Peptide-PEG Amphiphiles as Cytophobic Coatings for Mammalian and Bacterial Cells

Brief Communication

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Summary

Amphiphilic macromolecules containing a polystyrene-adherent peptide domain and a cell-repellent poly(ethylene glycol) domain were designed, synthesized, and evaluated as a cytophobic surface coating. Such cytophobic, or cell-repellent, coatings are of interest for varied medical and biotechnological applications. The composition of the polystyrene binding peptide domain was identified using an M13 phage display library. ELISA and atomic force spectroscopy were used to evaluate the binding affinity of the amphiphile peptide domain to polystyrene. When coated onto polystyrene, the amphiphile reduced cell adhesion of two distinct mammalian cell lines and pathogenic *Staphylococcus aureus* strains.

Introduction

Cytophobic, or cell-repellent, coatings are highly desirable for use in proteomics, cell culture technologies, and biologically integrated medical devices because the lifetime, reliability, and performance of many medical implants, diagnostics, and high-throughput screening formats are hindered by protein adsorption or cellular adhesion [1–10]. For example, bacterial cell colonization and biofilm formation on implanted or inserted medical devices are common in clinical practice and contribute to adverse outcomes. Catheters alone account for hundreds of thousands of nosocomial infections each year, resulting in significant cost and burden

to the health care system [1]. Current methods to prevent biological fouling on surfaces include plasma treatment, biotin-avidin conjugation strategies, phospholipids, self-assembled monolayers on transition metal coatings, chemically grafted poly(ethylene glycol) (PEG), albumin adhesion, polyclonal antibodies, and antibiotics [11–18]. Of these approaches, surface modification with PEG has met with success for the prevention of cell and protein adhesion [16, 19–27]. However, chemically grafting this macromolecule to a surface often requires surface preparation and multistep chemical procedures. Due to this and a variety of other issues, PEG coatings have not achieved in vivo clinical use. Herein we describe the synthesis of a prototypical peptide-poly(ethylene glycol) amphiphile, the application of the amphiphile to plastic surfaces, and the inhibition of subsequent human and bacterial cell adhesion to the coated surface.

Results and Discussion

These amphiphilic macromolecules possess two distinct domains: a hydrophilic PEG domain for cell repulsion and a relatively hydrophobic peptide domain selected for specific surface binding. The specific surface binding peptides were identified using peptide phage display, a combinatorial biological technique that selects high-affinity peptides for a specific target through iterative screens [28–33]. Although its original successes came from the recognition of biological targets and its uses for biotechnology and drug discovery [34–37], phage display has recently proven to be a valuable technique for identifying specific peptides that bind to a variety of inorganic and polymeric surfaces [38–48].

Phage display operates through affinity selection of phage-encoded peptides [28–32]. In this study, two peptide libraries (X_6PX_6 and X_6YX_6, where X represents one of the 20 amino acids encoded by synthetic NNK codons) were expressed separately on the pIII coat protein and used to identify polystyrene binding peptides. This combinatorial library (10^{10} total phage screened; 10^8 complexity) was screened against a polystyrene target polymer using a well of a microtiter plate (CoStar, Corning). Polystyrene is widely used in cell culture and diagnostic technologies and is a suitable material with which to demonstrate proof of concept due to its availability and good optical qualities. It is important to note that polystyrene plates available from laboratory suppliers come in two main types: tissue culture polystyrene (TCPS) and regular native polystyrene (PS), which was the substrate used in the screen. The TCPS surfaces have been treated with a high-energy process in order to develop a surface that is more hydrophilic and negatively charged, which are general characteristics conducive to cellular attachment.

Once the PS binding phage clones were identified, the base sequence of the DNA insert in the phage genome was located and translated to yield the corresponding amino acid sequence that was displayed on the phage surface. Eighteen peptide sequences were identified

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(Table 1; 1–18). The compositions of these peptides provide insight into which amino acids are important for PS affinity. The percent occurrence for each residue in the isolated sequences from the PS panning was compared to those randomly picked from the initial library population or from the theoretical amino acid frequency. Almost all of the amino acids occur at frequencies similar to those in their respective libraries; for example, approximately 1% occurrence was found for R, C, and Q; 3% for N, H, Y, W, and M; and 7% for A, G, and P. In contrast, I, T, V, and particularly K are strikingly underrepresented. E and F are significantly enriched with 100% higher frequency than expected. Among the aromatic residues (F, Y, H, and W), F is the preferred hydrophobic residue. The presence of hydrophilic residues likely balances the hydrophobic properties of the selected peptides.

The relative binding strengths of these on-phage peptides to PS were determined by treating the bound phage with a horseradish peroxidase-anti-M13 monoclonal antibody conjugate and then with the chromogenic agent 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (Table 1). Given the large error associated with determining these values, we have grouped the PS binders into three categories: weak, medium, and strong. The affinity constant (k_{aff}) for four single peptides was estimated using an ELISA-type assay (see the Supplemental Data available with this article online) and included a relatively weak binder (1), a medium binder (15), and two strong binders (17 and 18). The values ranged from 6.7×10^4 to 7.3×10^6 M^{-1}, and are shown in Table 2. If the same experiment is performed on TCPS, we observe an order of magnitude decrease in the affinity constant. For example, 18 had an affinity of 7.3×10^6 M^{-1} on PS and 3.0×10^5 M^{-1} on TCPS. As discussed earlier, the phage library-screening assay was performed on native PS, and the observed increase in the binding constant to native versus TCPS highlights the ability to find peptide sequences that possess affinity for a particular surface even among closely related substrates.

Atomic force spectroscopy was used to further characterize and quantify the adhesion strengths of a strong binder, peptide 17, to PS. For these experiments, a C-terminal cysteine derivative of peptide 17, 19, was prepared and chemisorbed to gold-coated cantilevers. We then measured the adhesion (pull-off) force between four model surfaces (TCPS, native PS, methyl-terminated self-assembled monolayer [SAM] on a gold surface, and carboxylic acid-terminated SAM on a gold surface) and the cantilever tip functionalized with peptide 19 under aqueous conditions. In order to ensure intersample consistency, the same cantilever was used to measure the forces on all model surfaces. The spring constants were determined by the thermal noise fluctuation method. Figure 1 shows the distribution of adhesion forces for the four model surfaces. An average adhesion force of 672 ± 271 pN was obtained from approximately 250 force curves taken at several different locations on the native PS (water contact angle 79°) surface, and an adhesion force of 60 ± 41 pN was obtained from approximately 550 force curves for the tissue culture PS (water contact angle 56°), again verifying the specificity of the peptides to their target surfaces. To gauge the magnitude of this strong, noncovalent interaction, we per-

formed additional experiments on hydrophilic (carboxylic acid-terminated SAM, water contact angle 14°) and hydrophobic (methyl-terminated SAM, water contact angle 114°) surfaces and calculated forces of 93 ± 42 pN and 2502 ± 1006 pN, respectively. On the acid-terminated SAM, in which there should be minimal hydrophobic surface-peptide interactions, only a small adhesion force was observed, while a large adhesion force was measured on the methyl-terminated SAM. It is important to note that hydrophobic interactions alone are not the sole explanation for the observed forces, as the more hydrophilic acid SAM has a much lower contact angle than the TCPS, yet the SAM surface has a higher average pull-off value ($p < 5 \times 10^{-38}$). In addition, we have conducted experiments on unrelated surfaces with intermediate surface energies and see little to no binding affinities for these PS peptides. Adhesion forces in control experiments between a PS surface and a bare gold-coated cantilever tip without peptide modification were negligible when compared to the adhesion forces with the PS binding peptide.

The peptide-poly(ethylene glycol) amphiphilic derivatives, 21 and 22, were synthesized to evaluate whether

Table 1. Peptides Isolated from Phage Display Libraries X$_6$PX$_6$ and X$_6$YX$_6$ and Their Relative Binding Strengths, Normalized to the ELISA OD of the Weakest Binder in the Panel of Peptides

No.	Peptide Sequence	RBS
	Weak Binders	1–15
1	FRMDFDYLYPSLP	
2	LNFMIFYLSLNPW	
3	FSYSVSYAHPEGL	
4	SVAFYDYLPTDLP	
5	LSFSDFYFSEGSE	
6	FAPMKSYGVSLPP	
7	LFGPIEYTQFLAN	
8	LFDAYWYSDTAMS	
9	PASLELYENLVAG	
10	GENFCPYSFFGCG	
11	YLSLHAYESFGGS	
12	FFGFDVYDMSNAL	
13	FYMPFGPTWWQHV	
	Medium Binders	30–100
14	LPHLIQYRVLLVS	
15	GFAWSSYLGTTVH	
16	FLSFVFPASAWGG	
	Strong Binders	>150
17	FFPSSWYSHLGVL	
18	FFSFFFPASAWGS	
19	FFPSSWYSHLGVL-SSG-C	
20	FFPSSWYSHLGVL-SSG	
21	FFPSSWYSHLGVL-SSG-PEG	
22	FFSFFFPASAWGS-SSG-PEG	

Italicized positions indicate nonvariable residues. RBS, relative binding strengths (±20%).

Table 2. Apparent Affinity Constants for Biotinylated Peptides Binding to PS

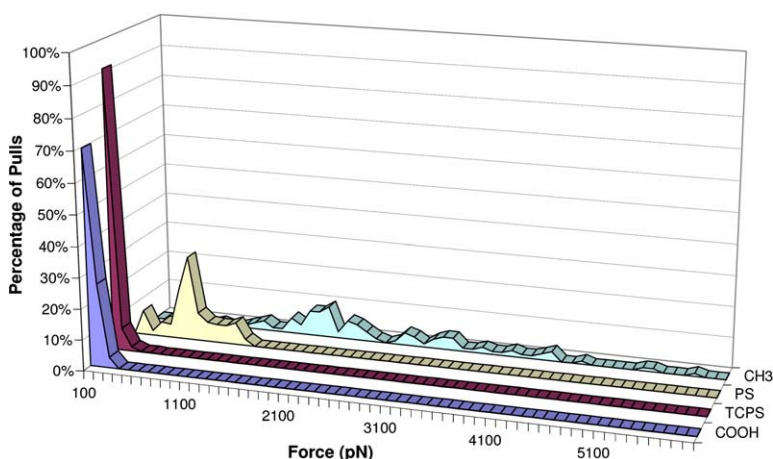| Peptide	No.	k_{aff} (M^{-1})	Model Fit (R^2)
FRMDFDYLYPSLPSSGK-biotin	1	6.62×10^4	0.997
GFAWSSYLGTTVHSSGK-biotin	15	1.78×10^5	0.996
FFPSSWYSHLGVLSSGK-biotin	17	1.86×10^6	0.996
FFSFFFPASAWGSSSSGK-biotin	18	7.30×10^6	0.989

Figure 1. Adhesion Strength between Peptide **19** and a Polystyrene Surface

Adhesion force distribution between a cantilever coated on one side with peptide **19** (FFPSSWYSHLGVL-SSG-C) and a carboxylic acid-terminated self-assembled monolayer on gold (blue), TCPS (red), a native PS surface (yellow), and a methyl-terminated SAM on gold (green).

these amphiphiles would reside at the critical interfacial site between the biologic and the plastic surface. We first measured the critical aggregation concentration (CAC) for **21** and **22** using a fluorescence titration method with pyrene. The CAC was determined to be 11.2 and 8.2 mM for **21** and **22**, respectively, confirming the amphiphilicity of these macromolecules.

Assembly of this amphiphile on the surface should alter the contact angle and reduce cell attachment if the peptide is interacting with the surface and the PEG is directed toward the cells. A cytophobic coating on a PS surface is of practical interest because control of cell adhesion on PS has many varied applications. We measured the contact angle for untreated TCPS and untreated native PS and compared those values to the corresponding peptide **21**- or **22**-coated TCPS and native PS surfaces. The surfaces were washed prior to measurement to remove any nonspecific interactions. Contact angles provide a macroscopic measure of the surface energy of the material-liquid interface. The contact angle changed from $55.7° ± 3.5°$ for uncoated TCPS to $41.7° ± 5.1°$ for **21**-coated TCPS, and $27.1° ± 4.8°$ for a coating of **22**. Likewise, the contact angle changed from $79.0° ± 3.6°$ for PS to $57.0° ± 4.0°$ for **21**-coated PS and $19.8° ± 3.0°$ for a coating of **22**. The decrease in the contact angle is consistent with hydrated PEGs at the surface. These data indicate that both **21** and **22** can coat both types of polystyrene surfaces and change their "wetabilities," with the slightly stronger binder (**22**) having a slightly larger effect.

The first cell attachment experiments were performed with human umbilical vein endothelial cells (HUVECs), because endothelial cells perform key roles in many of the tissues where implanted devices would be utilized such as the circulatory, pulmonary, renal, and digestive systems. We have performed experiments with other cell types, such as fibroblasts, and those data can be found in the Supplemental Data. A 0.1 mg/ml solution of **21** (21 μM) was prepared in phosphate-buffered saline (PBS) and added to the wells of a 96-well PS plate, along with a separate experiment of commercially available PEG 3400 in PBS (0.07 mg/ml; 21 μM) and a control of plain PBS. The coated plate was incubated at room temperature for 6 hr and the peptide solution was removed and the wells washed before cell seeding. Human endothelial cells (100 μl; $1.5 × 10^4$ cells per well) in medium

containing fetal bovine serum (FBS) were seeded onto either the plain PBS-treated wells (N = 9), off-the-shelf PEG 3400 wells (N = 9), or peptide-PEG amphiphile FFPSSWYSHLGVL-SSG-PEG (**21**)-coated wells (N = 9). After a 4 hr incubation period at 37°C, the wells were vigorously washed three times with excess PBS and then replenished with 100 μl of fresh medium and 20 μl of an MTS (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium) colorimetric proliferation assay solution (Promega). After a 2 hr incubation, the absorbance of the wells was read at 492 nm on a plate reader (Beckman Coulter AD 340C). The absorbance readings were converted to cell counts by using control wells of known cell counts run in parallel. The MTS assay showed the total cell number reduced from 4819 ± 668 for the PBS-treated wells to 216 ± 82 (p < $1 × 10^{-13}$) upon coating the PS with **21**. As expected, due to the PEG's inability to naturally coat PS (see Supplemental Data), the unbound PEG 3400 did not afford a reduction in cell attachment, 7535 ± 1720. Similar results were obtained using **21** with fibroblasts as well as with using amphiphile **22** with HUVECs (see Supplemental Data). Coating of the PS with the peptide alone without PEG, **20**, did not prevent cell attachment (see Supplemental Data). Experiments performed on TCPS plates show cellular attachment reductions on the order of a few fold, but unlike above, do not prevent all binding, likely due to the weakened peptide interaction with the TCPS surface. New phage display experiments are underway to select peptide sequences with an increased level of affinity for the TCPS surface to improve the binding of the amphiphile to this substrate.

The encouraging mammalian cell results prompted the evaluation of the cytophobic amphiphile as an antibacterial coating. We investigated whether amphiphile **21** would inhibit adhesion of *Staphylococcus aureus* on PS. All *S. aureus* strains used in this study are clinical isolates that form a classic biofilm complete with an exopolysaccharide matrix. *S. aureus* is a part of the normal flora but can also act as a pathogen by colonizing a variety of medical implants [49]. It is known that the first step in "slime" biofilm formation is bacterial cell attachment [50, 51]. Bacterial colonization of medical implants, known as biofilm formation, can be modeled in vitro by monitoring colonization of PS plates. Pathogenic strains

A

21   20   PBS

B
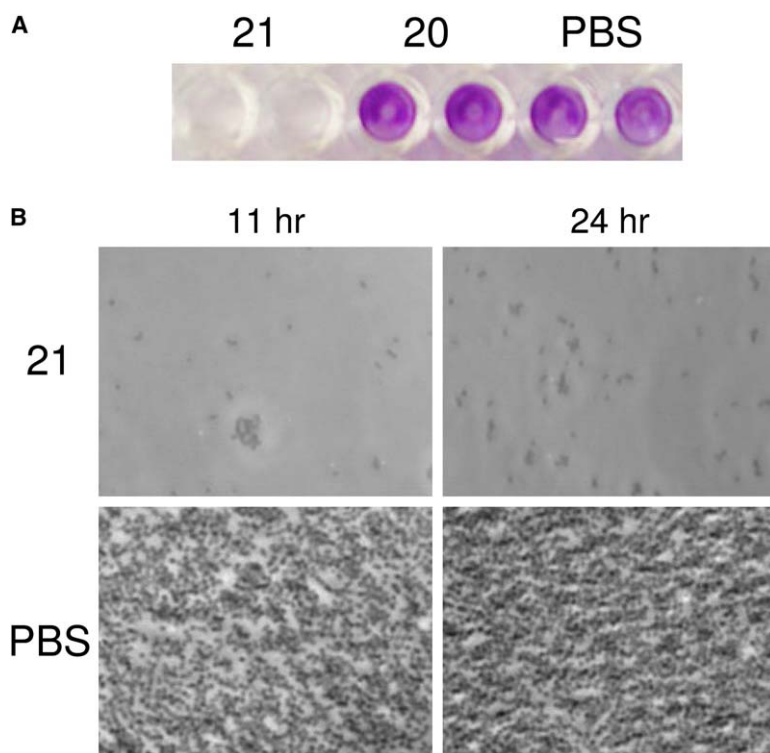
11 hr   24 hr

21

PBS

Figure 2. Prevention of Bacteria on Treated Surfaces

(A) Representative photographs of *S. aureus* seeded on treated PS surfaces. Bacterial attachment was assessed for wells treated with FFPSSWYSHLGVL-SSG-PEG (**21**), the control peptide FFPSSWYSHLGVL-SSG (**20**), and the untreated PBS-washed control. All samples were run in duplicate. The intensity of staining corresponds to the extent of bacterial colonization. Absorbance was measured on a plate reader and was 0.05 for **21**, 1.64 for **20**, and 1.07 for untreated PBS. (B) Top-down, phase-contrast micrographs of *S. aureus* seeded on PS surfaces treated with FFPSSWYSHLGVL-SSG-PEG (**21**) or PBS were assessed at 11 and 24 hr postinoculation.

of *S. aureus* (1×10^7 cells per well) were allowed to form a biofilm for 24 hr in PS plates coated with the amphiphile, **21**, the peptide alone, **20**, or the PBS untreated control. The formation of the biofilm was monitored by staining with the dye crystal violet (CV) as previously described [50, 51]. As shown in Figure 2A, treatment with amphiphile **21** significantly prevented colonization of the modified surface by *S. aureus* as evidenced by the reduced CV staining. The staining data were confirmed by direct microscopic inspection of the surface (Figure 2B). The gray areas are the PS surface and the dark areas are the *S. aureus* adhered to the PS. Similar results were observed with ten additional clinical isolates of *S. aureus* and two clinical isolates of coagulase-negative staphylococci (data not shown), suggesting that the effect observed may be generalized to other clinically relevant strains. The significant reduction in bacterial cell attachment within the first 24 hr is encouraging.

Significance

The design, synthesis, and evaluation of nonfouling peptide-PEG amphiphilic macromolecules are described. Application of this coating to a PS surface reduces both mammalian and bacterial cell adhesion in vitro. Both the PEG and PS binding peptide domains are required in the macromolecule, as neither the peptide nor the PEG alone inhibit cell binding when coated on the surface. The coating process is facile and requires only one relatively rapid incubation step to apply the amphiphile to a plastic surface. These cytophobic coatings are highly modular and adaptable, as the surface adhesion peptide can be interchanged with other unique adhesion peptides specific for a discrete surface. For example, we have also identified peptides, via phage display, with affinity for implant materials such as polycarbonate, nylon, stainless steel, and titanium [47, 52]. Studies are ongoing using both cytophobic and cytophilic coatings to direct cell attachment and cellular activity on native and nonnatural surfaces. Importantly, these interfacial biomaterials provide further motivation to design, evaluate, and optimize functional macromolecules for medical, biotechnology, and pharmaceutical applications.

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