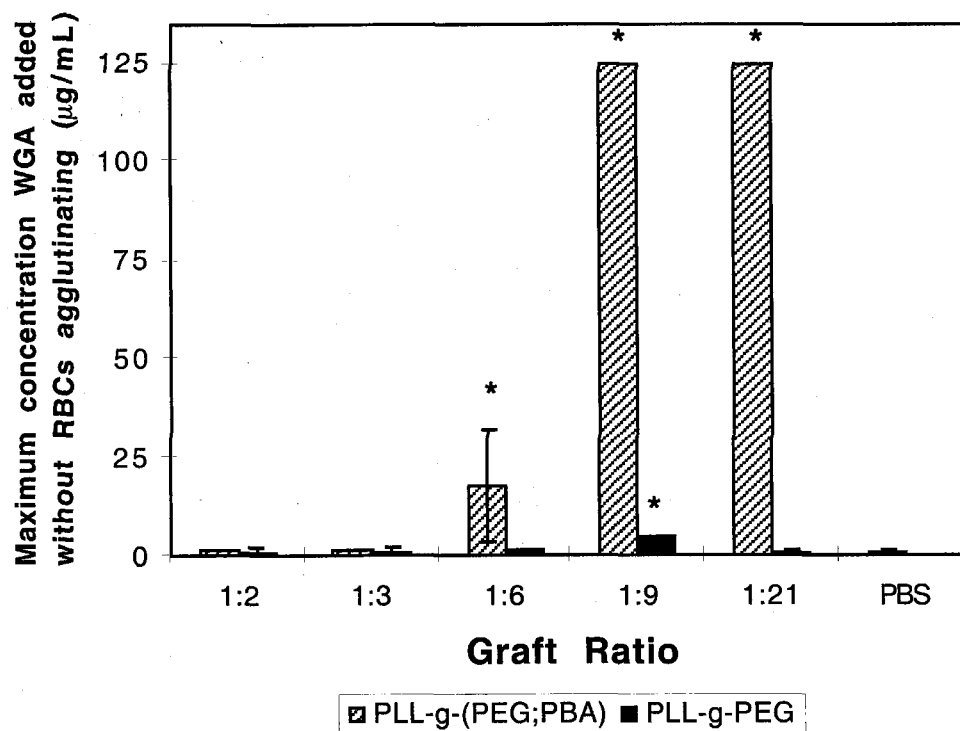
Figure 3.5. Effect of polymer pre-treatment of RBCs on the lectin-induced agglutination of RBCs.

Polymers were incubated with RBCs, and the highest concentration of WGA that could be added without the cells agglutinating was found (tested up to a maximum of 125 µg/mL). PBA-containing polymers with optimal graft ratios (PLL-g-(PEG;PBA) 1:9 and 1:21) strongly block agglutination by WGA. Assayed in PBS (pH 7.4), * = significantly different from PBS at 95% confidence using Student's t-test (mean ± sd, n ≥ 3; most error bars are too small to see).

(PEG;PBA) 1:9, P < 0.001). PLL-g-(PEG;PBA) 1:2 and 1:3 and all remaining PLL-g-PEG polymers did not affect the WGA agglutination of RBCs. The same results were obtained for the PLL-g-(PEG;PBA) polymers when the assay was conducted at a final pH of 8.7 (data not shown). No RBC hemolysis, morphology changes, or rouleaux formation was noted in any of the agglutination experiments. The polymers alone never caused RBC agglutination in these experiments. Because the copolymers contained a small amount of free PEG as a contaminant, a control experiment was performed to demonstrate that this
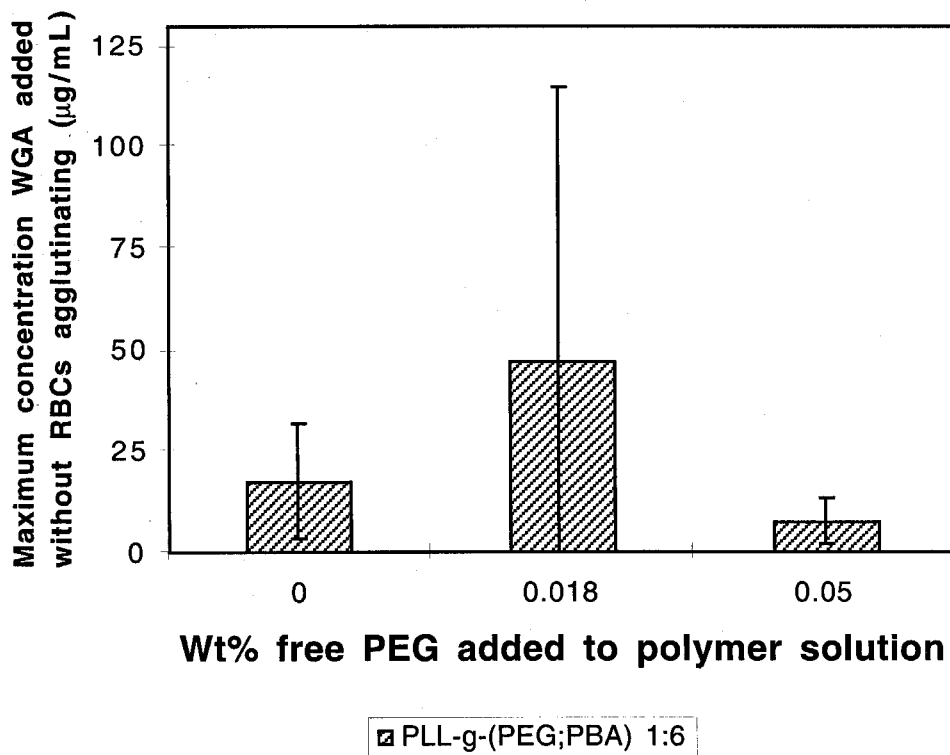
Figure 3.6. Effect of free PEG on WGA-induced agglutination of polymer-treated RBCs.

Free PEG was added to PLL-g-(PEG;PBA) 1:6, then RBCs were added, and the highest concentration of WGA that could be added without the cells agglutinating was found. The addition of free PEG, up to more than double the amount of free PEG present in the PLL-g-(PEG;PBA) polymers, had no effect on the WGA-induced agglutination in presence of a polymer. Assayed in PBS (pH 7.4), * = significantly different from PBS at 95% confidence using Student's t-test (mean ± sd, n ≥ 3).

free PEG did not influence agglutination (Figure 3.6). The addition of up to 0.05 wt% free PEG to PLL-g-(PEG;PBA) 1:6 solutions did not change the agglutination results (this represents a concentration of free PEG more than double that found in the PLL-g-(PEG;PBA) polymer solutions without added PEG). Also, this author (data not shown) and Elbert and Hubbell (Elbert, D. L. and Hubbell, J. A. 1998) have shown that solutions of 0.1 wt% PEG alone (MW 5 kDa, same experimental protocol) have no effect on WGA agglutination of RBCs in comparison with PBS controls.
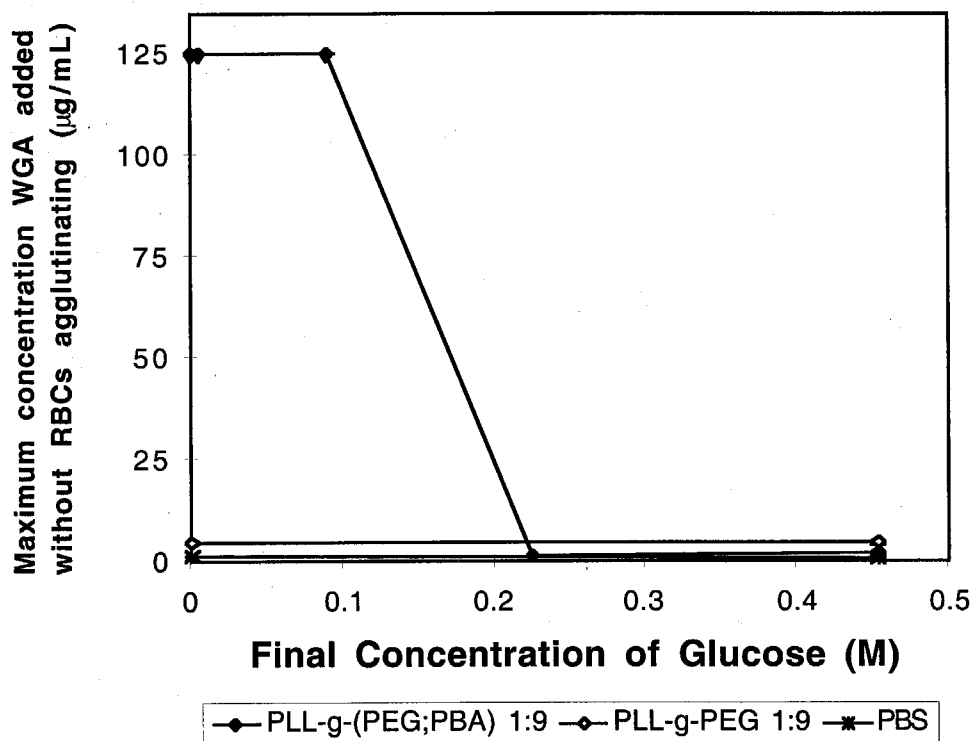
Figure 3.7. Effect of soluble glucose on WGA-induced agglutination of polymer-treated RBCs.

Glucose was added to polymers solutions, then RBCs were added, and the highest concentration of WGA that could be added without the cells agglutinating was found (tested up to a maximum of 125 µg/mL). Glucose can compete for PBA binding, and the presence of 0.23 M glucose prevented PLL-g-(PEG;PBA) 1:9 from blocking WGA agglutination. It should be noted that physiological concentrations of glucose are circa 5 mM, where the effect of PLL-g-(PEG;PBA) 1:9 was not blocked. Assayed in PBS (pH 7.4) (mean ± sd, n ≥ 3; most error bars are too small to see).

Prevention of RBC agglutination by polymer-mediated steric stabilization required that the PBA groups of the polymer could bind to RBC surface carbohydrate groups, as agglutination could be inhibited by pre-incubating the polymer with competing glucose (Figure 3.7). Glucose contains *cis*-diols which can bind PBA and hence should be able to block PBA binding to other carbohydrate groups. PLL-g-(PEG;PBA) 1:9 was pre-incubated with varying concentrations of glucose, RBCs were added, then varying concentrations of WGA were added. The presence of 0.23 M glucose (final concentration)

prevented PLL-g-(PEG;PBA) 1:9 from blocking RBC agglutination. This concentration is equivalent to approximately 500 α-glucose molecules per PBA moiety in the polymer. It should be noted that physiological concentrations of glucose (approximately 5 mM) did not diminish the ability of the polymer to prevent agglutination.

The prevention of RBC agglutination by PLL-g-(PEG;PBA) is a surface, rather than solution, effect since polymer-treated RBCs were not agglutinated by WGA even after unbound excess soluble polymer was removed from solution. For this experiment, polymers were incubated with RBCs, after which the RBCs were centrifuged and re-suspended in (polymer-free) PBS, twice. Then WGA was added to the RBCs for a final concentration of 125 μg/mL. RBCs treated with PLL-g-(PEG;PBA) 1:9 were not agglutinated, whereas RBCs treated with PLL-g-PEG 1:9 or PBS were agglutinated.

The amount of polymer necessary to prevent RBC agglutination is approximately a monolayer coating of the RBCs (Figure 3.8). This conclusion was reached by varying the concentration of PLL-g-(PEG;PBA) 1:9 in the polymer solution and then applying the solution to RBCs and adding WGA as before. It was found that using a solution of 0.025 wt% polymer was adequate to prevent RBC agglutination at the highest concentration of WGA tested. Assuming all of the polymer was adsorbed to RBC surfaces, this corresponds to approximately 500 ng polymer/cm^2 RBC surface (not including free PEG), which is near typical values for a monolayer of protein.

Antibody agglutination assays

The PLL-g-(PEG;PBA) polymers with optimal grafting ratios were also able to prevent RBC agglutination caused by antibody binding (Figure 3.9). Human type A RBCs were treated with polymer, then serial dilutions of anti-A mAb (from commercial blood typing antisera) were added, and the agglutination of the RBCs was scored using the protocol from the American Association of Blood Banks. A higher score or a higher titer mean that the RBCs were more easily agglutinated by the mAb. The ratio of mAb to RBCs
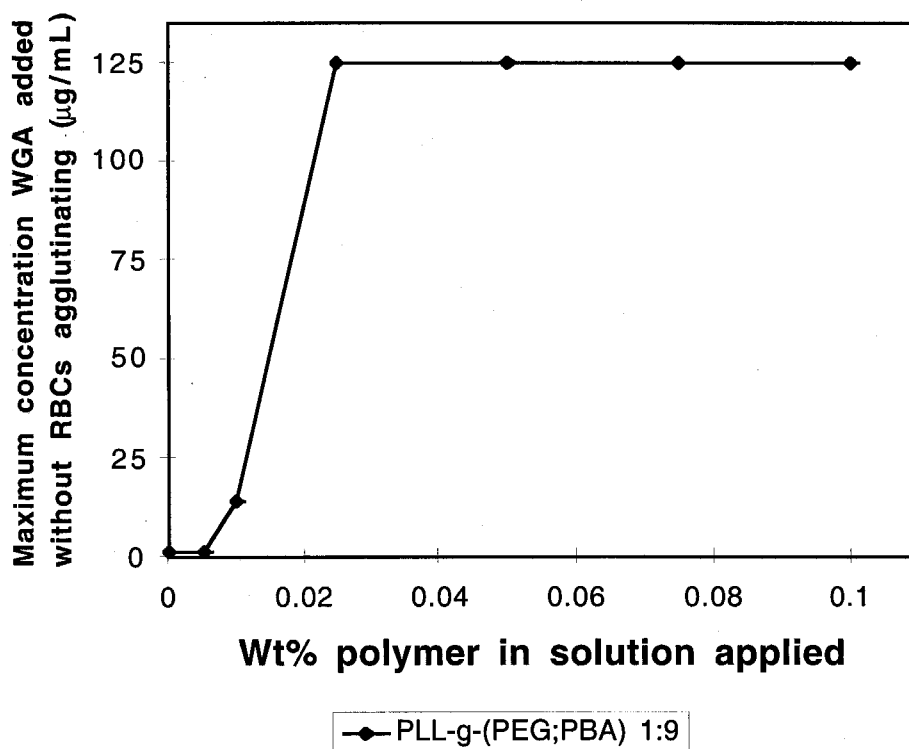
Figure 3.8.   Effect of polymer concentration on WGA-induced agglutination of polymer-treated RBCs.

Varying concentrations of PLL-g-(PEG;PBA) 1:9 were incubated with RBCs, and the highest concentration of WGA that could be added without the cells agglutinating was found (tested up to a maximum of 125 µg/mL). The lowest concentration of PLL-g-(PEG;PBA) 1:9 that still strongly prevented agglutination was 0.025 wt%, which corresponds to about 500 ng polymer/cm^2 RBC surface. Assayed in PBS (pH 7.4) (mean ± sd, n ≥ 3; most error bars are too small to see).

was chosen to match the ratios used previously by others who had covalently bound PEG

to RBCs (Scott, M. D. et al. 1997). PLL-g-(PEG;PBA) 1:9 and 1:21 greatly reduced the

agglutination of the treated RBCs by the mAb (P < 0.001 compared to PBS). PLL-g-

(PEG;PBA) 1:6 slightly reduced the agglutination of the treated RBC (P < 0.05 compared

to PBS, but was less effective than PLL-g-(PEG;PBA) 1:9, P < 0.01). PLL-g-

(PEG;PBA) 1:9 and 1:21 also greatly reduced the RBC titers (data not shown).
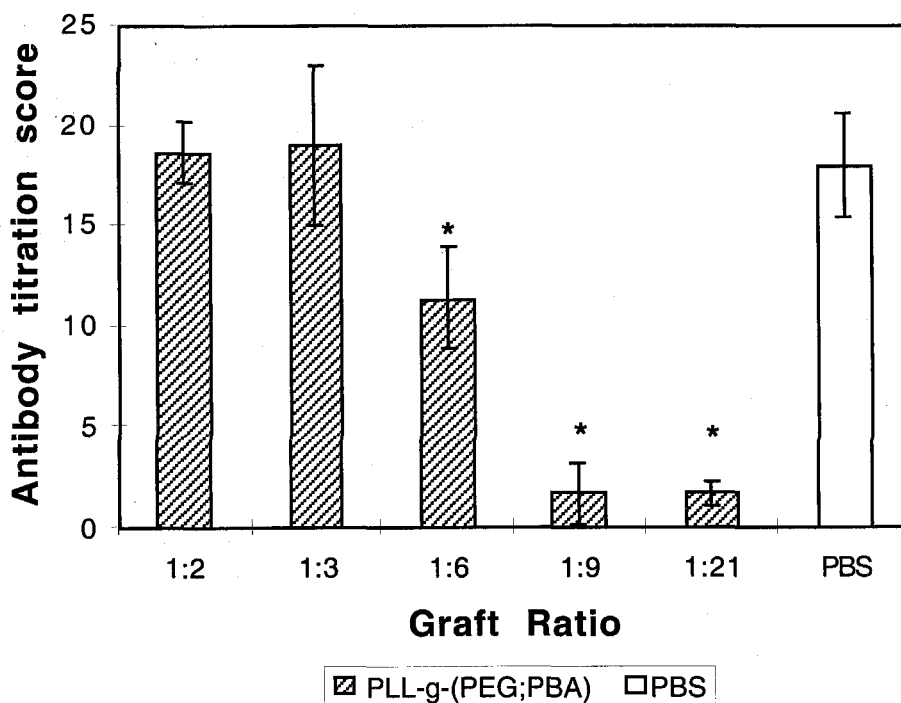
Figure 3.9. Effect of polymer pre-treatment of RBCs on the antibody-induced agglutination of RBCs.

Polymers were incubated with human type A RBCs, serial dilutions of anti-A mAb were added, and the agglutination response was scored, where a higher score indicates that the cells are more easily agglutinated. PLL-g-(PEG;PBA) 1:9 and 1:21 strongly blocked agglutination of the RBCs. Assayed in PBS (pH 7.4), * = significantly different from PBS at 95% confidence using Student's t-test (mean ± sd, n = 3).

3.4 Discussion

It was hypothesized that the ability of the PBA-containing copolymers to sterically stabilize biological surfaces would be governed by a balance of binding (via PBA) and non-binding (via PEG) interactions. It was found that the PEG grafting ratio of the PLL-g-(PEG;PBA) polymers controlled their ability to bind to *cis*-diol containing surfaces and their ability to prevent adhesion to polymer-treated surfaces. Experiments in which polymer binding to mannan resin was quantified revealed that copolymers with a higher PEG content chemisorbed less to the resin. This result indicates that the degree of PEG

grafting (not PBA content) controlled polymer binding to surfaces. This conclusion can be reached because the polymers with more PEG also contained more PBA binding moieties (and yet less polymer chemisorbed), because the polymers were tested well above their pKa (thus nearly all PBAs were in the tetrahedral binding form), and because electrostatics did not control binding. In addition, more PEG per gram of resin was bound to the resin by the PLL-g-(PEG;PBA) copolymers with lower amounts of PEG per molecule. Thus binding of PLL-g-(PEG;PBA) 1:3 to the resin was not limited by the amount of PEGylated copolymer on the resin but by the steric ability of the polymer backbone to bind to the resin surface. The low resin binding of all PLL-g-(PEG;PBA)s during competitive complexation with mannose and of all PLL-g-PEGs confirmed that ionic interactions of cationic amines and anionic PBAs with the resin were not substantially responsible for binding, but that a specific interaction between mannose mers and PBA caused binding. While a very low amount of PBA on the polymer backbone would be expected to decrease polymer binding to surfaces, the results indicate that 41 PBA mers per polymer provided sufficiently strong binding such that the degree of PEGylation controlled binding. Because the half-life of dissociation of the polymer with the fewest PBAs (PLL-g-(PEG;PBA) 1:21) from the resin is on the order of months, it is not surprising that number of PBAs on the backbone did not control binding. The dissociation experiments did show, however, that polymers with less PBA on the backbone (PLL-g-(PEG;PBA) 1:21) do dissociate faster than those with more PBA (PLL-g-(PEG;PBA) 1:9).

PLL-g-(PEG;PBA) copolymers with optimal grafting ratios were able to sterically block RBC agglutination induced by WGA and by a mAb. The binding of the PBA moiety was essential for this effect, since polymers without PBA and polymers with their PBAs blocked by free glucose were not able to prevent agglutination. Excess free PEG in solution did not effect the WGA-induced agglutination, either alone, in combination with PLL-g-(PEG;PBA) 1:6, or as seen in the lack of effect of PLL-g-(PEG;PBA) and PLL-g-PEG 1:2 and 1:3 polymers on agglutination. Likewise, the binding of the PLL-g-

(PEG;PBA) polymers to RBCs was not due to electrostatic interactions of the cationic amine groups or anionic PBA groups with the RBC surfaces. This conclusion can be reached because PLL-g-(PEG;PBA) polymers that had been competitively bound to glucose still have the same net charge and yet these, as well as the highly cationic PLL-g-PEG polymers, did not prevent RBC agglutination. Only a surface coating of polymer was required for steric stabilization by PLL-g-(PEG;PBA), since treated RBCs suspended in polymer-free PBS were still not agglutinated by WGA. Also, only approximately 500 ng/cm^2 of polymer was required to prevent agglutination, which is close to the surface concentration of polymer that would be expected to result in approximately monolayer coverage. Further confirmation of the steric stabilization mechanism is seen in that the binding sites of WGA on RBCs do not contain *cis*-diols, and WGA contains no carbohydrate component, hence the action of the polymers must be due to polymer binding to *cis*-diol groups on the RBC surfaces and sterically blocking WGA binding to any N-acetylglucosamine sites nearby. This mechanism cannot be confirmed in the case of the mAb, because the antigen binding site does contain a *cis*-diol (Wintrobe, M. M. et al. 1974). Because the PLL-g-(PEG;PBA) polymers had the same effect on WGA-induced agglutination of RBCs at pH 8.7 as at pH 7.4, this also provides confirming evidence that the pKa of the PBA moiety is below 7.4. The effectiveness of the polymers at pHs much lower than the pKa was not tested, as these pHs had adverse effects on both the mannan resin and on the RBCs.

That this surfactant is capable of sterically stabilizing underlying treated surfaces is supported by theoretical calculations. The investigations of Jeon et al. (Jeon, S. and Andrade, J. 1991; Jeon, S. et al. 1991) regarding prevention of protein-surface interactions via terminally attaching PEG upon the surface found that a high surface density of PEG was desirable, where the distance between surface-bound PEG molecules should be smaller than the Flory radius, in order to induce an extended "brush" configuration of the PEG molecules. Assuming from Figure 3.8 that 500 ng/cm^2 of PLL-g-(PEG;PBA) 1:9

was bound to the RBC surfaces (the amount may have been less than that, it is only known that such an amount was sufficient to prevent agglutination, whereas 200 ng/cm^2 was insufficient), the distance between PEG molecules is approximately 15 Å, which is much smaller than the Flory radius of approximately 47 Å. The estimations made by Jeon et al. regarding PEG interactions (including hydrophobic interactions) with a hydrophobic protein sphere found that the optimal distance between PEGs on the surface was approximately 10 Å to repel small proteins and approximately 15 Å to repel larger proteins. Szleifer's modeling of protein (lysozyme) adsorption to a PEG-grafted surface also found that a distance between PEGs of <16 Å was sufficient to block protein adsorption (Szleifer, I. 1997). Both of these values are well within the 15 Å range estimated for the PLL-g-(PEG;PBA) polymers in this work. The optimal surface for protein-resistance would also have the largest MW of PEG moieties possible; however, this parameter was not varied in these experiments.

The combination of the agglutination assays and the mannan binding assays reveals that PLL-g-(PEG;PBA) 1:9 and 1:21 are effective in preventing agglutination because, among the polymers tested, they are the ones that chemisorbed in sufficient quantities to stabilize the surfaces against biological recognition. Elbert and Hubbell found that a high molecular weight PLL backbone (MW 375 kDa) was required in order for PLL-g-PEG to prevent WGA-induced RBC agglutination (Elbert, D. L. and Hubbell, J. A. 1998). They also showed that PLL-g-PEG with a MW 375 kDa PLL backbone could adsorb to more complex biological surfaces (such as serum proteins on tissue culture polystyrene) in sufficient quantities to prevent cell adhesion, but PLL-g-PEG with a MW 20 kDa PLL backbone could not. By contrast, the addition of the PBA groups seems to render the MW of the polymer backbone less important. The PLL-g-(PEG;PBA) polymers have only a MW 24 kDa PLL backbone, with as few as 41 PBA binding moieties. The ability of these PBA-containing polymers to sterically prevent high affinity interactions, such as mAb binding, is presumably because PBA can form covalent bonds with the ubiquitous

carbohydrate groups on cell membranes, whereas the binding of PLL-g-PEG is due to weaker electrostatic attraction to net anionic surfaces that may be more heterogeneous in their charge distribution as well as partially shielded from electrostatic interactions at physiological ionic strength.

3.5 Conclusions

The ability of the PLL-g-(PEG;PBA) polymers to bind to surfaces and to sterically stabilize surfaces was tested. It was found that the degree of PEG grafting controlled the binding of the polymers to surfaces, where polymers containing less PEG bound in higher amounts to the surfaces. PLL-g-(PEG;PBA) polymers with optimal grafting ratios (1:9 and 1:21) spontaneously bound to RBC surfaces and blocked their agglutination by a lectin and by an antibody. The covalent binding of PBA to surfaces was specific and strong, resulting in more efficacy for shorter polymer backbones than could be acquired with electrostatic binding. The effectiveness of the PLL-g-(PEG;PBA) polymers required only a surface layer of polymer on the RBCs, with the PEGs spaced approximately 15 Å apart on the surface. These polymers may have direct application in treating RBCs before transfusion, to prevent immune reactions to the RBCs, as well as in many other applications where interactions with biological surfaces could advantageously be blocked.

3.6 Acknowledgments

4. Chapter 4

PLL-g-(PEG;PBA) Sterically Preventing Cell Adhesion to Surfaces *In Vitro*

4.1 Introduction

The PLL-g-(PEG;PBA) polymers synthesized in this work have been shown to chemisorb specifically to *cis*-diol-containing surfaces, and to spontaneously coat RBC surfaces and prevent protein binding to the RBCs at physiological pH. Another important application of the polymers would be coating surfaces and preventing cell adhesion to those surfaces. Investigations of the efficacy of the polymers in blocking cell adhesion to surfaces that are important in a number of clinically-relevant models will be reported in this chapter.

Cell adhesion to and migration upon ECM is important in a number of undesirable biological responses. Posterior capsule opacification occurs after cataract surgery when remaining lens epithelial cells migrate along the posterior lens capsule and proliferate (Apple, D. et al. 1992). The lens capsule is a basal lamina (a specialized ECM), thus if PLL-g-(PEG;PBA) could coat the capsule, it could potentially prevent lens epithelial cells from colonizing the posterior capsule and leading to visual opacification. In peritoneal adhesion formation, fibrin is deposited following damage to tissue induced during peritoneal surgery (diZerega, G. 1994; diZerega, G. S. 1997). These fibrin deposits can mature into permanent adhesions between organ surfaces leading to chronic pain, infertility, and bowel obstruction. Macrophage and fibroblast adhesion upon and infiltration into these deposits is involved, and the adhesions can form between the mesothelialized or demesothelialized peritoneal tissue surfaces. Coating of ECM surfaces with PLL-g-(PEG;PBA) might prevent the formation of permanent adhesions.

The interaction of PLL-g-(PEG;PBA) with serum proteins is also important at wound sites. Blood and serum is introduced to wound sites in virtually all surgeries, and this introduces important proteins such as fibronectin and vitronectin, which can interact with the exposed surfaces and promote cell adhesion and migration. Serum proteins are believed to be important in both posterior capsule opacification and peritoneal adhesion formation. Because serum contains many adhesive proteins to which cells can specifically bind via integrin receptors, the ability of the polymer to coat serum protein surfaces and block these specific interactions is important to investigate.

The PLL-g-(PEG;PBA) polymers could block cell adhesion to surfaces by coating the surfaces or also by coating the cell surface. Cells coated with polymer can be sterically prevented from interacting with adhesion proteins similar to how RBCs are prevented from interacting with lectins and mAbs, as described in Chapter 3. One issue in coating living cell surfaces, rather than solid surfaces, with polymer is that most cells, RBCs being a notable exception (Schekman, R. and Singer, S. J. 1976), are constantly turning over their membrane during the process of endocytosis. Thus the effect of the polymer coating could be more transient on cell surfaces than on ECM surfaces.

Glycoproteins and proteoglycans are abundant in the ECM, in serum, and on cell surfaces and as such represent a likely target for efficacy of PLL-g-(PEG;PBA) polymers in these models. Sialic acid residues frequently occupy the terminal position on carbohydrates of glycoconjugates, and usually contain hydroxyls at C7, C8, and C9 to which PBA can bind (Schauer, R. 1982). Other common terminal sugar residues which contain *cis*-diols include galactose, fucose, N-acetylgalactosamine, and mannose (Kobata, A. 1992). Proteoglycans such as heparan sulfate, chondroitin sulfate, heparin, and hyaluronate, however, contain very few *cis*-diols due to the content of the repeating sugars and their sulfation and acetylation patterns (Hardingham, T. E. and Fosang, A. J. 1992). Because of the complex mixtures of components present in all biological systems, it is

difficult to predict if the glycosylation patterns will be adequate to bind enough PLL-g-(PEG;PBA) to sterically stabilize the surfaces.

Tissue culture polystyrene (TCPS) represents a simpler and more static model system containing hydroxyls to which PLL-g-(PEG;PBA) polymers could bind. TCPS is polystyrene treated with a gas plasma (including oxygen) to generate a surface that is hydrophilic and that adsorbs a protein overlayer from serum that is hospitable to cell adhesion and proliferation in culture. The treatment generates a net anionic surface, and varies amongst manufacturers resulting in different surfaces. As revealed in our results, the TCPS surfaces used in this work contained a significant population of hydroxyls to which the PLL-g-(PEG;PBA) apparently could bind. Thus the TCPS surface was employed as a model hydroxyl-containing surface which also is illustrative of possible applications of the polymer to other hydroxyl-containing biomaterials surfaces for implantation or *in vitro* studies.

The ability of PLL-g-(PEG;PBA) copolymers to coat surfaces and prevent cell adhesion, spreading, and/or migration on these surfaces was investigated and is reported in this chapter (Figure 4.1). The PBA components of the polymer could bind to hydroxyl groups on the surfaces, thereby anchoring a dense PEG brush to the surface that could sterically prevent the interaction of cells or other adhesive proteins with the treated surface. The model surfaces include TCPS, adsorbed serum proteins, ECM, Matrigel components, and cell surfaces. *Rabbit lens epithelial cells (rLECs) were used, as this is the cell type* involved in posterior capsule opacification. The PLL-g-(PEG;PBA) polymers were found to coat these model surfaces and prevent cell interactions with the surfaces as a function of the PEG graft ratio. The polymers were most effective on TCPS and less effective on the more complex proteinaceous surfaces. The PLL-g-(PEG;PBA) polymers were more effective than the PLL-g-PEG polymers on all surfaces. The cytotoxicity of the polymers was also investigated *in vitro*. The PLL-g-(PEG;PBA) polymers showed evidence of time-
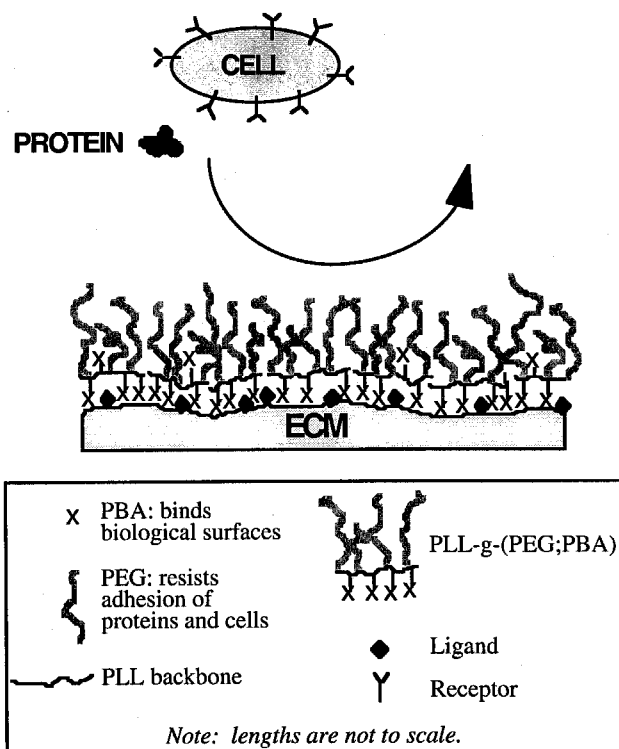
Figure 4.1. **Schematic of cell adhesion assays.**

The PBA moieties in the polymer backbone spontaneously assemble on hydroxyl-containing surfaces (here, extracellular matrix). The PEG chains are thus anchored to the surface and form a dense PEG brush. The PEG brush sterically inhibits interactions of other proteins and cells with the treated surface.

and dose-dependent toxicity; however, the mechanism was unclear and may have been affected by the artificial *in vitro* culture environment.

4.2 Materials and Methods

Polymer Synthesis

PLL-g-(PEG;PBA) and PLL-g-PEG polymers synthesized as described in Chapter 2 and were used in all assays. The graft ratio is defined as the number of PEG chains grafted to the PLL backbone divided by the number of lysine mers in the PLL backbone.

Cell Isolation

rLECs were isolated from the lens capsule of a Dutch banded rabbit. Betadine was dropped in the eye of a freshly sacrificed rabbit and the surgery was performed in a sterile manner. The cornea was removed and the iris was peeled back in 4 radial flaps. The lens was exposed via a continuous curvilinear capsulorhexis and the lens was expressed with pressure. Residual lens material was removed by rinsing with BSS balanced salt solution (Alcon Surgical, Fort Worth, TX) and aspirating with a syringe. An OPHTEC poly(methyl methacrylate) tension ring (Ophtec BV, Groningen, Holland) was inserted into the empty capsule using an EASY control microinserter (Ophtec). The capsule was then removed by cutting the zonules and cutting away the vitreous from the posterior capsule. The capsule was placed in a well of a 6 well TCPS plate and rinsed with 0.5 mL of balanced salt solution. 3 mL of medium was then added (Dulbecco's modified eagle medium supplemented with 10% fetal bovine serum, penicillin 100 U/mL, streptomycin 100 μg/mL, and amphotericin B 0.25 μg/mL, Gibco, Gaithersburg, MD). The lens was cultured at 37 °C in a humid atmosphere with 5% CO_2. The rLECs migrated and proliferated, coating the entire capsule within 1 week, and then grew over onto the TCPS substrate.

Cell Culture

rLECs were cultured in Dulbecco's modified eagle medium, supplemented with penicillin 100 U/mL, streptomycin 100 μg/mL, amphotericin B 0.25 μg/mL, and 10-20% fetal bovine serum (Gibco) (the cells recovered from freezing better in 20% fetal bovine serum, but the concentration could afterwards be reduced to 10%). For subculture, the cells were rinsed with Hank's balanced salt solution and then incubated with 0.05% trypsin, 0.53 mM EDTA, rt (Gibco). After dissociation, the trypsin was neutralized with an equal volume of medium, the cell suspension was centrifuged at 200 g for 5 min, the supernatant was aspirated, and the cells were suspended in fresh medium. Cells were split

1:5 for 7 passages and then split 1:2 to 1:4 and used up to a total of 20 passages. The doubling time of these cells is approximately 3 days. The cells are large and spread to a very high degree on TCPS surfaces, especially when at low confluence. A confluent monolayer has approximately 10^4 cells/cm^2. Cells were frozen at $5*10^5$ cells/mL in 10% dimethyl sulfoxide/90% medium.

Jurkat cells (a T cell lymphoma line that grows in suspension) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin 100 U/mL, streptomycin 100 µg/mL, amphotericin B 0.25 µg/mL, 2 mM L-glutamine, and 1 mM sodium pyruvate (BioWhittaker, Walkersville, MD). Cell concentrations were kept below 10^6/mL by diluting with fresh medium. The doubling time of these cells is approximately 24 h.

All cells were cultured at 37 °C in a humid atmosphere with 5% CO_2.

Cell-Surface Adhesion Assays: Coating the Surface

The ability of polymer-treated surfaces to block cell adhesion was assayed using protocols adapted from Elbert and Hubbell (Elbert, D. L. and Hubbell, J. A. 1998). All assays used culture medium with 10% serum unless otherwise noted. Surfaces to be treated with polymer were prepared as follows, using Falcon 24 well TCPS plates (VWR, Seattle, WA). The area per well of these plates is 2 cm^2. To test polymer and cell interactions with TCPS, the plates were used unmodified. To test interactions with adsorbed serum proteins, the wells were incubated with 250 µL fresh rLEC culture medium for 30 min. Then the medium was aspirated and the wells were rinsed with 1 mL PBS 3 times, the third time incubating in the PBS rinse for 45 min. To test interactions with ECM, rLECs were grown in the wells at least 11 days past confluence. The cells were then removed, leaving the ECM, by aspirating the medium, rinsing with 1 mL PBS, and then adding 0.5 mL 0.1 M NaOH. After 15 min, the solution was aspirated and the wells were rinsed with 1 mL PBS 3 times, the third time incubating in the PBS rinse for 45

min. To test interactions with Matrigel components, the Matrigel was diluted so that it no longer gelled. 0.4 mL Matrigel (Becton Dickinson, Bedford, MA) was mixed with 9.6 mL serum-free rLEC culture medium. 250 µL of this solution was added to each well and incubated 1 h, rt. Then the solution was aspirated and the wells were rinsed with 1 mL PBS 3 times, the third time incubating in the PBS rinse for 30 min.

The various surfaces were then treated with polymer. The final PBS rinses (as indicated in the above paragraph) were aspirated and 250 µL of a 0.1 wt% solution of the polymer in PBS was applied to the surfaces. After 5 min at rt, the solutions were aspirated. The wells were rinsed with 1.5 mL PBS 2 times, the second time incubating in the rinse for 5 min. Then 500 µL of rLEC medium was added to each well. In the case of the Matrigel component surface, 20% serum was used, all other assays used 10% serum. When it was desired to treat only half of a TCPS well with polymer, the left edge of the plate was placed on a block to hold it at an angle. 100 µL of 0.1 wt% polymer was applied to the right-hand side of the wells and incubated for 5 min. Then the solutions were aspirated and the right-hand side of the well rinsed with 100 µL PBS twice, the second time incubating the rinse for 5 min. Then the plate was placed flat again and 500 µL of rLEC medium was added to each well (after cells were seeded onto these plates, the wells were fed fresh culture medium every 3 - 4 days for over 1 month).

rLECs were then seeded at $2*10^3$ cells/cm^2 by adding 200 µL of medium containing cells into each well. The same suspension of cells was used to seed cells for all wells in each experiment (i.e., each graph shown below), to eliminate any variations in establishing cell density in the suspension. The plate was gently shaken to evenly disperse the cells, and then placed in the incubator on a sponge to dampen vibrations. After 4 or 24 h, non-adherent cells were removed by rinsing with 1 mL PBS twice, with gentle shaking. The cells were fixed and stained by adding 0.7 mL Wright stain (Sigma) for 10 min. Then 0.7 mL deionized water was added. After 10 min more, the stain was aspirated and the

wells were rinsed with 1 mL deionized water 3 times. Cells were counted by using an ocular grid eyepiece on a microscope with a 10X objective. The same three locations were counted in each well, for a total area of 0.028 cm^2 examined per well. The cell counts in these three locations were summed and treated as one data point per well (because location dependent density of the cells was noted in all cases). The observer was blind as to the treatment of each well during counting. Adherent cells were defined as any cells present at this point. Spread cells were defined as any cells extending pseuodopodia. When well spread versus poorly spread cells were distinguished, poorly spread cells were those that clearly had very few pseudopodia with the main body of the cell still concentrated in the center and an irregular shape. Well spread cells had spread considerably and had a fairly smooth shape.

The same cell adhesion assays were also performed when competitive inhibitors for PLL-g-(PEG;PBA) binding were also present in solution. TCPS surfaces were used. PLL-g-(PEG;PBA) solutions were prepared at 0.1 or 0.01 wt% in PBS. D-(+)-Mannose (0.1, 0.4, 1, or 5 M), NaCl (1.5 M or 5 M), mannan (1 wt%, from Saccharomyces cerevisiae, Sigma), or mannose + NaCl (1 M + 1.5 M or 5 M + 5 M) were added to these polymer solutions at the various concentrations listed. The solutions were incubated at rt for at least 30 min. The solutions were then applied to TCPS, rinsed, cells were seeded onto the surfaces, and spread cells were counted at 24 h as described above.

Cell-Surface Adhesion Assays: Coating the Cells

The ability of the polymer to coat cells and thus block cell adhesion to surfaces was also tested. Briefly, rLECs were rinsed in PBS, incubated with a polymer for 2 min, then either rinsed again and seeded onto TCPS, or seeded directly onto ECM with the polymer still remaining in the culture solution. Specifically, TCPS and ECM surfaces were prepared as described above, and 500 µL of culture medium was added to each well. rLECs were removed from their substrate using trypsin/EDTA, neutralized with an equal

volume of medium, and then centrifuged at 200 g for 5 min. The supernatant was aspirated, the cells were re-suspended in PBS, aliquotted into individual tubes for each polymer treatment, centrifuged again, and the supernatant was aspirated. In the first trial, cells were then treated with polymer for 2 min, centrifuged and re-suspended in culture medium, and then seeded onto TCPS. In this case, the cells were suspended in 0.1 wt% polymer solutions in PBS at $2*10^4$ cells/mL. After 2 min of incubation with the polymer, the cells were centrifuged at 100 g for 2 min. The supernatant was aspirated, the cells were re-suspended at $2*10^4$ cells/mL in culture medium, and seeded onto the TCPS substrate at $2*10^3$ cells/cm^2. In the second trial, cells were treated with polymer for 2 min, then medium was added and the cells were seeded onto ECM with the polymer still present in the solution. In this case, the cells were suspended in 0.1 wt% polymer solutions in PBS at $3*10^4$ cells/mL. After 2 min of incubation with the polymer, culture medium (20% serum) was added to reach a cell concentration of $2*10^4$ cells/mL. The cells were then seeded onto the ECM substrate at $2*10^3$ cells/cm^2. In both cases, after 24 h of incubation, the cells were rinsed, fixed, stained, and counted as described above.

A live/dead test was also performed on cells 2 h after treatment with polymer and seeding onto TCPS. The protocol described above for cell treatment and seeding onto TCPS was followed. Ethidium homodimer was used at 4 μM and Calcein AM was used at 2 μM according to the manufacturer's instructions (Molecular Probes, Eugene, OR). 2 h after the cells had been seeded onto TCPS, the non-adherent cells were removed from 3 wells, diluted with 50 mL PBS, and centrifuged at 100 g for 5 min. The supernatant was aspirated, and 638 μL of the live/dead reagent was added. 200 μL of cells were added per well in a new TCPS plate. The plate was covered with aluminum foil and incubated at rt for 30 min. The wells were then viewed on a fluorescence microscope, where live cells would appear green and dead cells would appear red upon excitation at 485 nm.

Cytotoxicity Assays

Cytotoxicity was assessed using MTT assays modified from Plumb et al. (Plumb, J. A. et al. 1989). For assays using adherent cells, confluent rLECs were used, with culture medium containing 20% serum, in 96-well flat bottom TCPS plates (Falcon). Each well containing cells was rinsed with 200 μL of PBS, then 17 μL of the polymer dissolved in PBS was added at double the final concentration. After 2.5 min, 17 μL of culture medium was added to the cells and polymer solution, and the plate was placed in the incubator (this volume was sufficient to keep the cells covered with fluid throughout the incubation). After the chosen incubation time (5 min or 4 h), the polymer solution was aspirated, the cells were rinsed with 200 μL PBS, and then fed 200 μL fresh culture medium and replaced in the incubator. After 1 day, the cells were fed 200 μL fresh culture medium. After 3 days total, the surviving cell numbers were assayed using MTT. MTT (Sigma) was dissolved at 5 mg/mL in PBS and sterile filtered through a 0.2 μm syringe filter. The cells were fed 200 μL fresh culture medium and 50 μL of MTT solution was also added. The culture plate was loosely wrapped in aluminum foil and replaced in the incubator. After 5 h, the supernatant was aspirated off of the cells. 200 μL of dimethyl sulfoxide and 50 μL of Sorenson's glycine buffer (0.1 M glycine, 0.1 M NaCl, pH 10.5) were added to each well and mixed. The absorbance of each well at 570 nm was then read using a plate reader. The absorbance levels of wells treated only with PBS were considered to be 100%, and the polymer-treated wells were referenced to that value.

For cytotoxicity assays with non-adherent cell cultures (Jurkats), the cells were cultured in suspension in 96 well U-bottom TCPS plates (Falcon). 100 μL of cells at $2*10^5$ cells/mL was placed in each well. 100 μL of the polymer dissolved in culture medium was added (at double the final concentration). After 4 h of incubation, 50 μL of MTT (5 mg/mL in PBS, filter sterilized) was added to each well. The culture plate was loosely wrapped in aluminum foil and replaced in the incubator. After 4 more h, the culture

plate was centrifuged at 200 g for 5 min and the supernatant was aspirated. 150 μL of dimethyl sulfoxide and 25 μL of Sorenson's glycine buffer was added to each well and mixed. The absorbance at 570 nm was then read using a plate reader. The absorbance levels of wells treated only with polymer-free culture medium were considered to be 100%, and the other wells were referenced to that value.

To test if the polymers were binding and potentially sequestering glucose in the cell culture media, the glucose concentration in the media was tested using an enzymatic assay. A glucose hexokinase assay kit was used (Sigma), where hexokinase converts glucose to glucose-6-phosphate, and glucose-6-phosphate dehydrogenase converts that product to 6-phosphogluconate while converting NAD to NADH. The consequent increase in absorbance at 340 nm can then be read. This assay is known to exhibit the highest specificity for glucose, and can be conducted in plasma (Bergmeyer, J. and Grassl, M. 1984). PLL-g-(PEG;PBA) 1:21 and 1:9 were dissolved in rLEC culture medium (20% serum) at 0.001 - 1 wt% and incubated for at least 5 min. 10 μL of sample was then combined with 1 mL of glucose assay reagent, shaken at rt for 15 min, and then the absorbance at 340 nm was read. The absorbance was compared to a glucose standard calibration curve and to culture medium without polymer. The absorbance due to phenol red and due to polymer at these concentrations was very negligible.

4.3 Results

Cell-Surface Adhesion: Coating the Surface

The ability of the polymers to bind to various model surfaces and prevent cell adhesion to those surfaces was tested. TCPS was used as a model surface containing hydroxyls for PBA binding. Adsorbed serum proteins provided a model of glycoproteins that could interact with polymer at a wound site either in solution or adsorbed to ECM. rLEC ECM and Matrigel were employed as models of ECM components, on which cell

binding and migration are potentially important in both posterior capsule opacification and peritoneal adhesion formation. Surfaces were incubated with a polymer solution in PBS, then rinsed and subsequently seeded with cells. Adherent and/or spread cells were counted after 4 or 24 h.

PLL-g-(PEG;PBA) polymers spontaneously coated TCPS surfaces and blocked cell adhesion to those surfaces. TCPS contains many hydroxyls to which PBA could bind (Table 4.1). PLL-g-(PEG;PBA) blocked cell adhesion to TCPS over a wide range of PEG graft ratios (Figure 4.2). PLL-g-(PEG;PBA) 1:21, 1:9, and 1:6 completely blocked cell adhesion ($P < 0.002$ v. PBS for each), and PLL-g-(PEG;PBA) 1:3 significantly reduced cell adhesion ($P < 0.006$ v. PBS). PLL-g-PEG (which can bind to TCPS via electrostatic attraction to the anionic surface) completely blocked cell adhesion only at graft ratios 1:9 and 1:6 ($P < 0.002$ v. PBS for each), and 1:21 significantly reduced adhesion ($P < 0.03$ v. PBS). Most cells that were adhered were spread, with the exception of cells on the PLL-g-

Table 4.1. ESCA analysis of TCPS surfaces.

Scan	Species	Content
Survey	C	81%
	O	17%
	Other	2%
High Resolution C$_{1s}$	285 eV (C-C, C-H)	86%
	286.5 eV (C-O-C, C-O-H)	12%
	Other	1%

Falcon 24 well TCPS plates were analyzed. Data shown is the average of 3 analyses. The data indicates that a significant amount of hydroxyls are present on the surfaces. If one were to assume that all oxygen species are present as either ethers (C$_{1s}$ at 286.5 eV), hydroxyls (C$_{1s}$ at 286.5 eV), or carboxylic acids (C$_{1s}$ other), the data indicates that approximately 7% of all carbons on the surface have a hydroxyl attached to them. It is likely that many of these hydroxyls will be located close enough to each other for PBA to be able to bind to them.
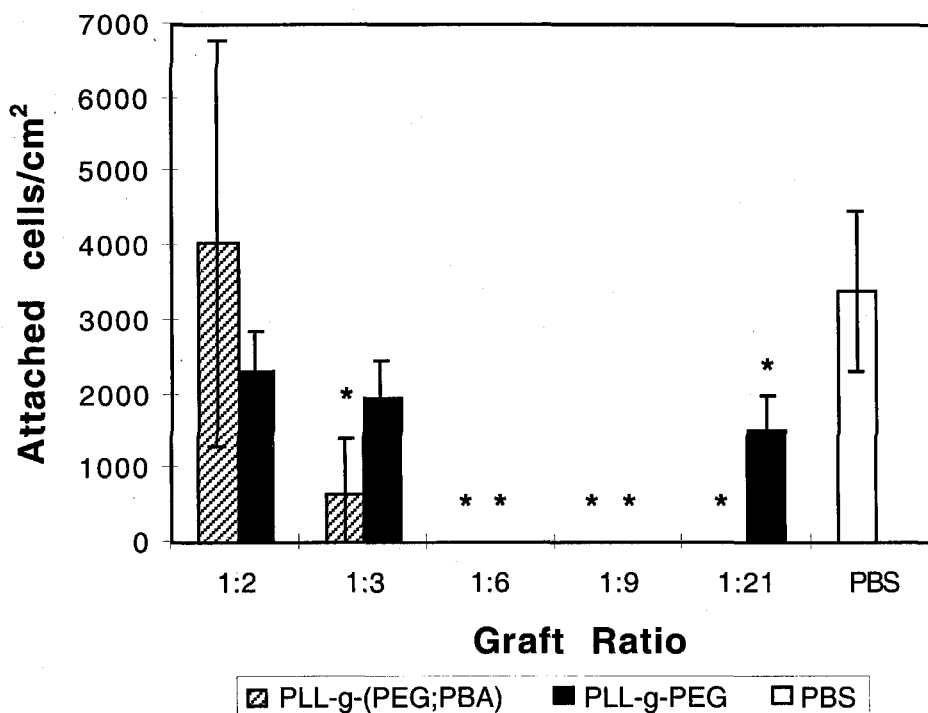
Figure 4.2. rLEC adhesion to polymer-treated TCPS at 24 h.

TCPS was incubated with 0.1 wt% polymer solutions in PBS (pH 7.4) for 5 min, then rinsed with PBS. Serum-containing medium was added and rLECs were seeded. After 24 h, non-adherent cells were washed away and adherent cells were counted. PLL-g-(PEG;PBA) completely blocked cell adhesion over a wide range of PEG graft ratios whereas PLL-g-PEG blocked cell adhesion over a narrower range of graft ratios. All adhered cells were well spread, except that most of the cells adhered to PLL-g-PEG 1:21-treated surfaces were unspread or poorly spread. * = significantly different from PBS at 95% confidence using Student's t-test (mean ± sd, n ≥ 3).

PEG 1:21-treated surfaces, where most adhered cells were unspread or extending only a few very small pseudopodia.

The mechanism of PLL-g-(PEG;PBA) binding to TCPS includes PBA-hydroxyl interactions as well as electrostatic interactions. Varying concentrations of soluble mannose, NaCl, or mannan were added to PLL-g-(PEG;PBA) 1:9 solutions before the solution was incubated with the TCPS surface. If the polymer binds to TCPS via PBA-hydroxyl interactions, mannose or mannan should competitively inhibit polymer binding to the surface. If the polymer binds to TCPS via electrostatic attraction between cationic

amines and/or anionic PBA with the plasma-treated TCPS surface, adding NaCl should inhibit polymer binding to the surface. If polymer binding is inhibited, seeded cells should adhere to and spread on the treated surface. When using 0.1 wt% polymer solutions, the addition of 0.1 - 5 M mannose, 1.5 - 5 M NaCl, or 1 wt% mannan was inadequate to permit any cell attachment. However, the addition of 1 M mannose plus 1.5 M NaCl allowed a small amount of cells to adhere to the surfaces, although 5 M mannose plus 5 M NaCl did not allow cell adherence (data not shown). When the polymer concentration was reduced to 0.01 wt%, the inhibitors became more effective (Figure 4.3). A mannose concentration of 5 M, or an NaCl concentration of 1.5 M or 5 M began to permit cell spreading on the surface, although all values were statistically less than the relevant PBS control. The 5 M NaCl solution allowed the most spreading of those solutions. It was noted that when solutions had NaCl added to them, cells adhered in patches to those treated surfaces, where there were distinct regions (on the order of 0.1 mm^2) where cells adhered and many larger regions where no cells adhered. However, when solutions contained 1 M mannose plus 1.5 M NaCl, cell spreading was equivalent to the PBS control (and evenly dispersed on the surface). Solutions containing 5 M mannose plus 5 M NaCl also permitted much cell spreading, although it was statistically less than the PBS control.

Cell adhesion to PLL-g-(PEG;PBA)-treated surfaces was prevented because of local steric blocking of adhesion, not because of toxic or solution effects. When one side of TCPS wells were treated with PLL-g-(PEG;PBA) 1:21 or 1:9, seeded cells grew to complete confluence on the untreated side of the well. However, the cells did not adhere to or migrate onto the treated side of the well, even after 1 month of culture with feeding of fresh medium twice a week (Figure 4.4). In addition, when cells were seeded onto TCPS wells that had been completely treated with PLL-g-(PEG;PBA) 1:21 or 1:9 and the cells did not adhere, those cells were still alive and competent to spread. If, after 2 h of incubation with the polymer-treated surface, the (non-adherent) cells were then removed and seeded
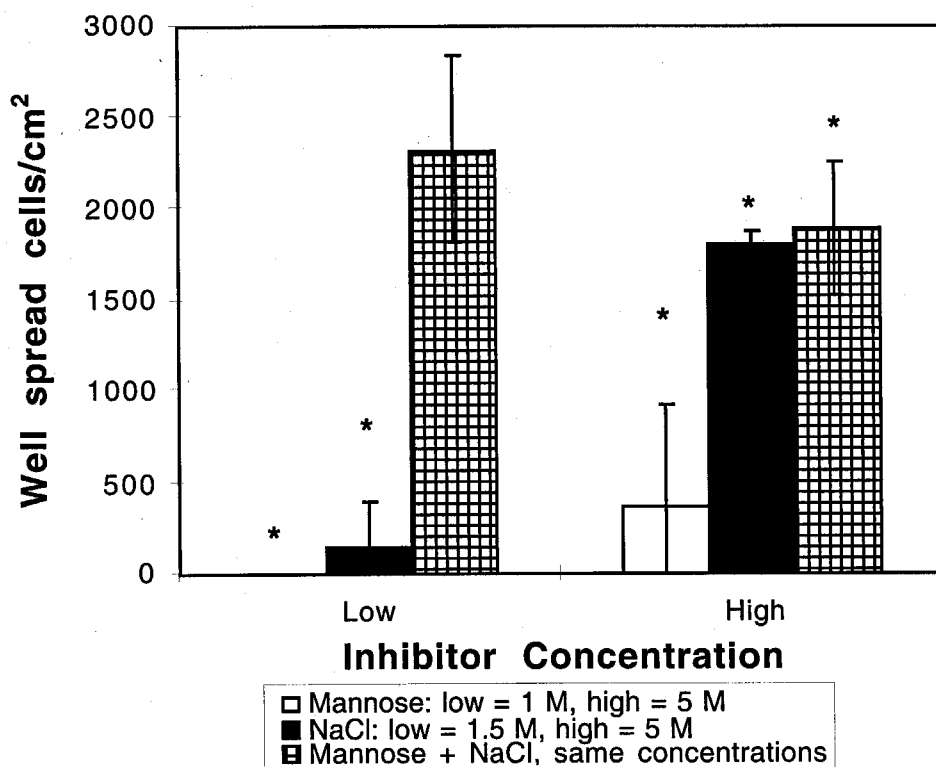
Figure 4.3. Inhibition of PLL-g-(PEG;PBA) binding to TCPS by soluble competitors.

PLL-g-(PEG;PBA) 1:9 was used at 0.01 wt% in PBS (pH 7.4). Mannose and/or NaCl was added to these solutions at varying concentrations and incubated for at least 30 min, rt. TCPS was then treated with these solutions, rinsed, and rLECs were seeded onto the surfaces. 5 M mannose, 1.5 M NaCl, 5 M NaCl, and the combination of 5 M mannose + 5 M NaCl all permitted some cell attachment to the surfaces, although all were statistically less than the relevant PBS control. Solutions containing 5 M NaCl allowed cells to adhere only in distinct patches. The combination of 1 M mannose + 1.5 M NaCl, however, allowed cells to adhere evenly with statistically the same density as surfaces treated with the relevant PBS control. * = significantly different from the relevant PBS control at 95% confidence using Student's t-test (mean ± sd, n = 3).

onto fresh TCPS in the same supernatant, the cells adhered, spread, and proliferated

normally.

When cells were seeded onto polymer-treated adsorbed serum protein surfaces,

only PLL-g-(PEG;PBA) 1:21 had a strong effect on blocking cell spreading. Surfaces

were created by incubating TCPS with serum-containing medium, then incubating those

proteinaceous surfaces with the polymer solutions, rinsing, and subsequently seeding cells.
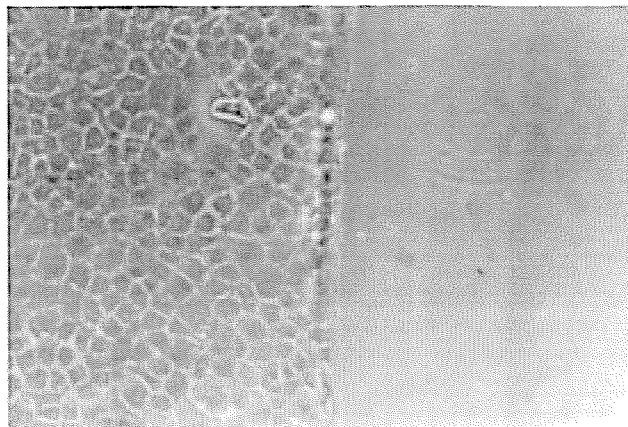
Figure 4.4. Lack of rLEC migration onto polymer-treated TCPS at 1 month.

The right half of a TCPS well was incubated with 0.1 wt% PLL-g-(PEG;PBA) 1:9 in PBS (pH 7.4) for 5 min, then rinsed with PBS. Serum-containing medium was added and rLECs were seeded. The well was fed fresh culture medium twice a week. The cells grew to confluence on the untreated side of the well, but did not migrate onto the polymer-treated side of the well even after 1 month. The same effect was seen when TCPS was treated with PLL-g-(PEG;PBA) 1:21. n = 3.

It was found that while all polymers allowed cell adhesion to the surface, some blocked cell spreading on the surface. Unspread cells and distinctly poorly spread cells were noted in some polymer-treated wells, whereas other wells had normal well spread cells (see Figure 4.5). At 4 h, serum protein surfaces treated with PLL-g-(PEG;PBA) 1:21 had very few well spread cells (Figure 4.6a, P < 0.001 v. PBS). Significant reductions in cell spreading were also seen for PLL-g-(PEG;PBA) 1:9 and PLL-g-PEG 1:21 treated surfaces (P < 0.03 and P < 0.02 v. PBS, respectively). However, there was no significant difference in the number of attached cells for any polymer treatment (Figure 4.6b). At 24 h, all attached cells were well spread, and no polymer reduced the number of spread cells (Figure 4.6c).
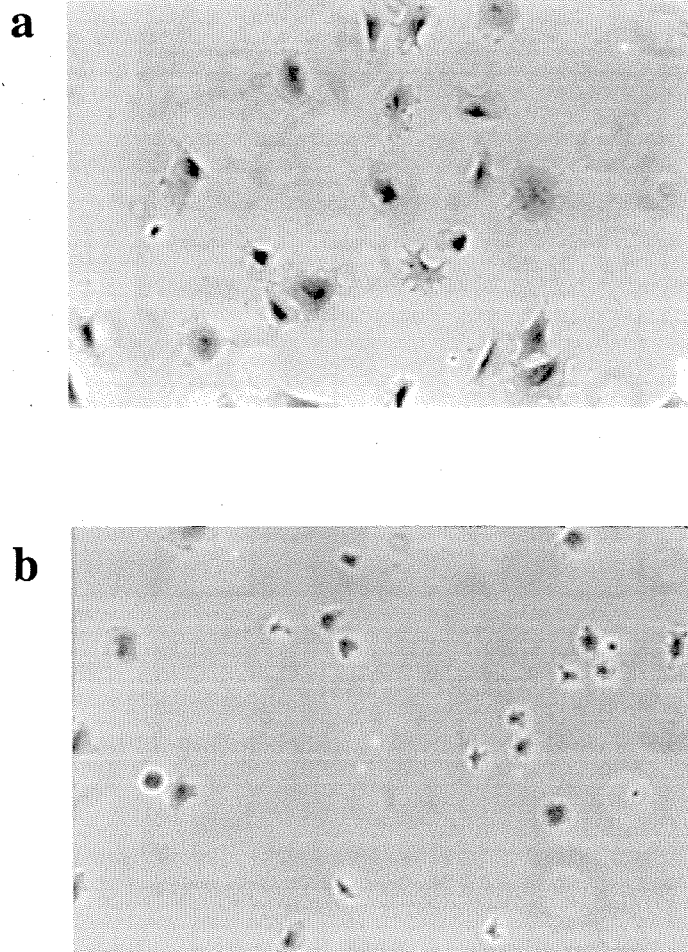
Figure 4.5. rLEC spreading on ECM at 4 h.

a. ECM treated only with PBS. b. ECM treated with PLL-g-(PEG;PBA) 1:21. Both photomicrographs are at the same magnification. Cells were well spread at 4 h when seeded onto ECM, but the cells were very poorly spread when the ECM was treated with PLL-g-(PEG;PBA) 1:21. The same morphologies were noted when the surface tested was adsorbed serum proteins.
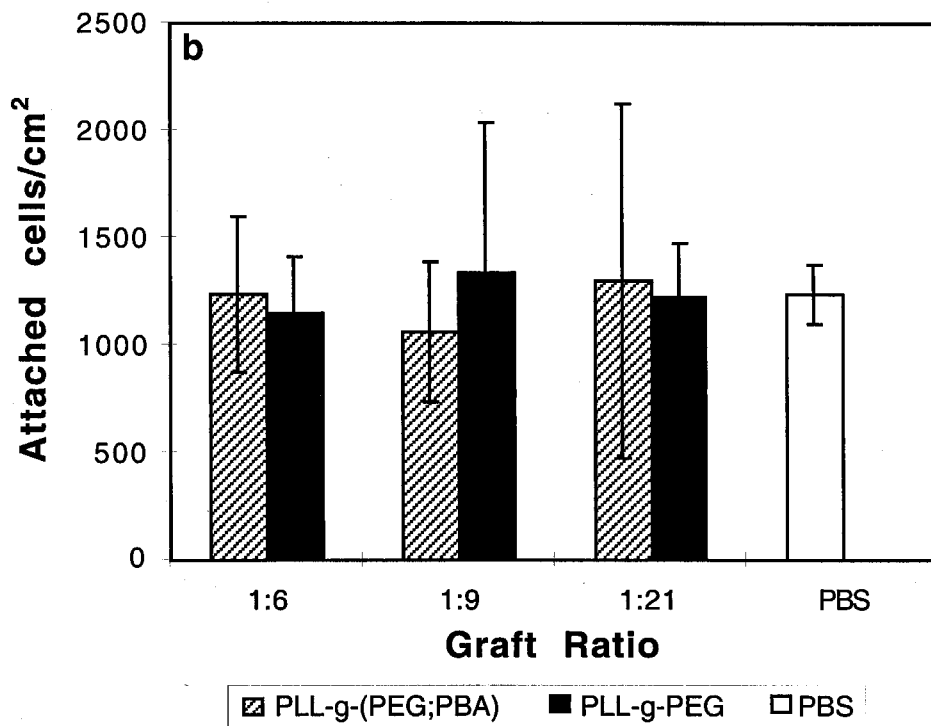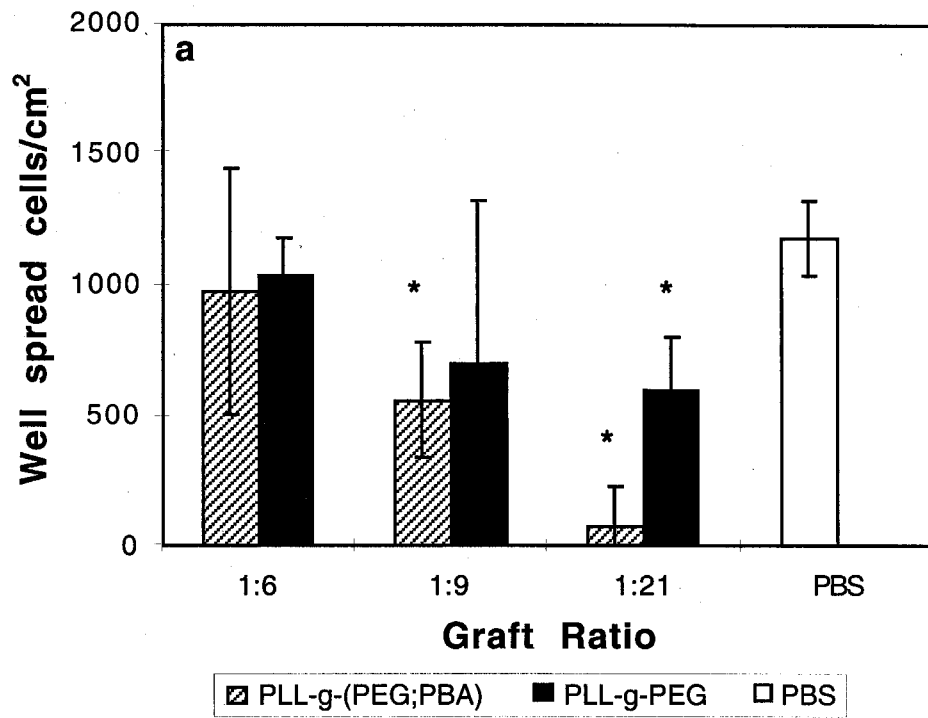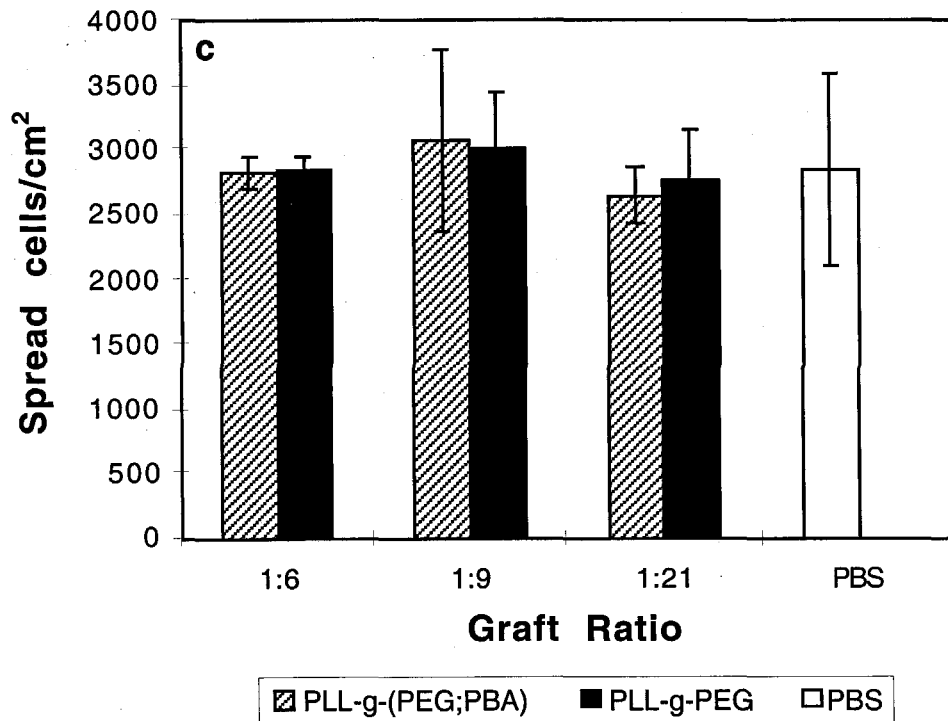
Figure 4.6. rLEC interaction with polymer-treated adsorbed serum proteins.

TCPS was incubated with serum-containing medium for 30 min, then rinsed with PBS. This surface was subsequently incubated with 0.1 wt% polymer solutions in PBS (pH 7.4) for 5 min, then rinsed with PBS. Serum-containing medium was added and rLECs were seeded. After 4 or 24 h, non-adherent cells were washed away and remaining cells were counted. a. Well spread rLECs at 4 h. b. Attached rLECs at 4 h. c. Spread rLECs at 24 h. PLL-g-(PEG;PBA) 1:21-treated surfaces had very few well spread cells at 4 h, but there were many attached cells at 4 h. By 24 h, all cells were well spread and no polymer significantly reduced cell attachment or spreading. * = significantly different from PBS at 95% confidence using Student's t-test (mean ± sd, n = 3).

When cells were seeded onto polymer-treated ECM, PLL-g-(PEG;PBA) 1:21 blocked cell spreading in a similar manner to that seen with serum proteins. ECM was revealed by removing confluent cells with 0.1 M NaOH. Then the ECM was rinsed and incubated with polymer, rinsed, and cells were subsequently seeded. Similar to the effects seen on polymer-treated serum proteins, it was found that some wells had unspread and/or

poorly spread cells. At 4 h, surfaces treated with PLL-g-(PEG;PBA) 1:21 had very few well spread cells (Figure 4.7a, $P < 0.00003$ v. PBS). However, PLL-g-(PEG;PBA) 1:21 treated surfaces supported a number of attached cells, even though they were still statistically less than control wells (Figure 4.7b, $P < 0.0003$ v. PBS). Significant reductions in cell spreading and attachment were also seen for some other polymer treatments, but the effect was much less dramatic. At 24 h, all attached cells were well spread, and no polymer reduced the number of spread cells (Figure 4.7c).

When cells were seeded onto polymer-treated Matrigel components, no polymer had a significant effect on cell attachment or spreading. Gelled Matrigel was not used, because its structure did not remain static during rinse steps and because the rLECs grew in network-like clumps on and retracted within the gel. Instead, Matrigel was diluted so that it no longer gelled, and was adsorbed onto TCPS. This resulted in what presumably was a monolayer coating of basal lamina components with no substantial thickness. This surface was then incubated with the polymer solution, rinsed, and cells were seeded. No reductions in cell attachment or spreading were seen on these polymer-treated surfaces at 4 h or 24 h, although only the 24 h time point was quantified (Figure 4.8).
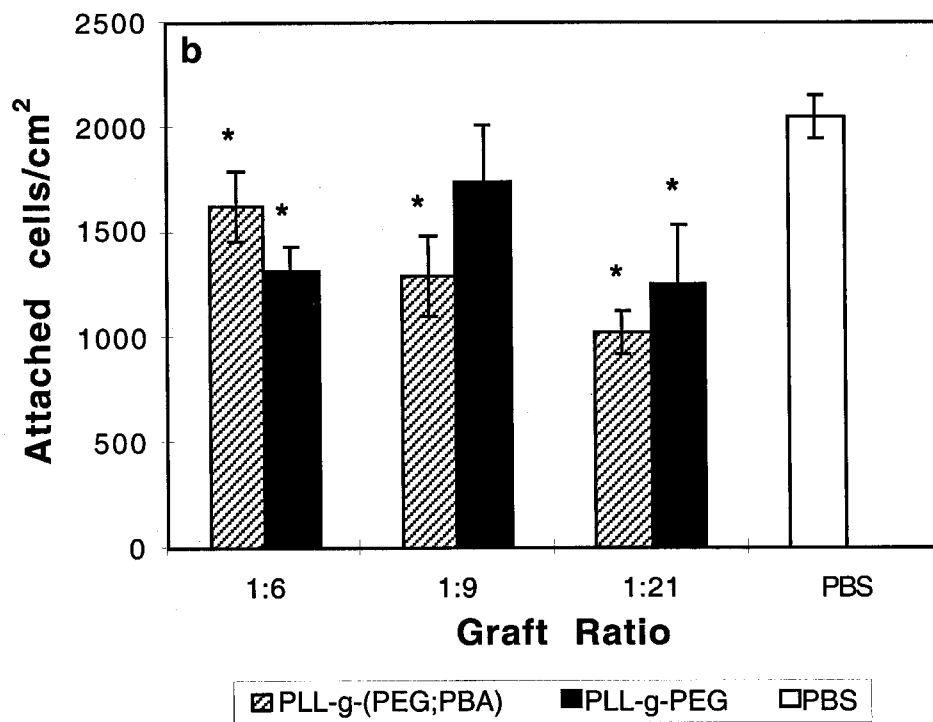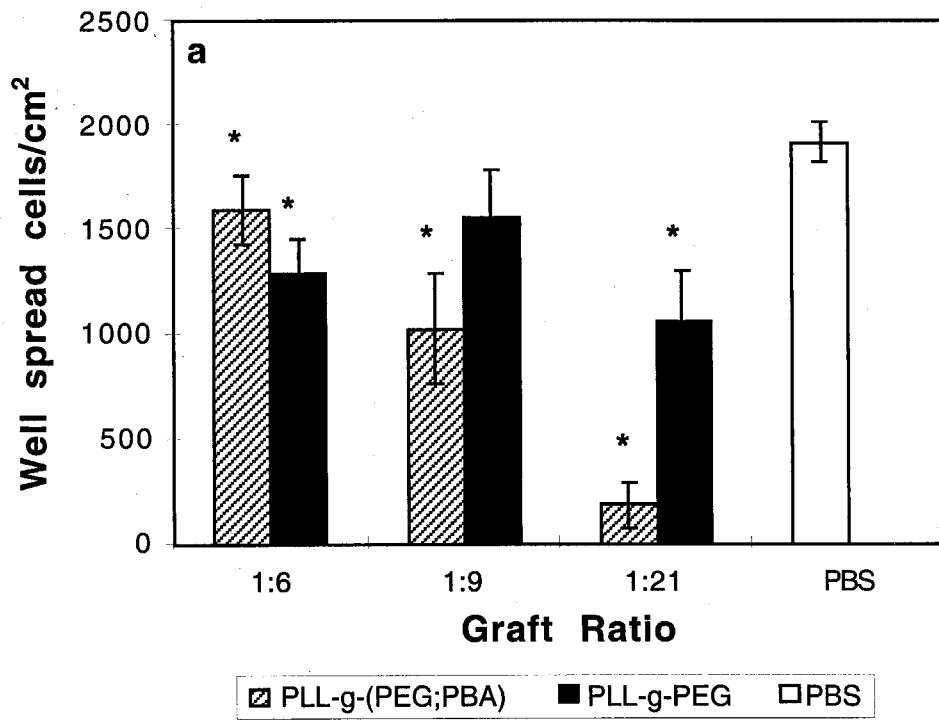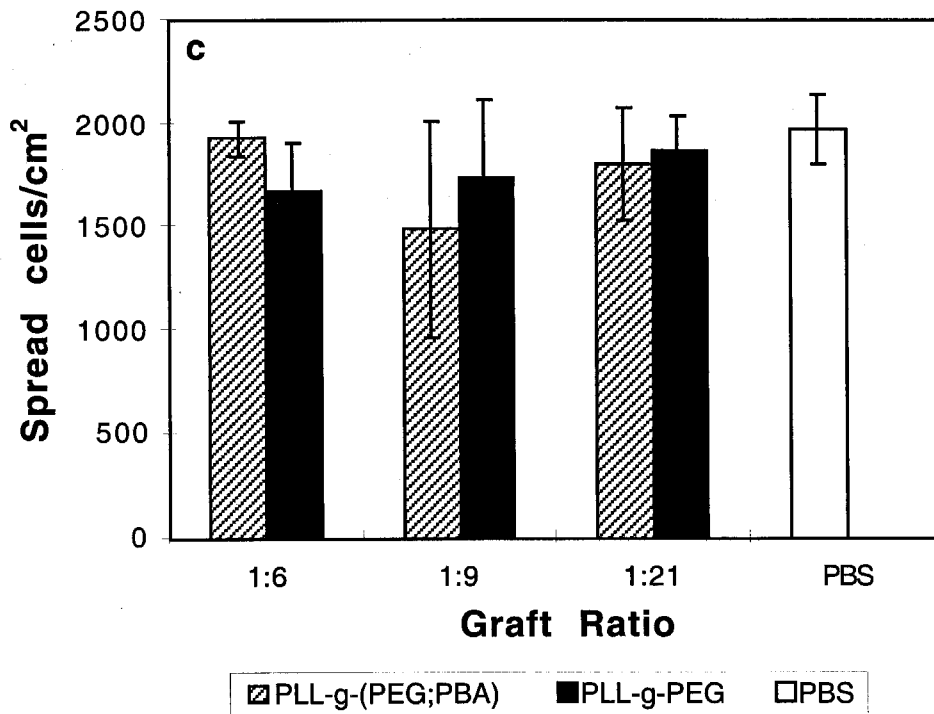
Figure 4.7. rLEC interaction with polymer-treated ECM.

rLECs were grown 11 days past confluence and then removed via 0.1 M ammonia treatment for 15 min. The remaining ECM was rinsed with PBS. This surface was then incubated with 0.1 wt% polymer solutions in PBS (pH 7.4) for 5 min, then rinsed with PBS. Serum-containing medium was added and rLECs were seeded. After 4 or 24 h, non-adherent cells were washed away and remaining cells were counted. a. Well spread rLECs at 4 h. b. Attached rLECs at 4 h. c. Spread rLECs at 24 h. PLL-g-(PEG;PBA) 1:21-treated surfaces had very few well spread cells at 4 h, but there were many attached cells at 4 h. By 24 h, all cells were well spread and no polymer significantly reduced cell attachment or spreading. * = significantly different from PBS at 95% confidence using Student's t-test (mean ± sd, n ≥ 3).
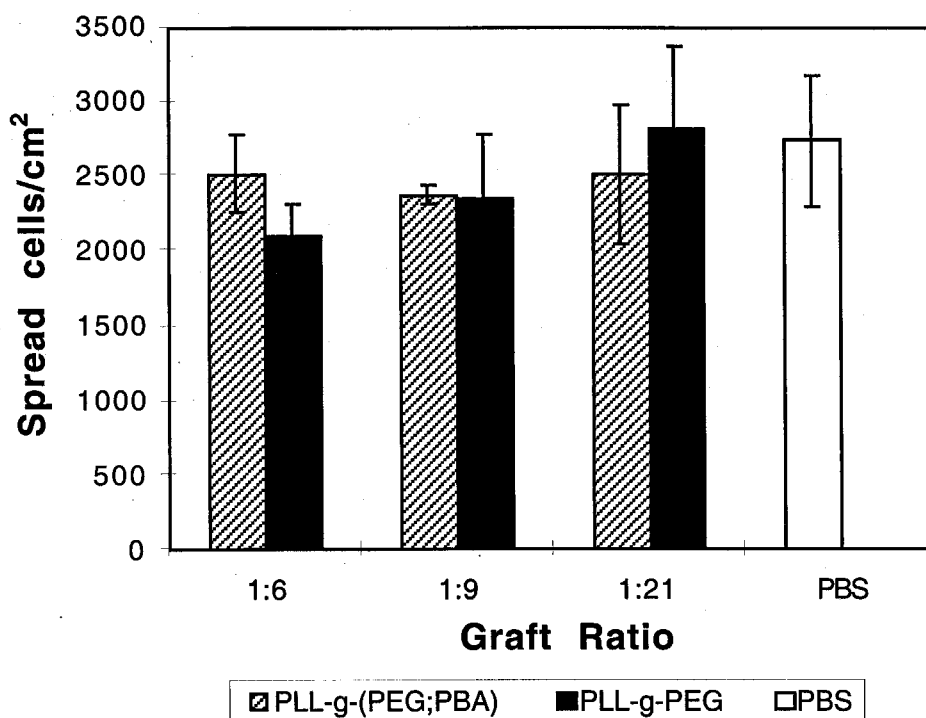
Figure 4.8. rLEC spreading on polymer-treated Matrigel components at 24 h.

Matrigel was diluted in serum-free medium so that it no longer gelled. This solution was applied to TCPS for 1 h and the surface was then rinsed with PBS. This surface was then incubated with 0.1 wt% polymer solutions in PBS (pH 7.4) for 5 min, then rinsed with PBS. Serum-containing medium was added and rLECs were seeded. After 24 h, non-adherent cells were washed away and remaining cells were counted. No polymer significantly reduced cell attachment or spreading on Matrigel components at 4 h or 24 h . * = significantly different from PBS at 95% confidence using Student's t-test (mean ± sd, n = 3).

Cell-Surface Adhesion: Coating the Cells

The ability of the polymers to bind to the surface of cells and thereby prevent adhesion of cells to TCPS or ECM was also tested. rLECs were rinsed in PBS and then incubated in 0.1 wt% polymer in PBS for 2 min. In the first assay, the polymer-treated cells were then centrifuged, re-suspended in culture medium, and seeded onto TCPS. Because it was feared that the repeated centrifuge steps were potentially killing the cells, in the second assay, the polymer-treated cells were simply diluted in culture medium and

seeded onto ECM (with the polymer still present in the solution). The number of spread cells was then counted at 24 h.

PLL-g-(PEG;PBA) and PLL-g-PEG 1:21 strongly blocked polymer-treated-cell spreading on TCPS at 24 h (Figure 4.9). PLL-g-(PEG;PBA) and PLL-g-PEG 1:9 also blocked spreading to a lesser extent. Approximately all cells that were adhered were well spread. However, many of the PLL-g-(PEG;PBA) 1:21- and PLL-g-PEG 1:21-treated non-adherent cells appeared phase dark, granular, and perhaps dead. Even wells containing PBS-treated cells had low spread cell counts (they were seeded at $2*10^3$ cells/cm^2) and many cells that appeared dead. To perform the protocol, the cells were centrifuged and re-suspended a total of 3 times, twice centrifuging in PBS. It is possible that the extended manipulations of the cells caused mechanical damage or that the extended exposure to PBS (not serum-containing medium) was not well tolerated. Centrifuging the cells while coated with polymer might increase the stress, or induce polymer bridging upon pellet formation. Also, the cell pellets were so small for each polymer or PBS treatment that it was difficult to aspirate the supernatant and be assured that no cells were aspirated at the same time. Thus while testing the next surface (ECM), it was decided to not centrifuge out the free polymer after treatment, but to leave the free polymer in solution while cell spreading was tested.

PLL-g-(PEG;PBA) and PLL-g-PEG 1:21 also strongly blocked polymer-treated-cell spreading on ECM in the presence of polymer at 24 h (Figure 4.10). Approximately all cells that were adhered were well spread. Again, however, many non-adherent cells appeared dead, including PBS-treated cells. Thus it was decided to assay the viability of the polymer- and PBS- treated cells.

Performing the live/dead test on polymer-treated cells revealed very few cells at all, live or dead (data not shown). The cells were treated with polymers and seeded onto TCPS as described for the first experiment, then the live/dead test was performed 2 h after cell
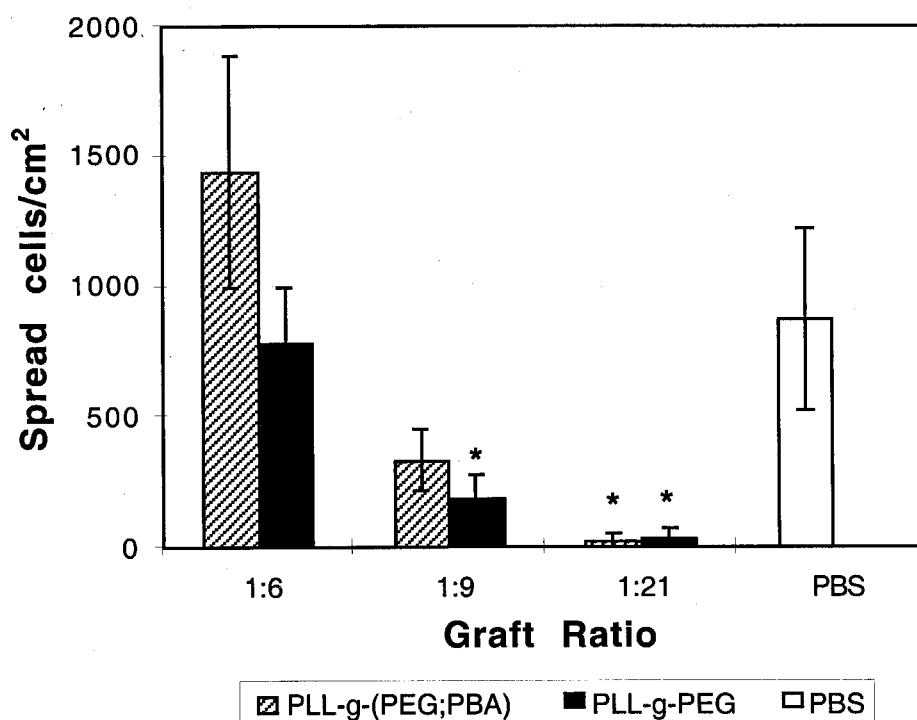
Figure 4.9. Polymer-treated-rLEC spreading on TCPS at 24 h (no polymer in solution).

rLECs were rinsed in PBS and then suspended in 0.1 wt% polymer solutions in PBS. After 2 min, the cells were centrifuged and re-suspended in culture medium, then seeded onto TCPS. At 24 h, all adhered cells were well spread and were counted. Treatment of cells with PLL-g-(PEG;PBA) and PLL-g-PEG 1:21 and, to a lesser extent, 1:9 strongly prevented cell adhesion to TCPS. However, many of the non-adherent cells appeared dead, thus the mechanism of adhesion prevention was unclear. Wells containing cells treated with PBS also had many cells that appeared dead, and a low % of spread cells (cells were seeded at 2000 cells/cm^2).

seeding. The live/dead test requires rinsing and centrifuging the cells to remove serum esterases, then adding a reagent that will turn live cells green and dead cells red when viewed with a fluorescent microscope. Very few red or green cells were seen, and phase contrast light microscopy also revealed extremely few cells per well (including in PBS-treated wells). In a control well where cells had not been treated with polymer or PBS, many more cells were seen. Thus the additional rinsing/centrifuging steps seemed to really destroy the cells (if the cells are so damaged that their membranes are no longer intact at all,
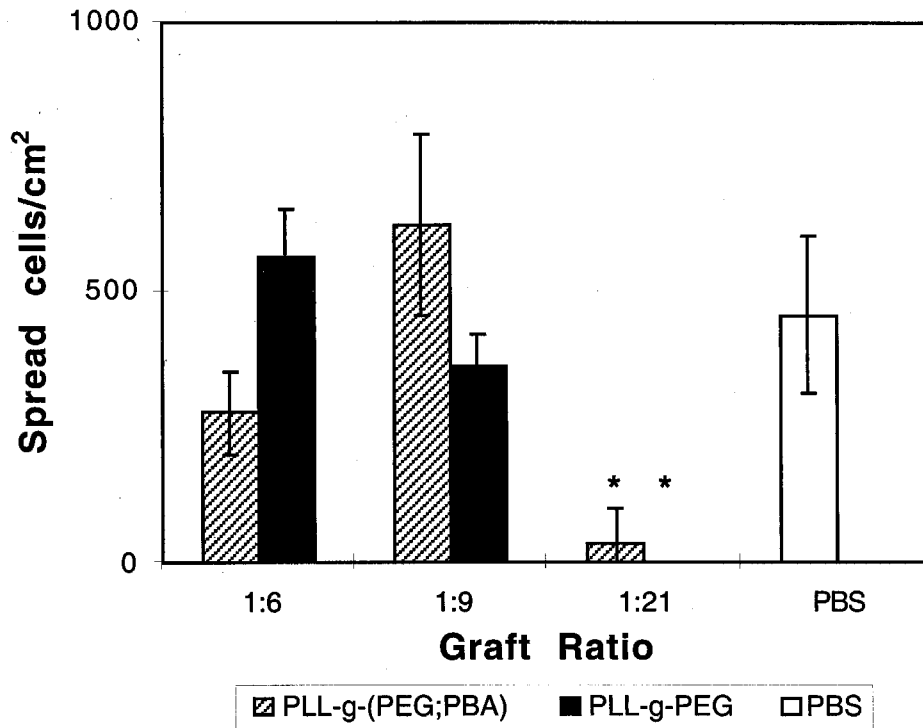
Figure 4.10. Polymer-treated-rLEC spreading on ECM at 24 h, with polymer in solution.

rLECs were rinsed in PBS and then suspended in 0.1 wt% polymer solutions in PBS. After 2 min, the cells were diluted in culture medium, then seeded onto ECM with the free polymer still present in solution. This protocol utilized 1 less centrifuge and re-suspension step than for the assay in Figure 4.9. At 24 h, all adhered cells were well spread and were counted. Treatment of cells with PLL-g-(PEG;PBA) and PLL-g-PEG 1:21 strongly prevented cell adhesion to TCPS. However, many of the non-adherent cells appeared dead, thus the mechanism of adhesion prevention was unclear. Wells containing cells treated with PBS also had many cells that appeared dead, and a low % of spread cells (cells were seeded at 2000 cells/cm^2).

the membrane will not be able to hold the red dye and be viewed). The percentage of live versus total cells seen for each polymer treatment was not considerably different than for PBS-treated cells. While the ability of the polymer to bind to cell surfaces and sterically block cell adhesion versus the polymer merely killing the cells was unclear, it was decided to assay the cytotoxicity of the polymers.

Cytotoxicity

The cytotoxicity of the polymers was tested *in vitro* using an MTT assay modified from Plumb et al. (Plumb, J. A. et al. 1989). This is a standard assay in which a dye is reduced by live but not dead cells, yielding a water insoluble purple formazan product which can be quantified by reading the absorbance at 570 nm. The polymer toxicity was first tested using adherent rLECs. The rLECs were grown to confluence before polymer treatment, because otherwise the polymer could coat the cells as well as the bare culture substrate and prevent replicating cells from being able to re-adhere to the surface. The cells were then incubated with the polymer. After 5 min or 4 h, the cells were rinsed and cultured in medium for 3 days before the numbers of cells were quantified with MTT. This allowed the assay to identify toxic effects that might take longer than a few hours to potentially kill the cells.

When applied to rLEC monolayers, PLL-g-(PEG;PBA) 1:9 had very low toxicity, but PLL-g-(PEG;PBA) 1:21 had apparent toxicity at high concentrations and long exposure times (Figure 4.11). After 2 h of exposure to PLL-g-(PEG;PBA) 1:21, the cells were observed to round up from the monolayer and become phase bright, in a concentration-dependent manner. By 4 h, the amount of surface coverage by the cells was approximately 2% for the 1 wt% treatment, 25% for the 0.1 wt% treatment, 70% for the 0.01 wt% treatment, and 100% for the 0.001 wt% treatment. This effect also occurred for the PLL-g-(PEG;PBA) 1:9 cells with 4 h of exposure, but to a much smaller degree. No cells were observed to be free-floating (i.e., completely non-adherent), thus it was possible that some cells exposed to high concentrations of PLL-g-(PEG;PBA) 1:21 were lysed. However, it is likely that the large numbers of rounded loosely-adherent cells were subsequently washed away during rinsing steps and thus were counted as "dead" when they may have been alive but detached from the surface. When the same assay was conducted using the corresponding PLL-g-PEG polymers, time and dose dependent toxicity was found for both PLL-g-PEG 1:21 and 1:9 (Figure 4.12). However, cells that were killed by PLL-g-PEG
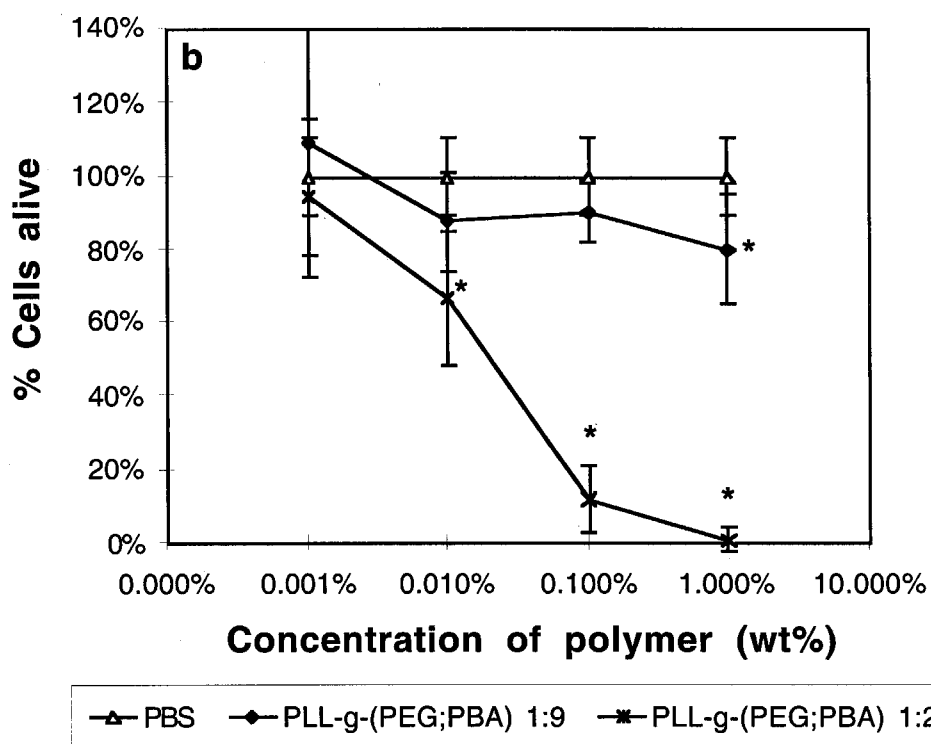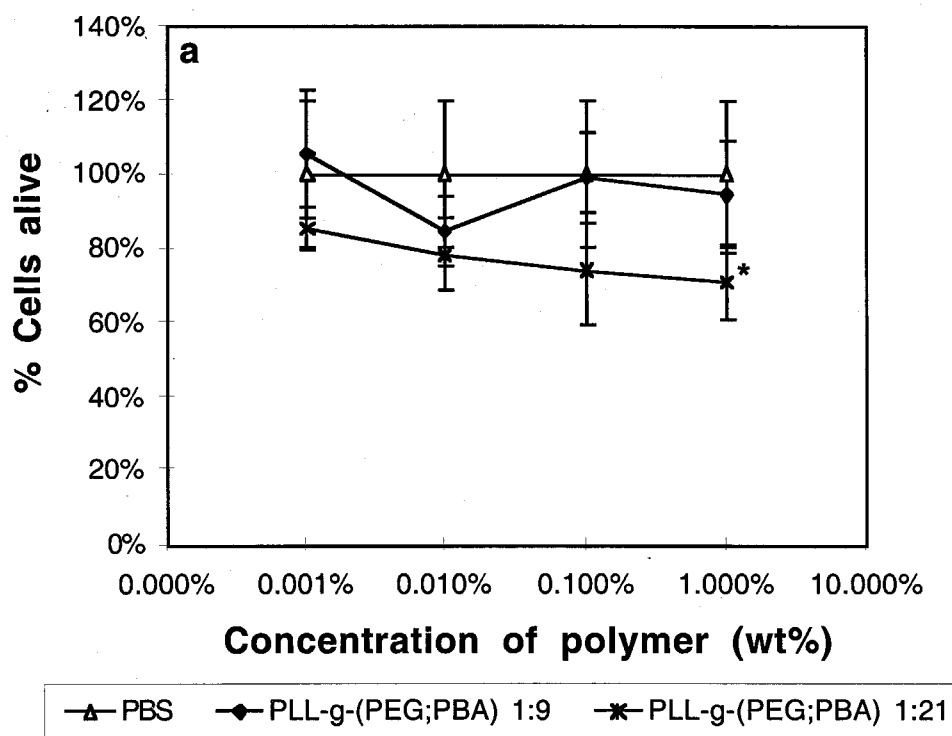
Figure 4.11. Cytotoxicity of PLL-g-(PEG;PBA) polymers on rLECs *in vitro*.

(Figure 4.11)
rLECs were grown to confluence, then rinsed with PBS. The cells were then treated with varying concentrations of polymers in PBS (pH 7.4) at double the final concentration for 2.5 min, then diluted with media to the final concentration. After 5 min or 4 h of exposure, the cells were rinsed and incubated in culture medium for 3 days. Then the final cell density was quantified using MTT and compared to the PBS control wells. a. Confluent rLECs incubated with polymer for 5 min. b. Confluent rLECs incubated with polymer for 4 h. Cells treated with high concentrations of polymer or for long incubation periods rounded up and became phase bright, with the effect being much stronger for PLL-g-(PEG;PBA) 1:21. The cells may have been washed away during rinse steps due to their low degree of adhesion to the surface. Cells treated with PLL-g-PEG 1:9 or 1:21 showed a completely different response, turning grainy and phase dark, and retaining adherence and high confluence on the surface. * = significantly different from PBS at 95% confidence using Student's t-test (mean ± sd, n = 3 for each polymer concentration and 9 for PBS).

(or PLL) became phase dark and grainy and remained adherent to the cell culture substrate in a clearly different response than seen for the PLL-g-(PEG;PBA) polymers. Thus it was decided to test the toxicity of the polymer on cells cultured in suspension to eliminate cell adhesion as a factor in the assessment of toxicity.

Both PLL-g-(PEG;PBA) 1:21 and 1:9 exhibited significant cytotoxicity when applied to Jurkat (suspension) cells for a total of 8 h (Figure 4.13). The cells were incubated with the polymer in culture medium for 4 h, after which the MTT reagent was added and the cells incubated for another 4 h. The toxicity effect was dose dependent and strongest for PLL-g-(PEG;PBA) 1:21.

The toxicity effects were not due to the polymers binding and sequestering the glucose in the media. The glucose concentrations in the rLEC medium and the Jurkat medium were 21 and 11 mM, respectively. The PBA moiety concentrations in 1 wt% solutions of PLL-g-(PEG;PBA) 1:21 and 1:9 are 8 mM and 7 mM, respectively. Thus if all the PBA moieties in the polymer were binding free glucose in the culture media, a significant reduction in the key energy source for the cells would occur. The free glucose concentration in rLEC culture medium with 0.001 - 1 wt% PLL-g-(PEG;PBA) 1:21 or 1:9 added was tested, using an enzymatic assay specific for glucose. No significant change in the detected glucose concentration was found (data not shown).
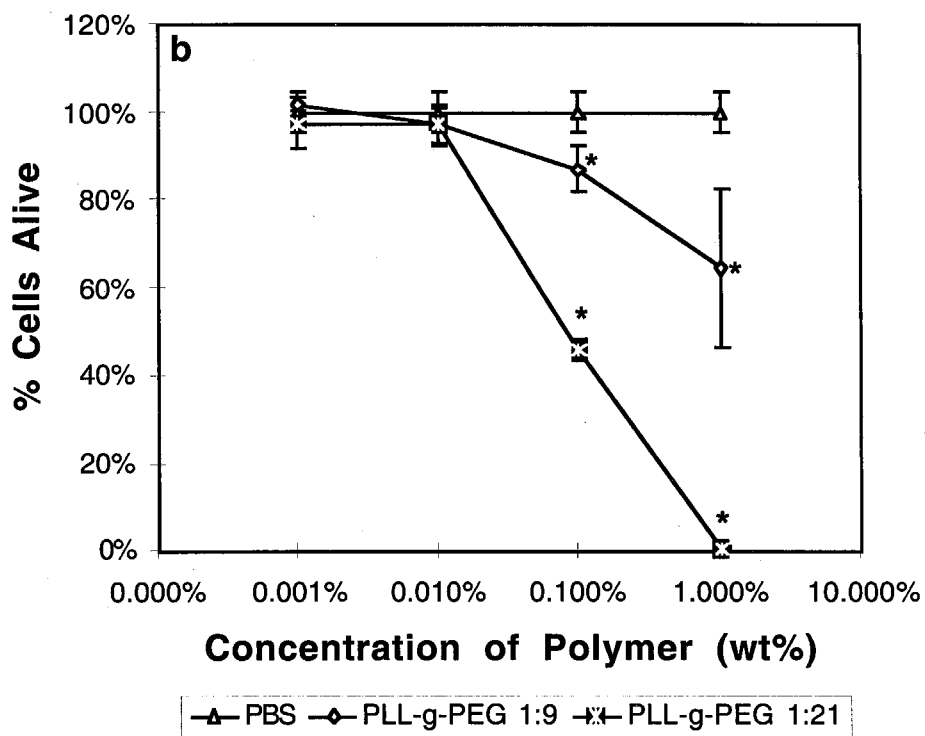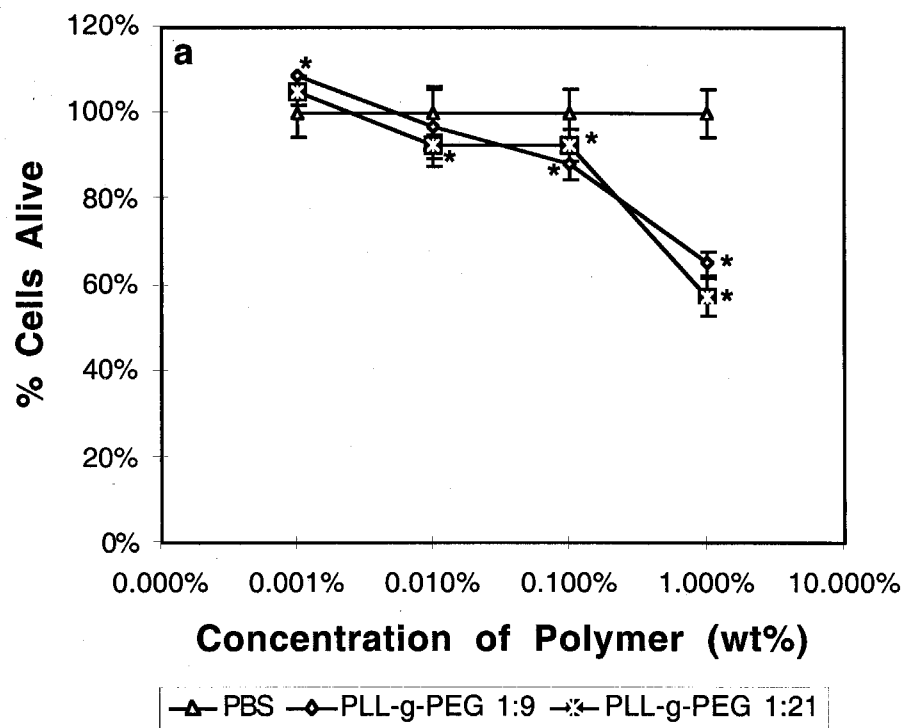
Figure 4.12. Cytotoxicity of PLL-g-PEG polymers on rLECs *in vitro*.

(Figure 4.12)
Cytotoxicity of the polymers was tested as described in Figure 4.11. a. Confluent rLECs incubated with polymer for 5 min. b. Confluent rLECs incubated with polymer for 4 h. Cells killed by PLL-g-PEG (or PLL, data not shown) turned grainy and phase dark, and retained adherence and high confluence on the surface. * = significantly different from PBS at 95% confidence using Student's t-test (mean ± sd, n = 3 for each polymer concentration and 11 for PBS).
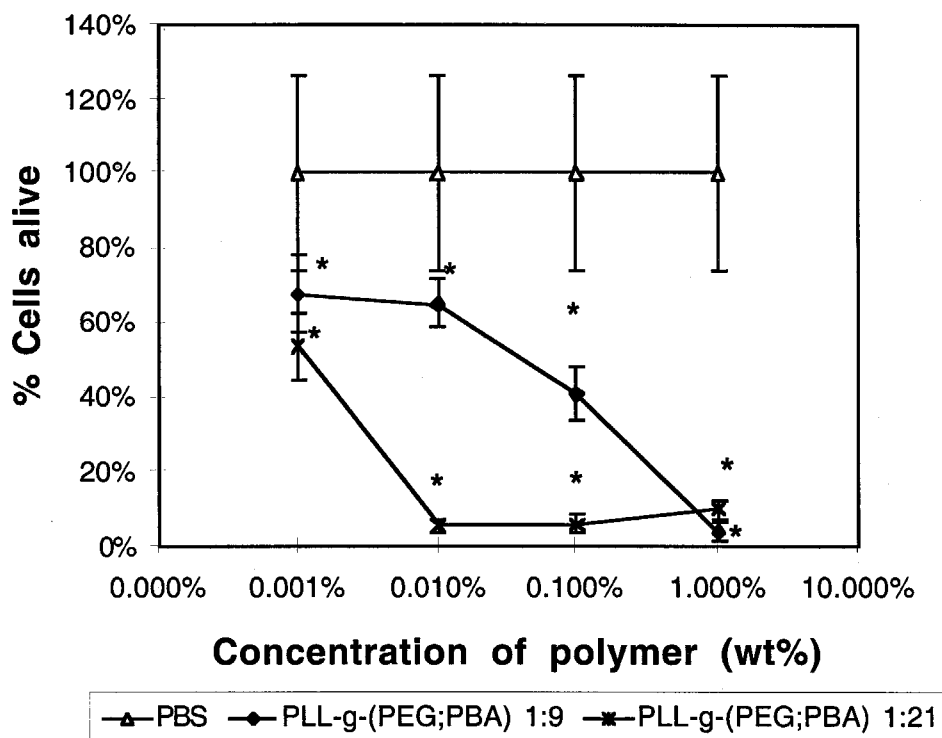
Figure 4.13. **Cytotoxicity of PLL-g-(PEG;PBA) polymers on Jurkats** *in vitro*.

Polymers were dissolved in culture medium at various concentrations and added to Jurkat suspension cells to reach the final concentration. After 4 h, MTT reagent was added and the cells were then incubated with the polymers for 4 additional h. The final cell density was then quantified by the absorbance of the reduced MTT dye. The absorbance of polymer-treated wells was compared to the polymer-free control wells. Both polymers showed dose-dependent toxicity, with the effect being much stronger for PLL-g-(PEG;PBA) 1:21. * = significantly different from PBS at 95% confidence using Student's t-test (mean ± sd, n = 4 for each polymer concentration and n = 12 for PBS).

4.4 Discussion

Cell-surface interactions are involved in a number of undesirable biological responses. The ability to block cell adhesion events by coating surfaces or the cells with PLL-g-(PEG;PBA) polymers is therefore an important possibility with these polymers. The efficacy of the PLL-g-(PEG;PBA) copolymers in relevant models was assayed as a function of their graft ratio and in comparison with PLL-g-PEG, and the cytotoxicity of the polymers *in vitro* was also tested.

Steric Stabilization of TCPS Surfaces

PLL-g-(PEG;PBA) polymers were found to spontaneously coat hydroxyl-containing surfaces and sterically block cell adhesion to the surfaces. Presumably the TCPS surface was sufficiently rich in hydroxyls that many were positioned appropriately for covalent complexation with PBA. PLL-g-(PEG;PBA) 1:21, 1:9, and 1:6 coated TCPS and completely blocked cell adhesion to the surface. This effect was not due to polymer toxicity nor to free polymer in solution, because non-adherent cells re-seeded on untreated surfaces behaved normally and because cells grew to confluence on untreated TCPS while not migrating onto polymer-treated TCPS. The fact that cells did not migrate onto the polymer-treated TCPS after a month of incubation shows that this treatment can be very long lasting, serum stable, and highly localized. The work in Chapter 3 showing that PLL-g-(PEG;PBA) dissociation from a diol-containing resin has a half life on the order of months correlates well with this persistent resistance to adhesion.

The PEG grafting ratio, not the number of PBAs on the backbone, controlled which polymers bound to the TCPS surfaces in sufficient amounts to block adhesion. PLL-g-(PEG;PBA) 1:21 was equivalent to or better than the other PLL-g-(PEG;PBA) polymers at blocking cell adhesion to surfaces, and had a long lasting effect even though it had the fewest PBA moieties on the backbone. Polymers with higher PEG grafting ratios also contained more PBA binding groups, but were equally or less effective at blocking

adhesion to the surfaces. This is consistent with the work in Chapter 3 which showed that polymers containing more PEG bound in lesser amounts to surfaces due to steric hindrance of the PEG side-chains, but that PLL-g-(PEG;PBA) 1:21 can bind enough PEG to surfaces to block adhesion to the surfaces. While PLL-g-(PEG;PBA) 1:6 presumably bound in lower quantities to the TCPS than PLL-g-(PEG;PBA) 1:21 did (less of the 1:6 polymer binds to a diol-containing resin than 1:21 does), the higher PEG content of PLL-g-(PEG;PBA) 1:6 seems to have compensated because the treated TCPS surface was still resistant to cell adhesion. However, PLL-g-(PEG;PBA) 1:3 and 1:2 presumably bound to the surface in such low quantities that there was not enough PEG present to block cell adhesion. Also, Elbert and Hubbell have previously reported that free PEG alone is inadequate to block cell spreading on TCPS or serum proteins (Elbert, D. L. and Hubbell, J. A. 1998).

The binding of PLL-g-(PEG;PBA) to TCPS surfaces involved PBA-hydroxyl binding as well as electrostatic attraction. Inhibition of polymer binding was much more effective with low concentrations of mannose plus NaCl than with high concentrations of either mannose or NaCl. While high concentrations of NaCl were more effective at inhibiting binding than high concentrations of mannose, the greater efficacy of the combination at low concentrations points to the consequential importance of the PBA-hydroxyl binding. Given the highly hydroxylated and highly charged nature of TCPS, it is not surprising that both interactions support binding of the polymer. Interestingly, polymer binding to the surface was more effectively blocked by low concentrations of mannose plus NaCl than by high concentrations of mannose plus NaCl. It may be that the extremely high concentrations of hydrophilic solutes in the latter case causes a salting out effect on the polymer, driving it to again bind to the surface. The patchy attachment of cells to surfaces treated with polymer plus NaCl may be due to the plasma treatment of the TCPS surfaces creating heterogeneous charge distributions on the surface. The strong binding of the polymer to surfaces can again be seen in the concentrations required to block polymer

binding to TCPS. When the polymer was used at 0.1 wt%, this provided approximately 250 times the amount of polymer necessary to coat the TCPS well with polymer. At the same time, the mannose concentration with 5 M mannose was approximately 7000 times the concentration of PBA in the solution. Yet a polymer concentration of 0.01 wt% was required to block all polymer binding via mannose plus NaCl inhibition. The possibility of hydrophobic attractions between the polymer (i.e., the phenyl groups) and the surface was not investigated.

Steric Stabilization of Proteinaceous Surfaces

On the more complex proteinaceous surfaces, only PLL-g-(PEG;PBA) 1:21 strongly blocked cell spreading on adsorbed serum proteins and ECM, and all polymers tested had no effect on Matrigel components. PLL-g-(PEG;PBA) 1:21 had a strong effect in blocking cell spreading on adsorbed serum proteins and ECM at 4 h. But even at 4 h, a high extent of cell attachment to the surfaces was observed. By 24 h, the cells had spread normally upon these surfaces at densities as high as the vehicle control-treated wells. This effect is likely due to a lower density of available *cis*-diols for PBA binding on these surfaces and consequently a lower amount of polymer present on the surfaces.

It is interesting to consider the various biological molecules present and whether they would be expected to lead to stronger or weaker binding of the PLL-g-(PEG;PBA) copolymers. The dominant components of basal lamina are the heparan sulfate proteoglycan perlecan, type IV collagen, laminin, and entactin; the most abundant glycoprotein in serum is albumin but a key adhesive protein is fibronectin. Perlecan is dominated by sulfated and acetylated groups that do not contain *cis*-diols, and occupies a large amount of space in the ECM due to its size and high ionic charge that attracts a large volume of hydration (Stringer, S. E. and Gallagher, J. T. 1997). Type IV collagen has only a few oligosaccharides concentrated at one end of the macromolecule (Paulsson, M. 1992). Laminin is heavily glycosylated, with 12 - 27 wt% carbohydrate, and often has

terminal galactose and sialic acid groups (Hughes, R. C. 1992; Mecham, R. P. 1991). Interestingly, the oligosaccharides on laminin are essential to promote cell spreading but not for cell attachment (Paulsson, M. 1992). Entactin is only 5 wt% glycosylated (Paulsson, M. 1992). Human serum glycoproteins dominantly contain terminal sialic acids groups, most being 3 - 7% sialic acid, and fibronectin and fibrinogen both contain sialic acids (Hughes, R. C. 1992; Schauer, R. 1982). However, albumin is not glycosylated during biosynthesis, although it can be glycated by glucose in the bloodstream (Bunk, D. M. 1997; He, X. M. and Carter, D. C. 1992). Thus, while there is a great abundance of *cis*-diol-containing terminal sugar groups in serum and in basal lamina, the dominant presence of perlecan and albumin along with regions of other glycoproteins that do not have glycans nearby may create significant areas where the PBA-containing polymers cannot bind. These polymer-free sites may provide locations for initial cell attachment, and after sufficient time cells may be able to secrete more proteins or remodel the ECM to create other areas that allow formation of focal adhesions and cell spreading.

The ability of cells to attach to proteinaceous surfaces treated with PLL-g-(PEG;PBA) may also be due to the fact that these surfaces were already coated with necessary adhesion proteins before the polymer was added. Cell adhesion to surfaces involves adhesion proteins adhering to a surface and cells then attaching to these proteins through integrin receptors. Adhesive proteins include fibronectin in serum and laminin and collagen in ECM (Alberts, B. et al. 1994). When a surface of adhesive proteins is coated with polymer, the polymer now must block the interaction of cellular integrins with the adhesive proteins. When TCPS is coated with polymer, the polymer need only block adsorption of adhesive proteins to the surface in adhesive conformations and then cell adhesion is blocked. But if less than a monolayer of polymer is present on a surface pre-coated with adhesive proteins, it is possible that a few cellular integrins can bind to exposed adhesive proteins, allowing initial cell attachment. Then the cell can secrete more proteins and remodel the surrounding ECM, eventually allowing the cell to spread. The surface-

bound polymer could dissociate from the surface if it is attached by only a few PBA groups, the polymer could slowly migrate to other areas, by unbinding and re-binding of bound PBA groups, or the polymer could remain on the surface between focal contacts of the cells or sandwiched amongst ECM components. It is possible that the lower amount of PBAs on the most surface-active polymers (PLL-g-(PEG;PBA) 1:21 and 1:9) contributes to the lesser efficacy of these polymers on the protein-containing surfaces, as they might dissociate more rapidly from the surfaces. However, the lower amounts of PBA also caused these polymers to have a less anionic charge at pH 7.4, which causes less electrostatic repulsion to the net anionic biological surfaces.

Model Surfaces

On all the model surfaces, PLL-g-(PEG;PBA) polymers were more effective than PLL-g-PEG polymers. PLL-g-(PEG;PBA) blocked cell attachment to TCPS over a wider range of PEG graft ratios than PLL-g-PEG did and PLL-g-(PEG;PBA) 1:21 was the most effective at blocking cell spreading on ECM and serum proteins. This is likely a consequence of the reversible covalent bonding between hydroxyls on the surface and PBA moieties in the polymer, and the subsequently low dissociation rate, in contrast with electrostatic binding between PLL-g-PEG and the anionic surfaces, which is hampered by the high ionic strength of the media. Data presented in Chapter 3 revealed that a monolayer coating of PLL-g-(PEG;PBA) 1:9 may have a density of 1 PEG every 15 Å, and a similarly mass density of PLL-g-(PEG;PBA) 1:21 would have 1 PEG every 16 Å. This distance between PEGs is adequate to force the PEG chains into a dense polymer brush and block protein adhesion to the surface. Thus, as long as a monolayer of PLL-g-(PEG;PBA) 1:21 or 1:9 is tightly bound to the surface, an adequate PEG density is present to block cell adhesion. With PLL-g-PEG, Elbert and Hubbell found that a PEG graft ratio of 1:7 was optimal for preventing cell spreading on TCPS when the PLL MW was 20 kDa. They hypothesized that at higher PEG graft ratios, less polymer adsorbed to the surface due to

steric hindrance, and at lower PEG graft ratios, the surface density of PEG was inadequate to prevent cell spreading. However, they also found that with a higher MW PLL backbone (375 kDa), PEG graft ratios of 3.5 to 14 were effective in the same assay. Thus it may be that PLL-g-PEGs with a 20 kDa PLL backbone are not bound tightly enough to the surface to resist desorption, whereas those with a 375 kDa backbone were more resistant to desorption and the PEG graft ratio was thus less important. Because the PBA moieties are covalently bound to the surface, a polymer with only a 20 kDa backbone is adequate to provide longer lasting binding than the electrostatically bound PLL-g-PEG. Of course, the density of *cis*-diols and of anionic charges on the surface will control the net strength of each binding interaction. However, on these model surfaces, the covalent binding of PBA was most effective.

The direct relevance of the model surfaces examined to *in vivo* applications is not immediately clear. The ECM generated in this study by incubating confluent cells with ammonia is not likely to be identical to ECM as found upon a denuded tissue surface *in vivo*. Besides being secreted under artificial conditions, the ECM may have been modified by the ammonia treatment. Common methods for removing cells to reveal their ECM in culture include ammonia treatment (Rutka, J. T. et al. 1986; Saelman, E. U. M. et al. 1994), detergent treatment (Gospodarowicz, D. et al. 1980), or EDTA treatment (Abrams, G. A. et al. 2000). While pilot trials with all of these treatment methods indicated low polymer efficacy on these surfaces, each treatment method is likely to alter the native ECM surface. Matrigel is another option for coating a surface with ECM components. Matrigel is isolated from the mouse Engelbreth-Holm-Swarm tumor and secretes the major components of basement membranes, including type IV collagen, heparan sulfate proteoglycan, laminin, and entactin, as well as growth factors and proteases (Paulsson, M. 1992). However, it is known that forms of some proteins such as laminin are different in Matrigel than in other tissues. In general, the glycosylation patterns of glycoproteins, proteoglycans, and glycolipids are known to be extremely diverse and to vary for different

species, tissues, ages, transformed states, and even within the same cells depending on the local environment (Dennis, J. W. et al. 1999; Hardingham, T. E. and Fosang, A. J. 1992; Hughes, R. C. 1992; Kobata, A. 1992; Paulsson, M. 1992). The greatest diversity is found in the terminal nonreducing sugars, which is where the PBA-containing polymers are most likely to interact due to steric reasons. For example, there are more than 30 variants of sialic acid, and murine sialic acids have more of their *cis*-diols blocked by acetylation than human sialic acids do. Thus, while these *in vitro* models may be useful for investigating the properties of the polymers, *in vivo* studies are necessary to truly evaluate the efficacy of the polymers in any given application.

The ability of the polymers to coat cell surfaces and thus block cell adhesion to other surfaces was also unclear. While both PLL-g-(PEG;PBA) and PLL-g-PEG 1:21 had similar strong effects in blocking cell adhesion to TCPS and to ECM, it appeared likely that this was due to cell killing. The assays suggested that the extended manipulation of the cells and holding of the cells in PBS may have caused significant cell death, and/or the inability to visualize the cell pellet may have contributed to aspiring cells during aspiration steps. The presence of the polymer may have also contributed to this effect. Alternatively, the apparent cytotoxicity of the polymers *in vitro* may likely have also caused cell death. The assays in Chapter 3 clearly showed the ability of the polymers to bind to RBC surfaces, and RBCs or other hardier cells may be a better model to test polymer binding to and steric stabilization of cell surfaces.

Cytotoxicity

Interpretation of the cytotoxicity assays also suffers from the artificial environment of an *in vitro* culture system. rLECs with high exposure to PLL-g-(PEG;PBA) 1:9 and especially 1:21 rounded up and were likely washed away during rinse steps, thus what appeared as "death" might have actually been "washing away." This response was intriguing because the cells appeared phase bright, intact, and healthy. It is possible that

they were undergoing mitosis, as PBA-containing polymers have been shown to induce mitosis of lymphocytes (Miyazaki, H. et al. 1993). This response was very different from the response of the rLECs to PLL-g-PEG 1:9 and 1:21, where a classic toxicity response was observed. The response of the rLECs to PLL-g-(PEG;PBA) requires further investigation to elucidate the full mechanism. When the polymer was incubated with Jurkat cells grown in suspension, significant toxicity was seen for PLL-g-(PEG;PBA) 1:21 and 1:9 at all concentrations. But the *in vivo* studies reported in Chapters 5 and 6 showed no evidence of toxicity when up to 4 mL of 1 wt% solutions of the polymers were applied to the peritoneal cavity or various sections of the eye. Thus it appears that the polymers are not toxic *in vivo*, at least in the applications considered in these experiments, and may only appear toxic *in vitro* as an artifact of the culture system.

While the polymers were not found to bind to glucose in the cell culture media, it is possible that they bound to some other components in the media, such as growth factors, that were essential for cell survival or proliferation. In fact, it would appear likely that the polymers did complex with some other components in the media, because the polymer would be fully expected to complex with the glucose in the media and yet appeared not to do so. Thus the PLL-g-(PEG;PBA) polymers may complex with components in cell culture media that may be continuously supplied or inessential *in vivo*. Also, the exact mechanism of MTT penetration into cells and reduction within cells is not known (Marshall, N. J. et al. 1995; Shearman, M. S. et al. 1995). Another possible reason for the apparent toxicity of the polymers to cells *in vitro* could be that the polymer coating on the cells blocks entrance of MTT into the cells.

However, there are a number of possible mechanisms by which PLL-g-(PEG;PBA) could be toxic, and all would correlate with 1:21 being more toxic than 1:9 since 1:21 binds to surfaces in greater amounts. One possible mode of toxicity is if the PLL-g-(PEG;PBA) polymers coated cell surfaces and sterically blocked the entry of necessary nutrients into the cells. Steric repulsion of molecules would be expected to be a function of MW, with larger

proteins being more likely to be repelled. Other researchers who have covalently bound enough PEG to RBC surfaces to block antibody binding to the surfaces showed that the RBCs retained normal ionic transport and permeability as well as normal morphologies and deformability (Murad, K. L. et al. 1999). However, the MW dependence of steric stabilization of surfaces treated with PLL-g-(PEG;PBA) was not tested. Another possible mode of toxicity would be the polymers binding to cell surface receptors, clustering them, and causing responses leading to apoptosis. Another possible mechanism could include polymer accumulation inside the cells, if a great amount of polymer is endocytosed (due to the polymer being bound to the cell surface and the cell undergoing endocytosis) and not subsequently released by degradation and/or exocytosis. Liquid-phase endocytosis of cell membrane-bound pluronics has been observed to lead to Pluronic accumulation inside the cells (Melik-Nubarov, N. S. et al. 1999). A final possibility is that PLL-g-(PEG;PBA) binding to the cell surfaces causes changes in membrane fluidity that somehow lead to cell death. Melik-Nubarov et al. have shown that Pluronic binding to cell surfaces can cause changes in membrane viscosity (Melik-Nubarov, N. S. et al. 1999). If the PLL-g-(PEG;PBA) copolymers synthesized in this work are indeed toxic by any of the above mechanisms, it may be possible to vary the polymer geometry to avoid the undesirable effects (i.e., using a shorter backbone to cause less clustering and uptake or varying the PEG MW and graft ratio to change the MW dependence of steric stabilization).

4.5 Conclusions

PLL-g-(PEG;PBA) polymers were found to spontaneously coat various surfaces and prevent cell adhesion to those surfaces. PLL-g-(PEG;PBA) was extremely effective at coating TCPS and blocking cell adhesion for over a month. This effect is localized, not due to toxicity, and is due to hydroxyl binding by PBAs as well as electrostatic binding by charged groups in the polymer and TCPS. Only PLL-g-(PEG;PBA) 1:21 blocked cell

spreading on adsorbed serum proteins or ECM, and this effect was transient. This was likely due to a low amount of *cis*-diols available for binding on these surfaces. On all the model surfaces, PLL-g-(PEG;PBA) was more effective than PLL-g-PEG. The ability of PLL-g-(PEG;PBA) to coat cell surfaces and thereby block cell adhesion to surfaces was unclear, as the cells seemed to be damaged by the handling necessary in the experiment and/or by toxicity of the polymer *in vitro*. Cytotoxicity assays showed apparent dose-dependent toxicity of the PLL-g-(PEG;PBA)s, especially 1:21 and especially with long exposure. However, the polymers did not appear toxic *in vivo* and seemed to bind to components in the culture medium other than glucose that may have been essential.

4.6 Acknowledgments

5. Chapter 5

PLL-g-(PEG;PBA) Prevention of Posterior Capsule Opacification *In Vivo*

5.1 Introduction

Posterior capsule opacification (PCO) is a common medical complication that involves cellular adhesion and migration on ECM, and thus represents a possible application for the polymers created in this work (Apple, D. et al. 1992; Ohadi, C. et al. 1991; Spalton, D. J. 1999; Tetz, M. R. and Nimsgern, C. 1999). PCO arises after up to 50% of cataract surgeries. A cataract is a clouding of the ocular lens as shown in Figure 5.1. The ocular lens consists of the capsule and the crystalline lens nucleus and cortex. The lens capsule is a thick periodic acid-Schiff positive basement membrane that is secreted by the lens epithelial cells during development. After development, the anterior capsule is lined with a layer of epithelial cells. These cells continuously migrate to the equatorial bow, at which they undergo mitotic division, elongate, and become filled with cortical fibers while the cells' nuclei gradually disappear. Thus the lens nucleus is acellular and grows slowly with age. The adult lens is also avascular and is not innervated.

In cataract surgery, a hole (rhexis) is cut in the anterior capsule, and the lens nucleus is removed, followed by clean up of the cortex. The rest of the capsule and many of the epithelial cells, however, remain. An intraocular lens (IOL) composed of polymers such as poly(methyl methacrylate), poly(hydroxyethyl methacrylate), silicone, or cross-linked phenylethyl acrylate and phenylmethyl methacrylate (AcrySof by Alcon) is then implanted in the capsule. In up to 50% of adult cases (and up to 100% of pediatric cases), PCO then begins to develop within a few years. It is believed that the remaining epithelial

cells proliferate, migrate onto the posterior capsule, and may undergo a myofibroblastic transformation. These cells can then form clusters of swollen opacified epithelial
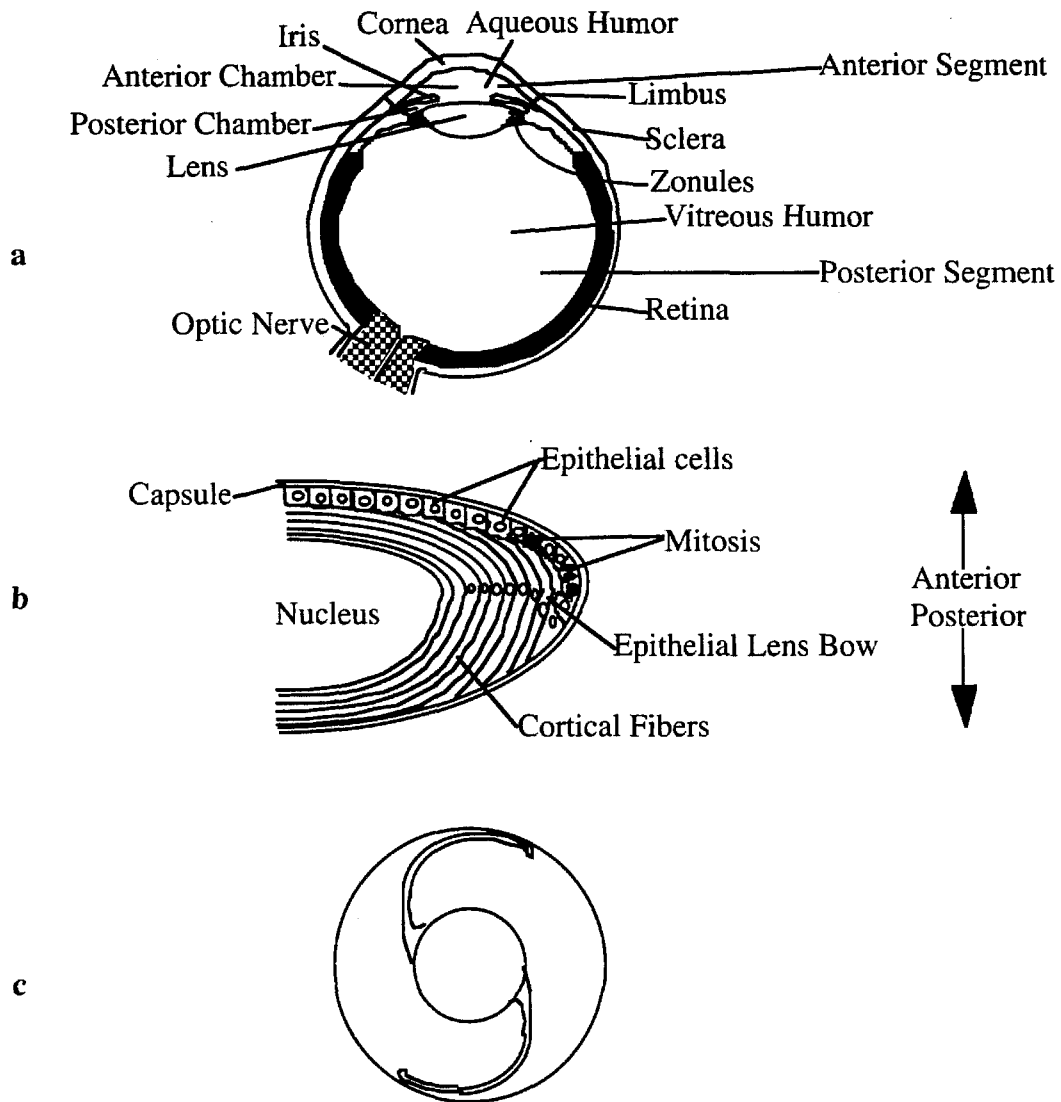
Figure 5.1. Schematics of eye, lens, and an intraocular lens.

a: Horizontal section of the right eye.
b: Horizontal close-up of the adult lens.
c: Vertical view of a typical intraocular lens (IOL).

Elschnig's pearls or deposit fibrous membranes which can also lead to wrinkling of the capsule. These components obscure the visual axis, leading to what is termed PCO, or

secondary cataract. The causes of the cellular responses leading to PCO are only poorly understood (Liu, C. et al. 1996). They may be directly related to the severity and duration of the disruption of the blood aqueous barrier and/or to inflammatory responses to the surgery (Nishi, O. 1999). Cytokines such as TNF-α, TGF-β, IL-1, and IL-6 may be involved in stimulating proliferation and myofibroblastic transformation (Kurosaka, D. and Nagamoto, T. 1994; Marcantonio, J. M. and Vrensen, G. F. J. M. 1999; Nishi, O. 1999; Quinlan, M. et al. 1997). Exposure to plasma proteins during the wound healing response may also stimulate epithelial cell proliferation (Saxby, L. et al. 1998). Type IV collagen, laminin, and fibronectin have all been implicated in promoting adhesion and migration of lens epithelial cells *in vitro* (Olivero, D. and Furcht, L. 1993). Understanding PCO is hampered by the long time required to develop the condition (typically 2 - 5 years for clinical significance) and the difficulty in quantifying the severity. *In vitro*, the lens epithelial cells have been seen to form a monolayer covering the posterior capsule within 7 - 14 days (Liu, C. et al. 1996; Quinlan, M. et al. 1997; Saxby, L. et al. 1998).

In most cases in the developed world, the treatment for PCO is capsulotomy of the posterior capsule via a Nd:YAG laser. Cataract extraction is one of the most common elective surgeries in the US, and is the most common in Americans aged 65 or older. Over 600,000 Nd:YAG surgeries were billed to Medicare in 1991, with total Medicare cataract expenses of $3.4 billion in 1991. Nd:YAG surgery is a fairly safe and successful procedure, with a complication rate < 1% and a serious complication rate < 0.1%. However, it is a frightening treatment for the patient and it can lead to complications including raised intraocular pressure, retinal detachment, cystoid macular edema, damage to the IOL, uveitis, endothelial cell trauma, vitreous prolapse, corneal edema, and hyphema. In the developing world, the costs of the Nd:YAG laser are too high. Cataract extraction and IOL implantation is often precluded because of the likelihood of PCO and the inability to treat it. Clearly, reducing or eliminating the incidence of PCO would have great implications.

Currently, the only clinical approaches to reducing the incidence of PCO are surgical techniques: atraumatic surgery, good cortical cleanup, varying the size of the rhexis; and using IOLs with improved design and placement: almost all lenses are now biconvex due to the "no space, no cells" theory, and capsular bending rings and certain materials have been theorized to yield better responses. Current areas of investigation to prevent PCO include local delivery of cytotoxic agents (Behar-Cohen, F. et al. 1995; Behar-Cohen, F. et al. 1995; Bretton, R. H. et al. 1999), antiproliferatives (Hepsen, I. F. et al. 1997; McDonnell, P. et al. 1988; Nishi, O. et al. 1996; Power, W. et al. 1994; Tetz, M. R. et al. 1996), integrin blockers (Nishi, O. et al. 1997; Nishi, O. et al. 1996), or gene therapy agents (Malecaze, F. et al. 1999), and varying the physical interactions between the IOL and the capsule (Assia, E. et al. 1999; Hara, T. et al. 1995; Nishi, O. and Nishi, K. 1999) (Nishi, O. 1999). Most applications of drugs in the capsule are hampered primarily from damage that is induced to the rest of the ocular structures.

The polymers developed in this work might well be able to coat the posterior capsule following cataract surgery and prevent the formation of a secondary cataract. After removal of the lens and cortical cleanup, an aqueous solution of the polymer could be injected into the capsular space before closing the eye. The polymer could spontaneously assemble on the posterior capsule surface (as well as on the lens epithelial cell surfaces) and thereby prevent lens epithelial cells from migrating onto the posterior capsule. While the polymer would be expected to remain on the capsule for a long time (due to low turnover of the ECM and long-lasting binding), it would eventually hydrolyze along the backbone and/or dissociate from the surface. But while the development of clinically significant PCO typically occurs on the order of years, as discussed above, it is likely that the initial inflammatory and healing responses following surgery are what precipitates the condition. Epithelial cell outgrowth can begin within a few days. The polymer may only need to inhibit migration during the first week or two following surgery in order to prevent the cascade of events leading to PCO.

In this chapter, the application of PLL-g-(PEG;PBA) polymers to PCO prevention *in vivo* is investigated. An exploratory trial applying PLL-g-(PEG;PBA) or PLL-g-PEG 1:9 or 1:21 to two rabbit eyes each was conducted, with a duration of 7 weeks. A further trial applying PLL-g-(PEG;PBA) 1:9 was then conducted, with an n of 4 and a duration of 14 weeks. Applications of PLL-g-(PEG;PBA) 1:9 and 1:21 were also conducted in the posterior segment to test for adverse effects. The experiments suffered from difficulty in achieving significant PCO in control rabbits. However, the application of PLL-g-PEG was found to induce serious inflammation in most of the animals. In contrast, PLL-g-(PEG;PBA) administration seemed to induce no inflammatory or toxic responses. Resolution of the *in vivo* model in our hands is necessary to truly test the efficacy of the polymer in this model.

5.2 Materials and Methods

All animal procedures were performed at the University of California, San Francisco Medical Center in accordance with their animal care and use guidelines. In all cases, only one eye was treated per animal.

In the first trial, adult New Zealand white albino rabbits (2 - 5 kg) were used, with an n of 2 for each treatment. The rabbits were anesthetized with ketamine at 35 - 50 mg/kg and Xylazine at 5 - 10 mg/kg by intramuscular injection. Betadine 5% (Escalon Ophthalmics, Skillman, NH) was applied to the eye and the procedures were performed in a sterile manner under an operating microscope. The capsule was accessed via a limbal incision. Healon (1 wt% sodium hyaluronate, Pharmacia Ophthalmics, Monrovia, CA) was then injected to maintain anterior chamber depth for the capsulectomy. The base structure of hyaluronate does not contain *cis*-diols. An anterior capsulectomy was performed using a bent 27 gauge needle and forceps. The lens material was then removed by phacoemulsification with irrigation with BSS balanced salt solution (Alcon Surgical, Ft.

Worth, TX). The Healon can be assumed to have been washed out by the irrigation involved in this step. 200 µL of 1 wt% polymer in Dulbecco's PBS (pH 7.4, Gibco, Gaithersburg, MD) was then applied to the posterior capsule with an anterior chamber cannula or a needle. The polymers applied were PLL-g-(PEG;PBA) 1:9 or 1:21, PLL-g-PEG 1:9 or 1:21, or the vehicle control; solutions had been sterile filtered with a 0.2 µm Supor Acrodisc filter (Gelman, VWR, Seattle, WA). After 5 min, the incision was then closed with a 7-0 Vicryl suture (no lens was implanted and the polymer solution was left in the eye). 100 µL Ophthetic cephazolin (antibiotic) and 100 µL Ophthetic Kenalog (steroid) were then injected subconjunctivally. The eyes were examined visually at 5 days, 2 weeks, 1 month, and 7 weeks for evidence of inflammation and of PCO. The rabbits were sacrificed after 7 weeks. The eyes were then fixed in 10% buffered formalin and processed for histopathological examination.

In the second trial, Dutch belted rabbits (4 - 5 pounds) were used, with an n of 4 for each treatment. The rabbits were anesthetized with ketamine at 35 - 50 mg/kg and Xylazine at 5 - 10 mg/kg by intramuscular injection. Betadine 5% was applied to the eye and the procedures were performed in a sterile manner under an operating microscope. The capsule was accessed via a limbal incision. Viscoat (less than 4 wt% sodium chondroitin sulfate and less than 3 wt% sodium hyaluronate, Alcon) was then injected to maintain anterior chamber depth for the capsulectomy. The base structure of chondroitin sulfate does not contain *cis*-diols. An anterior capsulectomy was performed using a bent 27 gauge needle. The lens material was then removed by phacoemulsification with irrigation with BSS Plus balanced salt solution (Alcon). The Viscoat can be assumed to have been washed out by the irrigation involved in this step. A finer cortical cleanup was then also performed, using an Irrigation and Aspiration handpiece. 300 µL of 1 wt% polymer in Dulbecco's PBS was then applied to the posterior capsule with a 27 gauge anterior chamber cannula. The polymers applied were PLL-g-(PEG;PBA) 1:9 or the vehicle control; these solutions had been sterile filtered with a 0.2 µm Supor Acrodisc filter (Gelman, VWR,

Seattle, WA). After a minimum of 1 min, the incision was then closed with a 7-0 Vicryl suture (no lens was implanted and the polymer solution was left in the eye). 100 μL Ophthetic cephazolin (antibiotic) and 100 μL Ophthetic Kenalog (steroid) were then injected subconjunctivally. The eyes were examined visually at 5 weeks, 8 weeks, and 14 weeks for evidence of inflammation and of PCO. The rabbits were sacrificed after 14 weeks. The eyes were then fixed in 10% buffered formalin and processed for histopathological examination.

The polymers were also applied to the posterior segment to test for adverse responses. In 1 trial, 100 μL of PLL-g-(PEG;PBA) 1:9 or 1:21 at 1 wt% in Dulbecco's PBS was injected intravitreally into New Zealand white albino rabbits. There was an n of 1 for each treatment. In another trial (targeting proliferative vitreoretinopathy, a model which was later abandoned), a vitrectomy was performed in Dutch banded rabbits. Subsequently, 200 μL of PLL-g-(PEG;PBA) 1:9, 1:21, or no polymer at 1 wt% in Dulbecco's PBS was applied to the retina. The polymer was allowed to incubate for 5 min before suturing the wound closed. 6 - 10 h later, 100 μL of Dulbecco's PBS containing 10^5 human scleral fibroblasts labeled with SpDiI (Molecular Probes, Eugene, OR) was injected into each eye. There was an n of 1 for each polymer and an n of 2 for the PBS control. Following both trials, the eyes were examined visually at 4 days, 2 weeks, and 1 month.

5.3 Results

The results of the first *in vivo* PCO trial are given in Table 5.1. The lens was removed from two rabbit eyes for each treatment. PLL-g-(PEG;PBA) and PLL-g- PEG 1:9 and 1:21 and the PBS vehicle control were tested. 200 μL of 1 wt% polymer solutions were administered to the posterior capsule and incubated for 5 min before suturing the wound closed. No IOLs were implanted. The eyes were inspected periodically, and the rabbits were sacrificed after 7 weeks for histological examination of the capsules.

Table 5.1. Results of first *in vivo* PCO trial.

Treatment	5 Days	2 Weeks	1 Month	7 Weeks	Histology
PLL-g-(PEG;PBA) 1:9 (A,B)	A - Mild corneal opacity, normal. B - Inflammation and corneal opacity.	A - Normal. B - Inflamed, extremely swollen eye, cloudy cornea. Blocked outflow pathways of eye? Sacrificed.	A - Normal.	A - Could not see any PCO with microscope. Sacrificed.	A - No cells migrating. B - No sections successfully obtained.
PLL-g-(PEG;PBA) 1:21 (C,D)	C & D - Mild corneal opacity, normal.	C & D - Normal.	C & D - Normal.	C & D - Could not see any PCO with microscope. Sacrificed.	C - Very minimal cell migration. D - Lots of lens material, no cell migration.
PLL-g-PEG 1:9 (E,F)	E & F - Inflammation and corneal opacity.	E - Normal F - Inflamed, extremely swollen eye, cloudy cornea. Blocked outflow pathways of eye? Sacrificed.	E - Normal, maybe a little PCO?	Found dead in cage. Probably unrelated to eye.	E - No sections because found dead in cage. F - Lots of cell migration.
PLL-g-PEG 1:21 (G,H)	G & H - Inflammation and corneal opacity.	G & H - Inflamed, extremely swollen eyes, cloudy corneas. Blocked outflow pathways of eye? Sacrificed.			G - Capsule incomplete in sections. H - Lots of fibrous proliferation on posterior capsule periphery, less in center.
PBS (I,J)	I & J - Mild corneal opacity, normal.	I & J - Normal.	I & J - Normal.	I & J - Could not see any PCO with microscope. Sacrificed.	I - Not enough lens capsule in sections. J - Some fibrous proliferation on posterior capsule.

Two New Zealand white albino rabbits were used per treatment (one eye per animal). An anterior capsulectomy was performed and the lens material was removed by phacoemulsification. 200 µL of 1 wt% polymer in PBS (pH 7.4) was applied to the lens capsule and incubated for 5 min before closing the wound. The eyes were examined periodically by microscopy and sacrificed after 7 weeks. The cells in the table list the results for both or each eye of the treatment group. Most of the PLL-g-PEG treatments and 1 PLL-g-(PEG;PBA) 1:9 treatment caused severe inflammation and high intraocular pressure. The efficacy of the treatment in the remaining eyes was difficult to assess, as the control animals did not develop significant PCO.

None of the animals, including control animals, developed significant PCO in this trial. Some cell migration and/or fibrous proliferation was seen in the histology, but none was significant enough to allow a definitive comparison. Both animals treated with PLL-g-PEG 1:21, 1 animal treated with PLL-g-PEG 1:9, and 1 animal treated with PLL-g-(PEG;PBA) 1:9 developed a strong inflammatory response at 2 weeks. The eyes were extremely swollen, inflamed, and had cloudy corneas. The high intraocular pressure may have resulted from blocked outflow pathways of the eye (the aqueous humor is constantly secreted in the posterior chamber and drained in the anterior chamber, such that it is refreshed approximately every 2 h in rabbits). The other animals had normal eyes with no inflammatory or toxic responses noted.

The results of the second *in vivo* PCO trial are given in Table 5.2 and a photograph of each eye at 14 weeks is given in Figure 5.2. The lens was removed from four rabbit eyes for each treatment: PLL-g-(PEG;PBA) 1:9 or the PBS vehicle control. 300 μL of 1 wt% polymer solutions were administered to the posterior capsule and incubated for 1 min before suturing the wound closed. No IOLs were implanted. The eyes were inspected periodically, and the rabbits were sacrificed after 14 weeks for histological examination of the capsules.

Again, none of the animals developed strong PCO. However, some weak indications of PCO were seen in some animals, and this effect was seen equally in both the PLL-g-(PEG;PBA) 1:9-treated and the control animals. Histologically, all of the eyes had a large amount of cortex present on the posterior capsule -- about 50% of the volume of the original lens. It was unclear if the surgery did not adequately remove the lens material or if a large amount of material had been deposited following the surgery. The eyes all looked very quiet histologically, with no inflammatory or toxic responses seen.

The trials applying polymers to the posterior segment did not indicate any significant adverse responses to PLL-g-(PEG;PBA) 1:9 or 1:21. 100 μL of 1 wt% PLL-g-(PEG;PBA) 1:9 or 1:21 was injected intravitreally into one eye each. No adverse
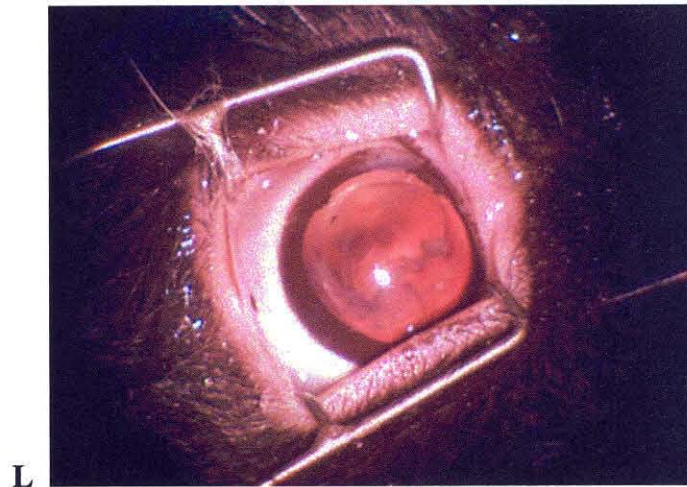
Table 5.2. Results of second *in vivo* PCO trial.

5 weeks	8 weeks	14 weeks
PLL-g-(PEG;PBA) 1:9 treated animals:		
L - Quiet, with some retained cortex. Clear posterior capsule.	Cells growing from retained cortex into center of capsule. Posterior capsule plaque visible, some PCO.	Quiet, some lens cortex present. Capsule clear, very slight opacity.
M - Quiet, with some retained cortex. Clear posterior capsule.	Iris irregular, some retained cortex at inferior pupillary margin. No PCO seen, but eye not well dilated.	Diffuse opacification of corneal cortex, vascularization of periphery of cornea. Can't see capsule very well.
N - Quiet, with some retained cortex. Some PCO.	Some retained cortex. Some PCO.	Animal missing from its cage (probably died).
O - Died shortly after surgery - probably from anesthetic.		
PBS treated animals:		
P - Quiet, with some retained cortex. Some PCO.[a]	Some retained cortex. Some PCO.	Quiet, some lens cortex present. Some mild opacity of posterior capsule, capsule adherent centrally.
Q - Quiet, with some retained cortex. Clear posterior capsule.[b]	Center of lens capsule clear, some retained cortex at margin. No PCO seen.	Quiet, some lens cortex present. Capsule clear, very slight opacity.
R - Some retained cortex. Slight adverse response. Clear posterior capsule.	Lots of iris atrophy, not well dilated. Subepithelial scar on cornea. No PCO seen.	Mild corneal haze, good red reflex. Some lens cortex present. Capsule clear, view hazy.
S - Damage to iris from surgery, hard to view because iris poorly dilated. Lots of retained cortex. Didn't see any PCO.	Very poorly dilated, view very cloudy. Can't see capsule.	Some corneal opacity, some superior iris atrophy. Eye relatively quiet. Lens cortex present. Can't see capsule.

[a] Surgery may have created a hole in the anterior or posterior capsule.
[b] Surgery created a rip in the posterior capsule.
Four Dutch belted rabbits were used per treatment (one eye per animal). An anterior capsulectomy was performed and the lens material was removed by phacoemulsification. 300 µL of 1 wt% polymer or PBS (pH 7.4) was applied to the lens capsule and incubated for 1 min before closing the wound. The eyes were examined periodically by microscopy and sacrificed after 14 weeks. Each row in the table describes the observations for one eye. The efficacy of the polymer treatment was difficult to assess, since no animal developed strong PCO. No inflammatory or toxic responses to the polymer treatment were observed.

L
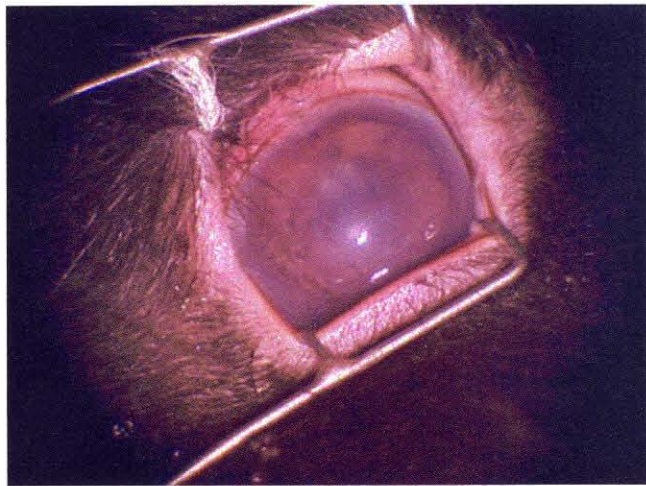
M
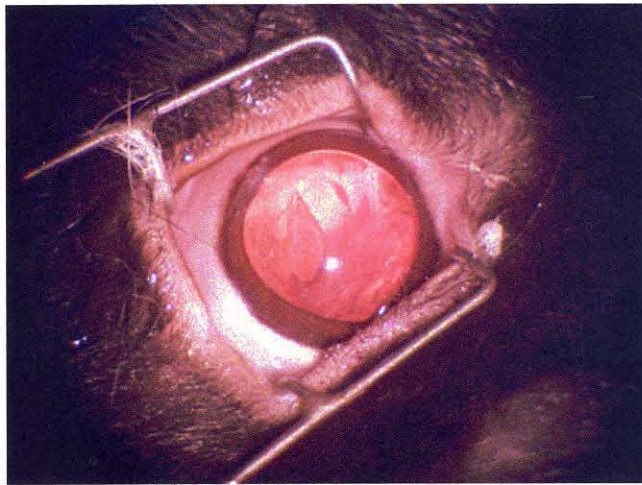
P
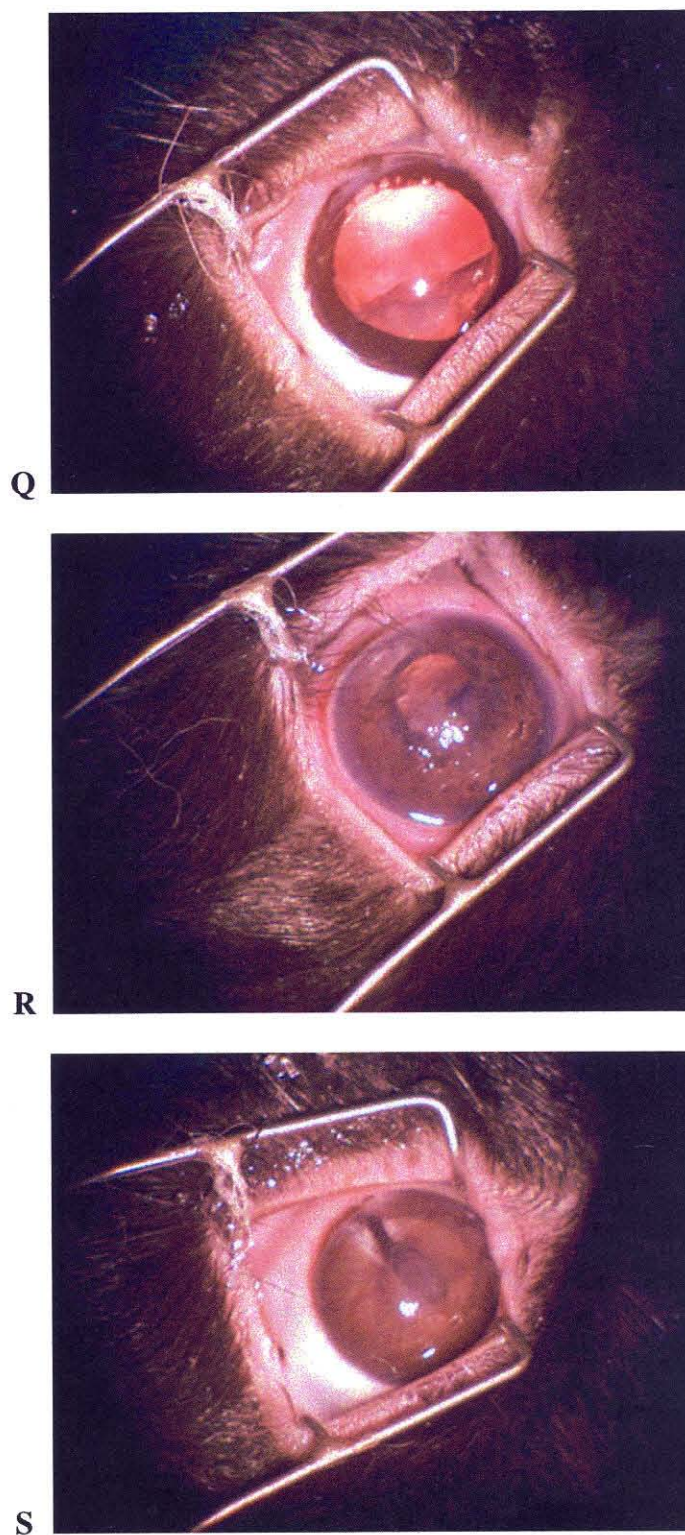
Figure 5.2. Photographs of eyes at 14 weeks in second *in vivo* PCO trial.
L and M were treated with PLL-g-(PEG;PBA) 1:9, P - S were treated with the vehicle control. See Table 5.2 caption for experimental summary.

responses were noted at 4 days or 2 weeks, though some possible vitreous hemorrhaging was noted in the eye treated with PLL-g-(PEG;PBA) 1:9 at 1 month. 200 μL of PLL-g-(PEG;PBA) 1:9, 1:21, or no polymer at 1 wt% in PBS was also applied to retinas following vitrectomy. The polymer-treated eyes appeared no different from the PBS-treated eyes at 4 days, 2 weeks, and 1 month.

5.4 Discussion

The *in vivo* trials suffered from the lack of significant PCO development in the control animals. Numerous researchers have conducted PCO experiments in rabbits, and a survey of 14 papers all showed significant PCO formation within 1 to 3 months (i.e., scores of at least 2 on a scale of 0 - 4, whereas the animals in this work would have scored a 1 at most). The methodology in this work does not differ significantly in rabbit type, age, surgical approach, or quantitative assay methodology from these other works. However, most of those other studies did involve implantation of an IOL following lensectomy. Assia et al. have noted that the anterior and posterior sections of capsules without IOLs tend to adhere together, and that this seems to block cell migration into the central region of the capsule (Assia, E. et al. 1999). Thus the lack of IOL implantation in the studies in this work may have hindered the development of PCO.

However, these studies did provide some information about the performance of our polymers *in vivo*. PLL-g-PEG seemed to induce a strong inflammatory response, where the intraocular pressure became so high as to necessitate an early sacrifice of the animals. The reason for this is unknown, but it could be due to the conventional toxicity that was seen with the PLL-g-PEGs *in vitro* as reported in Chapter 4 (PLL alone is known to be cytotoxic, and the residual free amines present on PLL-g-PEG may cause toxicity). In contrast, PLL-g-(PEG;PBA) 1:9 and 1:21 seemed not to be toxic or inflammatory. In the PCO trials, one animal experienced inflammation when treated with PLL-g-(PEG;PBA) 1:9

in the first trial; however, none of the four animals treated with this polymer in the second trial showed any adverse responses. Applications of PLL-g-(PEG;PBA) 1:9 or 1:21 to the posterior segment also did not seem to induce significant adverse responses in the animals, though the n was only 1 for each application. The *in vivo* results also imply that application of the polymer does not interfere with wound healing. This is important, being that all surgeries induce wounds that must heal, and the polymer could potentially coat surfaces that need to adhere in order for wounds to close.

5.5 Conclusions

The efficacy of PLL-g-(PEG;PBA) in preventing PCO formation was not elucidated in these studies, due to difficulty in forming significant PCO in control animals. However, PLL-g-(PEG;PBA) 1:9 and 1:21 seem to not elicit any adverse responses when applied to the posterior capsule, to the retina, or intravitreally. In contrast, application of PLL-g-PEG 1:9 and 1:21 to the posterior capsule tends to induce a strong inflammatory response and high intraocular pressure.

5.6 Acknowledgments

6. Chapter 6

PLL-g-(PEG;PBA) Prevention of Peritoneal Adhesion Formation *In Vivo*

6.1 Introduction

Peritoneal adhesion formation is a common consequence of abdominopelvic surgery and involves interactions between cells, ECM, and fibrin that might be interrupted by application of PLL-g-(PEG;PBA) copolymers. Peritoneal adhesion formation occurs after 55 - 100% of peritoneal surgeries and is a major source of complications such as infertility, bowel obstruction, and chronic pain (diZerega, G. S. 1997). Adhesion formation occurs at the surface of the peritoneum, and surface interactions control the formation as well as the prevention of adhesions. The peritoneum contains two distinct layers of collagen, large amounts of glycosaminoglycans, an abundant vasculature, fibroblast-like cells, and is lined with mesothelial cells overlying a basal lamina.

During surgery in the peritoneal cavity, events such as tissue abrasion, desiccation, ischemia, infection, and foreign body exposure can lead to peritoneal inflammation, bleeding, and demesothelialization of the peritoneum; these events are believed to begin the biological cascade resulting in adhesion formation (diZerega, G. 1994; diZerega, G. S. 1997; Holmdahl, L. 1997; Risberg, B. 1997). Increased vascular permeability leads to release of a serosanguineous exudate rich in fibrinogen and inflammatory cells. Coagulation of fibrin occurs within 3 h as a natural healing response, and a balance between deposition and fibrinolysis (initiated by tissue plasminogen activator and urokinase plasminogen activator) determines whether the fibrin matrix becomes permanent. If this fibrin matrix forms between the damaged surfaces of two organs, an adhesion will result via cell infiltration into the matrix, angiogenesis, and collagen expression. The normal

peritoneum is fibrinolytic as regulated by the mesothelium, but fibrinolysis is typically depressed in ischemic tissues. The systems of coagulation, fibrinolysis, kinin/bradykinin, arachidonic acid metabolism, and complement are all involved in mediating the response. Cellular elements include polymorphonuclear neutrophils initially, and then macrophages predominate, covering most of the wound surface in a monolayer at 2 days. Macrophages are involved in regulation of the response, including secretion of chemical messengers and recruitment of new mesothelial cells which re-mesothelialize the wounded surface within 5 - 7 days. Macrophages present may modulate tissue fibrinolytic activity. The new mesothelial cells are believed to derive from islands of cells that attach throughout the wound and proliferate, not from a migration of cells from the wound margin. Persistence of fibrinous attachments for 3 days can result in permanence. Non-degraded fibrin is invaded by macrophages and fibroblasts, collagen is deposited, and the final adhesion is highly vascularized connective tissue often coated by a monolayer of mesothelial cells. Much is still unknown about the peritoneum and the process of adhesion formation, and the complexity of the system and difficulty in finding predictive animal models has confounded efforts to find preventative measures.

Peritoneal adhesion formation is a serious problem with great economic impact. The cost of surgery and hospitalization for lysis of adhesions performed in the US in 1988 was estimated to be $1.2 billion, and this figure does not include outpatient costs or lost productivity (Ray, N. F. et al. 1993). The only clinical approach to treating adhesions is adhesiolysis coupled with adjuvants, which has marginal efficacy in preventing the recurrence of adhesions and thus offers limited hope to sufferers.

Methods to reduce the incidence of adhesion formation have been reviewed and include improved surgical technique and the use of adjuvants such as drugs and physical barriers (Risberg, B. 1997). Minimizing tissue ischemia, tissue manipulation and foreign body (such as glove powder) introduction is believed to reduce the incidence of adhesion formation, but is typically inadequate to completely prevent adhesions. Administration of

drugs such as anticoagulants, nonsteroidal anti-inflammatory agents, antihistamines, calcium channel blockers, corticosteroids, cytostatics, proteolytics, and motility promoters have been investigated. Pharmacological efficacy suffers from difficulty in achieving long-lasting administration to the site and from the drugs interfering with normal wound healing. The instillation of concentrated solutions of macromolecules such as hyaluronic acid (Sepracoat™, Genzyme), chondroitin sulfate, dextran, PEG (Nagelschmidt, M. et al. 1998), or carboxymethylcellulose (Wurster, S. H. et al. 1995) has been hypothesized to reduce adhesion formation by either a lubricating effect on the tissue surfaces or by hydroflotation of the internal organs, but the actual efficacy is questionable. Saline or Ringer's lactate (Iaco, P. D. et al. 1994) have also been administered. Gel barriers placed on peritoneal surfaces include hyaluronic acid-carboxymethylcellulose (Seprafilm™, recently marketed by Genzyme), photopolymerized PEG gels (Hill-West, J. et al. 1994), and thermally-gelled PEG-polypropylene oxide gels (Leach, R. E. and Henry, R. L. 1990). Solid barriers include expanded poly(tetrafluoroethylene) (Preclude™, Gore) and oxidized regenerated cellulose (Interceed®, Ethicon) in clinical use, as well as PEG-carboxymethylcellulose films (Rodgers, K. E. et al. 2000) and fibrin sealant which can aid hemostasis as well as providing a barrier (Iaco, P. D. et al. 1994). The PLL-g-PEG polymers developed by Elbert and Hubbell have also been shown to reduce adhesion formation (Elbert, D. L. and Hubbell, J. A. 1998). However, in spite of all the research in the field, peritoneal adhesion formation remains a critical and costly issue in the clinic.

The PLL-g-(PEG;PBA) polymers developed in this work might be capable of interrupting peritoneal adhesion formation by binding to ECM or cellular surfaces and blocking adhesion of other macromolecules and cells to those surfaces. This hypothesis is investigated in this chapter in an *in vivo* model of adhesiolysis, representing the treatment that would be provided clinically following initial adhesion formation. PLL-g-(PEG;PBA) 1:9 was applied as a lavage immediately following lysis of surgically pre-induced adhesions, and the recurrence of adhesions was monitored. In addition, a specific *in vitro*

model of PLL-g-(PEG;PBA) interrupting macrophage adhesion to a mesothelial monolayer and the possibility of the PLL-g-(PEG;PBA) interacting with fibrin during gelation are both investigated. Administration of PLL-g-(PEG;PBA) 1:9 was found to prevent adhesions from becoming worse following lysis. PLL-g-(PEG;PBA) 1:21, and to a lesser degree PLL-g-PEG 1:9 and 1:21 were found to reduce IC-21 macrophage adhesion to RM4 mesothelial cells. No polymer was found to have a significant effect on the storage modulus of fibrin gels formed in the presence of polymer.

6.2 Materials and Methods

Polymer Synthesis

PLL-g-(PEG;PBA) and PLL-g-PEG polymers synthesized in Chapter 2 were used in all assays. The graft ratio is defined as the number of PEGs grafted to the PLL backbone divided by the number of lysine mers in the PLL backbone.

Cell-Cell Adhesion Assays

IC-21 mouse macrophages and 4/4 R.M.-4 rat mesothelial cells were purchased from ATCC (Manassas, VA). IC-21 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin 100 U/mL, streptomycin 100 µg/mL, and amphotericin B 0.25 µg/mL (Gibco). IC-21 cells were subcultured by rinsing with PBS, then incubating in PBS at 37 °C. The cells were then suspended in the PBS and at least double that volume of medium was added. Cells were split 1:2 to 1:4. RM4 cells were cultured in F-12K nutrient mixture, Kaighn's modification, supplemented with 15% fetal bovine serum, penicillin 100 U/mL, streptomycin 100 µg/mL, and amphotericin B 0.25 µg/mL (Gibco). RM4 cells were subcultured by rinsing with 0.25% trypsin (Gibco), incubating at rt until detached, then suspending in fresh medium and split 1:2 to 1:5. All cells were cultured at 37 °C in a humid atmosphere with 5% CO_2.

The ability of the polymers to block adhesion between polymer-treated macrophages and a polymer-treated monolayer of mesothelial cells was assayed in a protocol adapted from Elbert and Hubbell (Elbert, D. L. and Hubbell, J. A. 1998). RM4 mesothelial cells were grown to 8 days past confluence in 24 well TCPS plates (Falcon) and 0.8 mL RM4 culture medium was present in each well. IC-21 macrophages were removed from their culture substrate into PBS, and 1 wt% polymer in PBS was added to bring the total polymer concentration to 0.2 wt%. $1.2*10^5$ IC-21 cells in 200 µL were seeded onto the RM4 cell layer (already containing the RM4 medium) and 200 µL of 1 wt% polymer was added to bring the culture medium to 0.2 wt% polymer. The plate was gently shaken to evenly disperse the cells, and placed in the incubator on a sponge. After 1 h, the supernatant was aspirated and the wells were rinsed 3 times with 1 mL PBS to remove non-adherent cells. 0.5 mL RM4 medium was then added to each well to keep adherent IC-21 cells from detaching from the surface. At this point, the adherent IC-21 cells were round, phase bright, and readily distinguishable from the RM4 cells. The number of IC-21 cells present in the same three locations per well were then counted using a 10X objective and an eyepiece containing an ocular grid, and the values were summed and treated as one data point per well. The observer was blind as to the treatment of each well during counting.

Fibrin Gel Rheology

The rheological properties of fibrin gels formed in the presence of polymers was tested using a Bohlin Instruments CVO120 High Resolution Rheometer. Fibrinogen, polymer, thrombin, and Ca^{2+} concentrations were set to equal those that would be present if plasma mixed 1:1 with a 1 wt% polymer solution in the peritoneal cavity. Fibrinogen from human plasma (~50% protein, Fluka, Milwaukee, WI) was dissolved in Tris-buffered saline (TBS: 33 mM Tris, 137 mM NaCl, 3 mM KCl, pH 7.4) at 11 mg protein/mL. This solution was dialyzed against TBS at rt for 24 h using a 6 - 8 MWCO Spectra/Por

membrane (VWR, Seattle, WA). The solution was then filtered through a 0.2 μm syringe filter and diluted to 9.6 mg fibrinogen/mL, using the known extinction coefficient of fibrinogen at 280 nm. This solution was stored at rt to prevent protein precipitation and was used within 1 week. Polymers were dissolved at 1 wt% in HEPES-buffered saline (HBS: 10 mM HEPES, 137 mM NaCl, pH 7.4) and filtered as described in the polymer synthesis section. Thrombin from bovine plasma (~50% protein, Sigma) was dissolved at 8 U/mL in TBS. Thrombin aliquots were stored at -20 °C and used within 8 h of thawing, and never re-frozen. $CaCl_2$ was dissolved at 100 mM in deionized water. For the assay, 25 μL fibrinogen solution, 0.6 μL $CaCl_2$ solution, and 50 μL polymer solution were mixed in a micro centrifuge tube. This mixture was pipetted onto the rheometer plate and then 25 μL of the thrombin solution was added and swirled in. The 20 mm upper plate was immediately lowered onto the mixture with a 200 μm gap, and thus a 20 mm parallel plate configuration was used for testing. The mixture was kept humid by placing it in an enclosed humid atmosphere while it gelled at 25 °C for 45 min. Then the elastic modulus of the resulting gel was found using the oscillating mode at 0.5 Hz, with 0.05 strain and an initial guess stress of 0.5 Pa. The average of ten readings on each gel was taken for each data point. A strain amplitude up to 0.37 and a frequency up to 1 Hz were found to be within the linear region for these gels.

In Vivo Adhesiolysis Model

The effect of a polymer lavage on the formation of peritoneal adhesions was tested in a rat adhesiolysis model. The animal housing and the procedures were evaluated and approved according to Swiss federal laws by the veterinary authority of the Canton of Zurich. 16 female white OFA rats were randomly divided into 2 groups: vehicle control or polymer treatment. The vehicle control was HBS and the polymer treatment was 1 wt% PLL-g-(PEG;PBA) 1:9 in HBS. The surgeon was blinded as to the treatment of each rat during surgery and evaluation.

The surgeries were performed as follows. After an accommodation time in the housing facility, anesthesia was induced and maintained with halothane/O_2. In dorsal recumbence, the abdominal region was clipped and prepared for aseptic surgery. After a midline celiotomy, the uterine horns were exposed and the vasculature of the arcade of the horns was systematically cauterized using bipolar electrocautery. The most proximal and the most distal large vessels on each horn were not cauterized. Following this, the antimesenteric surface of each horn was cauterized at four spots 1 mm in diameter. Following injury, the abdomen was closed using monofilament sutures and skin staples. Analgesia was provided by an injection of Buprenorphine (0.1 mg/kg sc.). On the seventh postoperative day (*week 1*), the formed adhesions were lysed at laparotomy. The extent and grade of adhesions were measured and scored before lysis. The adhesions were then carefully separated by sharp or blunt dissection. Following lysis, the rats were randomly assigned to the polymer or control group. 4 mL of treatment solution was instilled into the abdomen and the animals were closed as described above. On the seventh day following lysis (*week 2*), the animals were sacrificed by CO_2 asphyxiation and the extend and grade of adhesions was measured and scored again.

An adhesion was defined as an inability to visualize the mesometric vessels entering the uterus. The *extent* of adhesions was defined as the percentage of the length of the uterine horn involved in any adhesions. The values for the left and right horn were averaged and treated as one data point. A *grade* representing the *severity of the adhesions* was assigned 0 - 5, where 0 = no adhesions, 1 = small adhesions at the site of cauterization, 2 = small adhesion connecting the horns, 3 = horns adhered together, 4 = small adhesion to another organ, 5 = severe adhesion to another organ (i.e., bladder, intestine, etc.). The extents of adhesions at week 1 (i.e., before adhesiolysis) versus week 2 (i.e., after adhesiolysis and treatment with either vehicle control or polymer) for each treatment were compared by the Mann-Whitney U test. The grades of severity at week 1 versus week 2 for each treatment were compared by the χ^2 test. Because the χ^2 test should

not be used when the expected values of the cells are much less than 5, the data was collapsed into a 2x2 grid. The outcome values for the grid were either grade ≤ 1 or grade > 1.

6.3 Results

Cell-Cell Adhesion

The ability of the polymers to coat cell surfaces and block cell-cell adhesion was tested in an *in vitro* model relevant to postsurgical abdominopelvic adhesion formation. IC-21 macrophages were incubated with 0.2 wt% polymer and then seeded onto a monolayer of RM4 mesothelial cells in medium which also contained 0.2 wt% polymer. After 1 h, the number of adherent IC-21 cells was counted (at this time point, the IC-21 cells had not yet spread and were thus phase bright and distinguishable from the RM4 cells).

PLL-g-(PEG;PBA) 1:21 reduced the number of adherent IC-21 cells by more than 90% (Figure 6.1). PLL-g-PEG 1:21 and 1:9 also significantly reduced the number of adherent IC-21 cells. However, the viability of the non-adherent IC-21 cells was not assayed. The RM4 cells remained confluent throughout the assay and showed no signs of toxicity.

Fibrin Gel Rheology

The mechanical properties of fibrin gels form in the presence of polymers were assayed by measuring the elastic modulus (storage modulus) of the resulting fibrin materials. A higher elastic modulus indicates that a material is stiffer, and thus might be more difficult to degrade both mechanically and enzymatically *in vivo*. Fibrinogen,
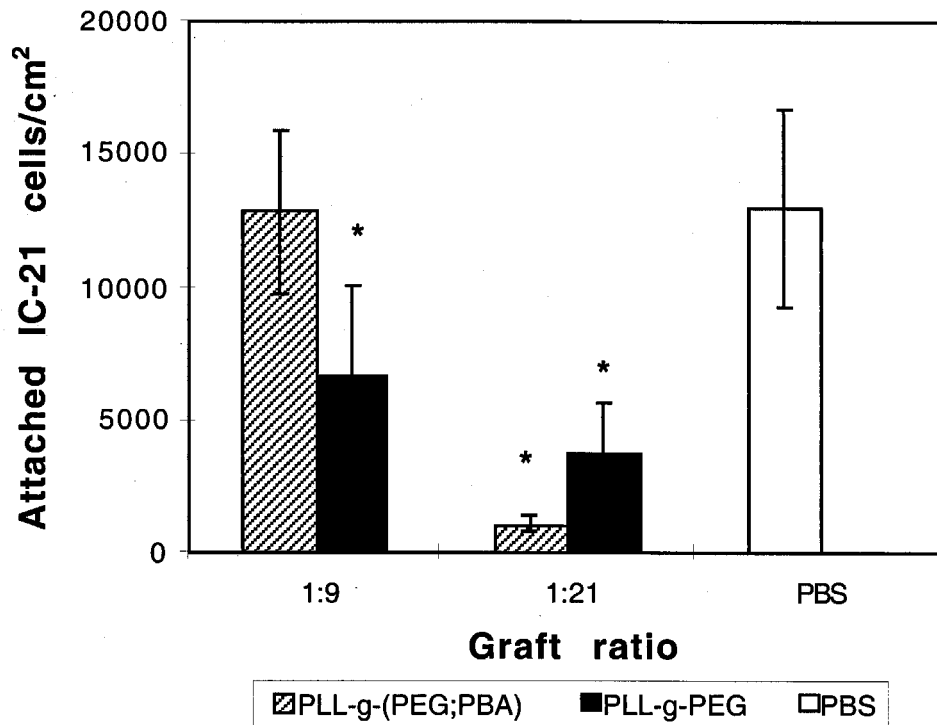
Figure 6.1. Polymer-treated IC-21 spreading on polymer-treated RM4 cells at 1 h.

IC-21 macrophages were suspended in 0.2 wt% polymer in PBS. They were then seeded onto confluent RM4 mesothelial cells in serum-containing medium, and the supernatant was brought to 0.2 wt% polymer. After 1 h, non-adherent IC-21 cells were washed away and remaining IC-21 cells were counted (the IC-21 cells were unspread and phase bright at this time, and easily distinguished from the RM4 layer). PLL-g-(PEG;PBA) 1:21 and PLL-g-PEG 1:21 and 1:9 significantly reduced the number of adherent IC-21 cells. However, the viability of the IC-21 cells was not assayed. * = significantly different from PBS at 95% confidence using Student's t-test (mean ± sd, n = 4).

calcium, the polymers, and thrombin were combined in concentrations that would be found if plasma mixed 1:1 with 1 wt% solutions of the polymer in the peritoneal cavity. These four components were combined and gelled *in situ* in a humid atmosphere, on a parallel plate apparatus at 25 °C for 45 min. These parameters were chosen, as previous work has indicated that fibrin gel elastic moduli are plateaued by this point (Gunther, A. et al. 1995; Scrutton, M. C. et al. 1994) and it was found that lower temperatures and gelation times reduced problems with the gels drying. If the gels were formed without a humid

atmosphere (in ambient air), they were found to dry out quickly with immense unpredictable increases in elastic modulus. Gels formed at 25 °C for 45 min in the humid atmosphere were found to remain moist and provide reproducible modulus values.

No polymer was found to significantly change the elastic modulus of fibrin gels formed in the presence of the polymer (Table 6.1). PLL-g-(PEG;PBA) 1:9 did increase the storage modulus when compared to a polymer-free control, but this change was not statistically significant. The moduli values found in this work were about 1/10 of the values found by Elbert and Hubbell (Elbert, D. L. and Hubbell, J. A. 1998) when applying PLL-g-PEG 1:6 or HBS. Elbert and Hubbell used the same fibrinogen, calcium, polymer, and thrombin concentrations. However, the batches and preparations of proteins were different and their gels were formed for 10 min at 37 °C without the use of any device to maintain humidity during gelation.

Table 6.1. Elastic moduli of fibrin gels formed in the presence of polymers.

Polymer	Elastic Modulus (Pa)	P	n
PLL-g-(PEG;PBA) 1:9	10.5 ± 5.4	0.12	6
PLL-g-(PEG;PBA) 1:21	6.1 ± 1.1	0.83	3
PLL-g-PEG 1:9	6.4 ± 1.8	0.92	5
monomethoxy-PEG (MW 5 kDa)	7.5 ± 4.6	0.68	3
HBS	6.5 ± 2.9		7

Elastic modulus ± sd, P values versus HBS using Student's t-test. Fibrin gels were formed in the presence of various polymers, at 25 °C for 45 min. No polymer significantly changed the elastic modulus of the gels. PLL-g-(PEG;PBA) 1:9 did increase the modulus but it was not statistically significant.

In Vivo Adhesiolysis Model

The ability of PLL-g-(PEG;PBA) 1:9 to reduce postoperative adhesion reformation in a model of postoperative abdominopelvic adhesiolysis was tested *in vivo*. Adhesions were induced in rats by cautery of the vascular arcade supplying the uterine horn and then allowed to develop for 1 week. The rats were then re-opened and the extent and grade of any adhesions present was noted. The adhesions were then surgically lysed from the uterine horns and HBS or PLL-g-(PEG;PBA) 1:9 1 wt% in HBS was instilled in the peritoneal cavity. After 1 more week, the rats were sacrificed and the extent and grade of any adhesions present were again noted. The extent is the percent of the length of the horns involved in adhesions and the grade is a subjective score 0 - 5 of how severe the adhesions are.

Adhesiolysis alone, without polymer treatment, was found to worsen adhesions in the rat, whereas treatment with PLL-g-(PEG;PBA) 1:9 prevented the adhesions from worsening. Both the extent of adhesions and the grade of adhesions significantly worsened after adhesiolysis in the vehicle control animals. The extent of adhesions after lysis and HBS lavage changed from 53 ± 11 to 73 ± 17 and the changes in the grade of severity are shown in Figure 6.2a ($P < 0.05$ week 1 v. week 2, both for extent and for grade). In contrast, the extent and grade of adhesions did not significantly change pre-versus post-adhesiolysis when the animals were lysed and treated with PLL-g-(PEG;PBA) 1:9. The extent values for week 1 and week 2 were 66 ± 20 and 68 ± 21 and the grade values are shown in Figure 6.2b ($P > 0.5$ week 1 v. week 2, both for extent and for grade). Comparing week 1 (i.e., the adhesions just prior to adhesiolysis) to week 2 (after adhesiolysis, treatment, and healing) was chosen in part because the average extents and grades were higher for the polymer groups than for the control groups at week 1, before adhesiolysis and treatment. Thus the animals randomly assigned to the polymer group might characteristically exhibit lower fibrinolysis following surgery, and also the more protracted lysis procedure required to lyse these worse adhesions could induce greater
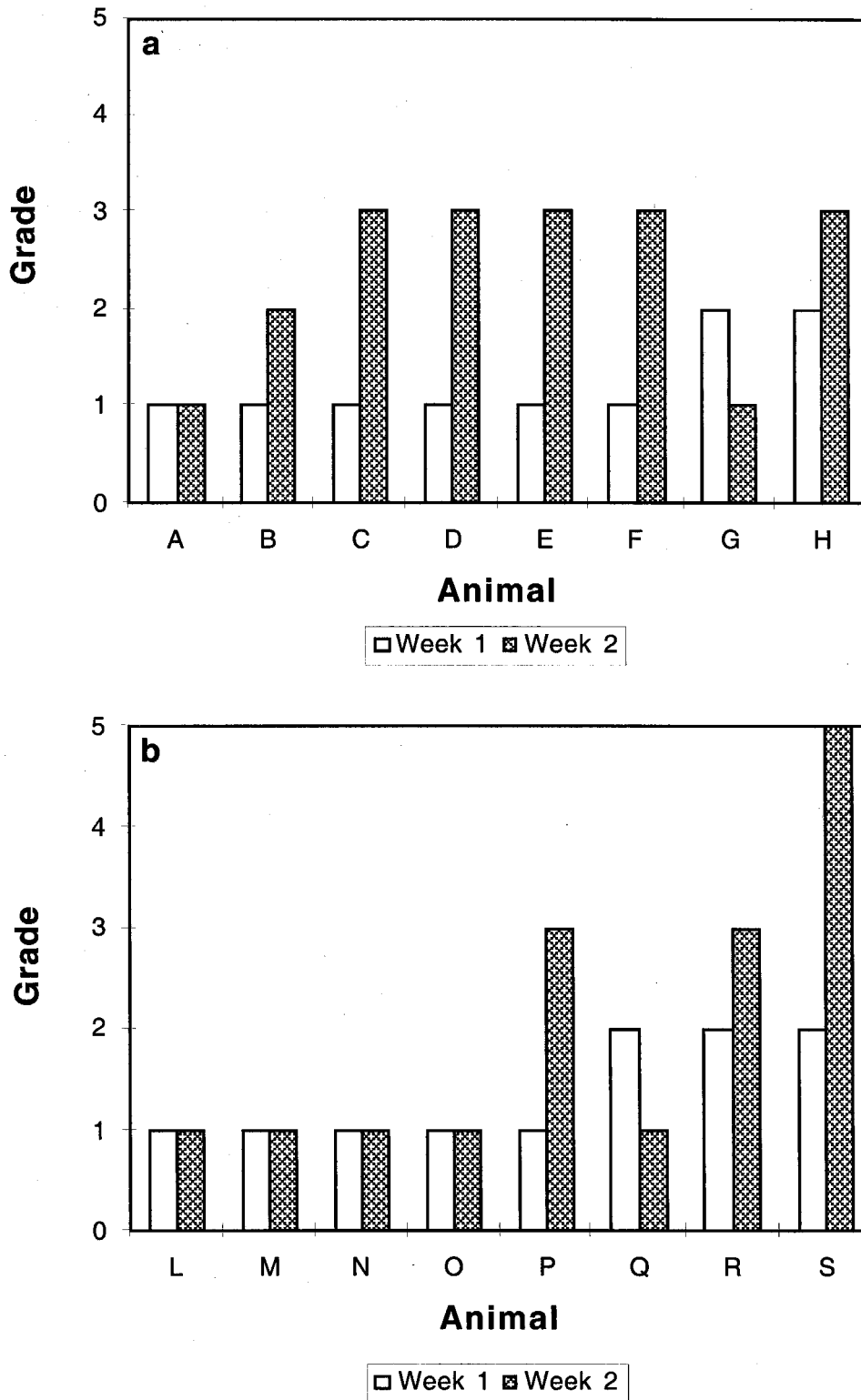
Figure 6.2. Grade of peritoneal adhesions before and after adhesiolysis and polymer lavage *in vivo*.

(Figure 6.2)
Adhesions were induced in rats by cautery of the uterine horns. 1 week after adhesion induction, the peritoneal cavity was examined, the adhesions formed were graded (week 1), and then surgically lysed. 4 mL of HBS or 1 wt % PLL-g-(PEG;PBA) 1:9 in HBS was then instilled in the peritoneal cavity and the cavity was subsequently closed. 1 week later, the cavity was re-examined and the adhesions reformed were graded (week 2). The grade representing the severity of the adhesions was assigned 0 - 5, where 0 = no adhesions, 1 = small adhesions at the site of cauterization, 2 = small adhesion connecting the horns, 3 = horns adhered together, 4 = small adhesion to another organ, 5 = severe adhesion to another organ (i.e., bladder, intestine, etc.). The grades at week 1 versus week 2 for each treatment were compared by the χ^2 test. Because the χ^2 test should not be used when the expected values of the cells are much less than 5, the data was collapsed into a 2x2 grid. The outcome values in the grid were either grade $\leq$ 1 or grade > 1. a. Rats treated with HBS. b. Rats treated with PLL-g-(PEG;PBA) 1:9. Rats treated with HBS following adhesiolysis developed significantly more severe adhesions (P < 0.05) whereas the adhesions in rats treated with PLL-g-(PEG;PBA) 1:9 did not worsen (P > 0.5). The same trend was seen in the extent of the adhesions.

intraperitoneal loads of fibrous exudate and inflammatory responses after the second

surgery. No gross tissue abnormalities or toxic responses were noted in any animals.

6.4 Discussion

PLL-g-(PEG;PBA) 1:9 showed some promise in a therapeutic model of blocking

postsurgical abdominopelvic adhesion reformation *in vivo*, but a mechanism of action is

not clear. PLL-g-(PEG;PBA) 1:21 strongly blocked IC-21 cell adhesion to RM4 cells, but

PLL-g-(PEG;PBA) 1:9 did not. However, given the *in vitro* cytotoxicity of PLL-g-

(PEG;PBA) 1:21 seen in Chapter 4, a possible toxicity effect of this polymer cannot be

ruled out even though no visible signs of toxicity were seen. Even though PLL-g-

(PEG;PBA) 1:9 was not effective in blocking cell-cell adhesion in this model, it was shown

in Chapter 4 to reduce cell spreading and adhesion on polymer-treated serum proteins and

ECM, to strongly block cell adhesion on a polymer-treated model hydroxylated surface,

and to coat cells and reduce their adhesion to TCPS. In addition, all of these *in vitro*

models are significantly removed from the true *in vivo* adhesiolysis response. However, it

would be difficult to synthesize a more appropriate model, especially given the poorly understood pathogenesis.

The effect of PLL-g-(PEG;PBA) 1:9 on adhesion reformation does not appear to involve an effect on the strength of the fibrin gel formed. PLL-g-(PEG;PBA) 1:9 did increase the storage modulus relative to controls, but the increase was not significant and the statistical error was large. It is possible that the polymer does interact with the fibrin in some cases, and that this gel strengthening effect opposes other beneficial effects of the polymer *in vivo*, leading to a less dramatic amelioration of the adhesions. The reason for any increased gel strength is unknown, but could involve the PBA groups on the polymer cross-linking fibrin strands.

Our results indicate that PLL-g-(PEG;PBA) 1:9 has a notable effect in resisting the reformation of adhesions, even though the adhesions were not completely ameliorated in this model. Others have seen more impressive results in prevention of adhesion reformation by treatment with poloxamers, carboxymethylcellulose films or solutions, or nonsteroidal anti-inflammatory agents, though methods of injury and analysis all differ (Cofer, K. F. et al. 1994; Rodgers, K. E. et al. 2000; Steinleitner, A. et al. 1991; Wurster, S. H. et al. 1995). Lysis of existing adhesions has been known to produce more severe adhesions in rat models (O'Sullivan, D. et al. 1991; Wurster, S. H. et al. 1995). It is this tendency for each subsequent surgery to induce formation of more/worse adhesions that makes the formation of peritoneal adhesions so deleterious and poses such a clinical dilemma. The adhesions in animals treated with a PLL-g-(PEG;PBA) 1:9 lavage following lysis statistically did not change. Thus the polymer shows promise, and could perhaps be improved by further customizing the grafting ratios and size of the copolymer. Future work should include controls such as the corresponding polymer without PBA or physical mixtures of the copolymer components. PEG alone has been shown to reduce the formation of adhesions, but most applications use very high doses (5 mL of 5 - 20 wt% PEG MW 4 kDa) (Elbert, D. L. and Hubbell, J. A. 1998; Nagelschmidt, M. et al. 1998;

Nagelschmidt, M. and Saad, S. 1997; O'Sullivan, D. et al. 1991). In addition, future work could test the polymer in the less challenging model of initial adhesion formation. Elbert and Hubbell have previously shown PLL-g-PEG 1:6 to reduce the initial formation of peritoneal adhesions, though the effect may have been due to a change in the strength and degradability of fibrin formed in the presence of this polymer (Elbert, D. L. and Hubbell, J. A. 1998). Importantly, PLL-g-(PEG;PBA) 1:9 was not seen to interfere with wound healing or to cause any toxic responses *in vivo*.

6.5 Conclusions

PLL-g-(PEG;PBA) 1:9 was shown to have a positive effect in blocking worsening of lysed peritoneal adhesions. PLL-g-(PEG;PBA) 1:9 did not significantly affect the elastic modulus of fibrin gels formed in its presence, and did not reduce adhesion of IC-21 cells to RM4 cells. PLL-g-(PEG;PBA) 1:21 did strongly block this cell-cell adhesion, but toxicity was not ruled out as a cause. Instillation of PLL-g-(PEG;PBA) 1:9 into the peritoneal cavity following adhesiolysis prevented the significant increase of adhesion extent and severity seen in the control animals. The mechanism of the effect is unknown, but possible mechanisms of efficacy were seen in Chapter 4. The healing response during peritoneal adhesion formation is very complex and the polymer might have interfered with any number of events involved in the cellular and biochemical cascade.

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7. Chapter 7

General Discussion and Conclusions

7.1 Major Conclusions

Cell-surface and cell-cell interactions in biological systems are involved in a number of pathophysiological responses. Such interactions proceed by multiple parallel pathways and as such are difficult to interrupt based on biochemical approaches, e.g., based on competitive inhibition of the receptor-mediated pathways utilized in recognition and attachment. One might be able to address such interactions using means that are strictly physicochemical in nature and do not rely on the details of biological recognition, e.g., colloidal steric stabilization as described herein. Such approaches might be particularly valuable after a surgical or catheter-based intervention, where the surgeon would have ready access to the site to be treated. Such a treatment should be capable of being applied *in vitro* or *in vivo* without damaging the treated cells or tissues, lead to a confluent intimate coating over all treated surfaces, and remain present in sufficient quantities to block adhesion over the required time frame. Thus it was sought to design a water-soluble graft copolymer that contained backbone groups that would strongly bind ubiquitous molecules on cell and extracellular matrix surfaces, consequently anchoring the grafted side chain non-binding groups to the surface and rendering the surface highly resistant to protein binding and cell adhesion. Such a polymer treatment would provide a molecular barrier to adhesion that could be applied by simply exposing the desired surfaces to an aqueous polymer solution.

Reported in this thesis is an example of designing a surfactant polymer that will spontaneously coat biological surfaces and block high-affinity interactions with the surface through a physicochemical mode of action. This surfactant copolymer contains PBA

moieties with an affinity for biological (e.g., cell or tissue) surfaces and PEG moieties with an affinity for aqueous solutions, causing the surfactant to spontaneously assemble on wet biological surfaces. The polymer spontaneously binds to *cis*-diol-containing surfaces at physiological pH, having a pKa circa 6. PLL-g-(PEG;PBA) polymers with optimal grafting ratios spontaneously coat RBC surfaces and block their agglutination by proteins. Optimal polymers also spontaneously coat TCPS, serum protein, and ECM surfaces and block cell adhesion, spreading, and migration upon these surfaces to varying extents. PLL-g-(PEG;PBA) 1:9 was shown to reduce the worsening of peritoneal adhesions following adhesiolysis. However, some of the polymers showed apparent dose- and time-dependent toxicity *in vitro* even though no adverse effects were noted *in vivo*, both intraperitoneally and intraocularly.

The differing interactions of PLL-g-(PEG;PBA) with cell versus proteinaceous surfaces are of interest in understanding mechanisms as well as in designing clinical applications. PLL-g-(PEG;PBA) seemed more effective at sterically stabilizing RBCs from protein adhesion than at sterically stabilizing proteinaceous surfaces from cell adhesion. This may result from the different glycosylation patterns on the surfaces, as RBCs are rich in sialic acid whereas many serum and ECM components lack appropriate sugar groups for PBA binding (He, X. M. and Carter, D. C. 1992; Jackson, R. L. et al. 1973; Marchesi, V. T. et al. 1972; Paulsson, M. 1992). In addition, cell membranes are fluid, thus the possibility exists that cell surfaces are rearranged upon binding by PLL-g-(PEG;PBA), enabling a more effective polymer distribution, whereas proteins coated onto TCPS do not offer bound polymer as many opportunities for rearrangement. It must again be emphasized that glycosylation is highly variable and dependent on species, age, and environment and all model assays may not represent true *in vivo* efficacy (Dennis, J. W. et al. 1999; Varki, A. 1992). However, given the current data it appears that the current PLL-g-(PEG;PBA) polymers would be best targeted to clinical applications that require steric stabilization of cellular, rather than ECM, surfaces. This conclusion must be taken with the

caveat that cellular endocytosis will effect the rate of polymer clearance from treated cellular surfaces.

The interaction of PLL-g-(PEG;PBA) with cells is important given the apparent cytotoxicity of the polymers *in vitro*. This fact complicated interpretation of some of the *in vitro* work. A significant number of possible mechanisms could account for an incorrect appearance of toxicity *in vitro*, or for true toxicity. The potential toxicity issue represents an important possible area for future investigations.

On all the model surfaces investigated in this work, PLL-g-(PEG;PBA) was more effective or effective over a broader range of PEG grafting ratios than PLL-g-PEG. Electrostatic binding via PLL-g-PEG is potentially hampered by the high ionic strength of physiological fluids and the zwitterionic nature of many biological molecules. The reversible covalent bonds formed by the PBA moieties likely provided much stronger binding that compensated for any heterogeneous lack of binding sites along surfaces. The binding of the PLL-g-(PEG;PBA) polymers in this work was so long-lasting that the size of the polymer backbone or the degree of PBA grafting could likely be reduced substantially while still maintaining binding efficacy.

The PLL-g-(PEG;PBA) polymers described herein show promise in sterically blocking adhesion to biological surfaces applicable in numerous medical conditions. The polymers can be applied by a simple aqueous lavage laparoscopically following medical interventions and show no deleterious effects at high doses *in vivo*. While many biological recognition events that could be beneficially blocked must be targeted in a manner that is highly specific, e.g., with a competitive antagonist, in others cases it may be beneficial to inhibit several parallel interaction pathways all at once. In these cases, the less specific approach of using a polymer that physicochemically and nonspecifically blocks interactions with treated surfaces could be beneficial.

7.2 Future Investigations

While this work has created an understanding of the function of PLL-g-(PEG;PBA) polymers in steric stabilization of biological surfaces, there exist many areas in which further investigation could provide additional insight into understanding both basic scientific as well as applied functions of the polymers. These include investigating the steric stabilization function of the polymers, varying the copolymer architecture for varied function, further probing the pKa of the PBA moieties, and elucidating the mechanism for the apparent *in vitro* cytotoxicity.

The repulsive properties of the PLL-g-(PEG;PBA) polymers on surfaces can be further investigated with regards to structure. It would be very interesting to coat surfaces such as TCPS with the current polymers and investigate the dependence of protein repulsion as a function of the MW of the protein. Using proteins with various pIs, tertiary structures, and flexibility would also be interesting. Varying the copolymer architecture and testing the resulting functions could also be investigated. Different MW PEG side-chains as well as star (branched) PEGs at various graft ratios could be used. The length of the PLL backbone as well as the degree of PBA grafting on the backbone could be varied; the data in this work strongly suggests that much smaller polymer backbones with fewer PBAs would still be effective because the PBA binding is so strong. Tailoring of the backbone could affect dissociation rates as well as many possible toxicity mechanisms. All of these investigations would provide further insight into designing copolymers containing PEG and PBA for steric stabilization of surfaces to specific applications.

The low pKa of the PBA moieties in the PLL-g-(PEG;PBA) polymers is very intriguing and invites further investigation. The high concentration of amines in the polymer backbone surrounding the moieties is likely the cause of the low pKa. This could be further investigated by coupling PBA to the ε-amine of lysine and oligo(lysines), as well as by varying the PBA grafting ratio on PLL-g-(PEG;PBA) and PLL-g-PBA copolymers. Other amines besides lysine could also be investigated. The effect on the pKa of having

one or two PBAs conjugated per amine could also be considered. In addition, 2-formyl- and 3-formyl-PBA are now commercially available. The effect of having the linker at the ortho or meta position versus the para position can be examined. These investigations could help elucidate the reason for the low pKa of these polymers as well as aiding in the design of other PBA moieties with desirable pKas.

The apparent toxicity of the PLL-g-(PEG;PBA) polymers *in vitro* is an important issue that could be further investigated. Additional live/dead and metabolic assays could be conducted to determine if the cells truly are dying *in vitro*. If the cells are indeed dying, applications of PLL-g-(PEG;PBA) 1:6, 1:3, and 1:2, and of PLL-g-(PEG;PBA) 1:21 or 1:9 pre-incubated with fructose, could be used to see if polymer binding to cell surfaces or sequestration of soluble components is involved in the toxicity. Additional studies could be performed to determine what components the polymers bind when incubated in serum-containing media without cells. Experiments testing the polymer repulsion of proteins of various MW (discussed above) could identify if the polymer may be sterically excluding vital nutrients from entering the cells. Fluorescent-labeling of the polymer followed by confocal microscopy could identify the location of the polymer on/in cells and whether the polymer accumulates in any location within the cell. The size of the backbone of the polymers could be greatly reduced to see if polymer cross-linking of cell surface receptors or polymer-induced changes in the cell membrane viscosity is occurring. If the cells are not dying *in vitro*, tests could be performed to determine if the polymer is inducing mitosis or other metabolic changes *in vitro*. Depending on the results of these studies, the polymer may not be toxic *in vivo* or the polymer geometry may be alterable to produce a less toxic form.

7.3 Clinical Applications

The PLL-g-(PEG;PBA) polymers developed in this work show promise for efficacy in a number of clinically important areas, by blocking adhesion of proteins and cells to cell, extracellular, and biomaterials surface. The current polymers could immediately be applied to a number of *in vivo* models and evaluated for efficacy and further studies. However, the results do indicate that the polymers may be more effective at binding to cell surfaces than to ECM surfaces due to differing glycosylation patterns on those surfaces.

Cell surface-based diseases may be the most likely candidates for the polymers. These include alloimmunization (Vichinsky, E. P. et al. 1990). The donor RBCs could instead simply be incubated with the PLL-g-(PEG;PBA) polymers before transfusion to prevent subsequent antibody binding. Functions of polymer-treated RBCs such as oxygen and carbon dioxide transport, glucose transport, and cell deformability should be tested. Another pathology involving cell surface adhesion is reperfusion injury (Carden, D. L. and Granger, D. N. 2000). Simply perfusing the ischemic organ's vasculature with a PLL-g-(PEG;PBA) solution before organ reperfusion could coat the vascular endothelial cells with polymer and block neutrophil adhesion to those cells. While the endothelial cells would eventually lose the polymer coating due to membrane turnover, the treatment could likely last long enough to block neutrophil adhesion during the initial superoxide and inflammatory state following reperfusion. Another possible area of application is in hyperacute rejection of porcine (and other non-primate) organ transplants. The α1-3galactosyl epitope contains a *cis*-diol to which PBAs can bind, thus providing a direct site for polymer blockage of antibody binding (Rother, R. P. and Squinto, S. P. 1996). A simple infusion of a solution containing the PLL-g-(PEG;PBA) polymers into the vasculature of the organ to be transplanted may reduce the tendency of pre-formed antibodies from the recipient to bind to the organ and initiate a hyperacute immune rejection response (the duration of this effect, as well as the possibility of tolerance, would need to

be considered). The PLL-g-(PEG;PBA) polymers could also be infused into the peritoneal cavity following tumor resection, to bind to free intraperitoneal tumor cells and prevent their attachment and metastasis to other parts of the body.

The PLL-g-(PEG;PBA) polymers were also shown to block some cell binding to proteinaceous surfaces and thus could be effective in diseases relevant to polymer binding to protein as well. In addition to the possible efficacy of the polymers in preventing PCO and peritoneal adhesion formation, another possible application is rheumatoid arthritis. The polymers could potentially interrupt three major deleterious events that occur in rheumatoid arthritis. Complexes of immunoglobulins (rheumatoid factors) could be prevented from aggregating by the polymer binding to the highly sialylated oligosaccharides on those glycoproteins (Rademacher, T. W. et al. 1988). Matrix metalloproteinase degradation of cartilage (Forre, O. et al. 2000) could be blocked by polymer binding to proteoglycans and collagen in the joints. The polymer could also coat cartilage and bone and prevent pannus formation and adhesion to those surfaces (Forre, O. et al. 2000).

In addition to these biological surfaces, the PLL-g-(PEG;PBA) copolymers were also found to sterically stabilize TCPS very effectively, and thus could be harnessed to coat this and other solid biomaterial surfaces that contain hydroxyls. The steric stabilization of TCPS by the polymers was facile to apply, long lasting, and easy to pattern. Preventing adhesion of proteins and cells to specific areas of tissue culture ware would be advantageous in many experiments. Preventing "fouling" of biosensors is also very desirable. In addition, biomaterials surfaces that contain appropriate hydroxyl groups could be coated with the polymer prior to implantation, thereby preventing a number of undesirable binding events *in vivo*. The application method is easy and fast, and thus would be amenable to automation and mass production.

7.4 Additional Functionalities

While these PLL-g-(PEG;PBA) polymers were designed specifically to sterically stabilize biological surfaces, their properties are also amenable to modifications that would confer different useful functions to the polymers. For instance, groups containing biological information could be grafted to the pendant termini of the PEG groups. For example, adhesive peptides such as RGD could be attached to thereby allow certain cells to attach to a polymer-treated surface while blocking the binding of undesirable proteins. Stents could be coated with such a polymer, helping to block thrombus formation while encouraging endothelialization of the stent. In another example, the MW, PEGylation, and pKa of the polymer could be harnessed as an anti-cancer treatment. A diol-containing anti-tumor drug could be complexed with the PBA moieties on the polymer backbone. Following intravenous administration, the polymer would preferentially accumulate in tumors due to enhanced permeability and retention (EPR) of macromolecules with high MWs (Maeda, H. et al. 2000). The PEGylation of the polymer would reduce its hepatic clearance and proteolysis of the PLL backbone. In the lower pH of the tumor environment and the even lower pH of tumor cell endosomes following endocytosis, the PBA boron centers could become trigonal and thereby release the bound drug. The pKa of the PBA moiety could be varied (by varying its local environment in the polymer or adding groups to the phenyl ring) to be suitable for release at the desired location. Targeting agents such as antibodies could be conjugated to the polymer to further direct it to tumor sites.

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9. Vita

Natalie Dawn Winblade was born in Seattle, Washington, to William and Donna Winblade. In 1991, she entered the University of Washington, where her worked included research into surface analysis of polymeric biomaterial films by ESCA and SIMS under the supervision of Buddy D. Ratner. In 1995, she received her Bachelor of Science in Chemical Engineering degree Magna Cum Laude from the University of Washington. She then entered the Department of Chemical Engineering at California Institute of Technology in 1995. She received her Master of Science degree in 1997, for research leading to the results in this thesis. Upon the move of her advisor, Jeffrey A. Hubbell, to the Swiss Federal Institute of Technology (ETH) Zurich, Switzerland, in 1997, she continued her thesis work with Prof. Hubbell and also began a collaboration with Allan S. Hoffman at the University of Washington, Seattle. This dissertation was typed by the author.