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Detection of Nisin and Fibrinogen Adsorption on Poly(ethylene Oxide) Coated Polyurethane Surfaces by Time-of-Flight Secondary Ion Mass Spectrometry (TOF-SIMS)

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Abstract

Stable, pendant polyethylene oxide (PEO) layers were formed on medical-grade Pellethane® and Tygon® polyurethane surfaces, by adsorption and gamma-irradiation of PEO-polybutadiene-PEO triblock surfactants. Coated and uncoated polyurethanes were challenged individually or sequentially with nisin (a small polypeptide with antimicrobial activity) and/or fibrinogen, and then analyzed with time-of-flight secondary ion mass spectrometry (TOF-SIMS). Data reduction by robust principal components analysis (PCA) allowed detection of outliers, and distinguished adsorbed nisin and fibrinogen. Fibrinogen-contacted surfaces, with or without nisin, were very similar on uncoated polymer surfaces, consistent with nearly complete displacement or coverage of previously-adsorbed nisin by fibrinogen. In contrast, nisin-loaded PEO layers remained essentially unchanged upon challenge with fibrinogen, suggesting that the adsorbed nisin is stabilized within the pendant PEO layer, while the peptide-loaded PEO layer retains its ability to repel large proteins. Coatings of PEO loaded with therapeutic polypeptides on medical polymers have the potential to be used to produce anti-fouling and biofunctional surfaces for implantable or blood-contacting devices.

Keywords

Nisin; fibrinogen; TOF-SIMS; protein adsorption; medical polymers; PEO-polybutadiene-PEO triblock surfactants; robust PCA

Introduction

Nisin is a small (3.4 kDa) cationic and amphiphilic peptide produced by *Lactococcus lactis*. It is a member of the lantibiotic family, with strong antimicrobial activity against Gram-positive bacteria such as *Listeria monocytogenes* and the *Staphylococci*. Unlike traditional chemical antibiotics, nisin kills bacteria by physically opening pores in the cell membrane, substantially reducing the opportunity to give rise to resistant strains. The structure of nisin is distinguished by five intramolecular rings formed by thioether linkages from the

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uncommon amino acids lanthionine (Lan) and 3-methylanthionine (MeLan), and the presence of the post-translationally modified amino acids dihydroalanine (Dha) and dihydrobutyric acid (Dhb). Its potential application for anti-infective coatings on medical devices has led to considerable interest in its adsorption and function at material surfaces [1].

Proteins adsorb quickly and quasi-irreversibly on hydrophobic or charged surfaces [2,3]. Such surfaces can be coated with a variety of pendant polymers [4], typically polyethylene oxide (PEO), PEO-methacrylate [5,6], or “bottlebrush” graft co-polymers of poly(L-lysine) and PEO (PLL-g-PEO) [7,8]. These coatings render the surface hydrophilic, and impose a steric and entropic barrier against adsorption of proteins and cells. However, we recently described the adsorption of nisin to hydrophobic model surfaces that were coated with the poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) triblock surfactant Pluronic® F108 [9]. In particular, ellipsometric measurement indicated that nisin integrates into the PEO brush in multi-layer quantities. This integration of small proteins into a brush is predicted when the chain spacing is larger than the effective diameter of the protein [10,11,12]. The PEO layer “entraps” small peptides, and offers a substantial resistance to desorption and elution of the peptides. The degree of entrapment and rate of desorption are dependent on the protein dimensions relative to the PEO chain length, surface chain density and, for adsorption to charged surfaces such as metal oxides, the solution ionic strength [11,13].

PEO layers can be readily formed by adsorption of Pluronic® F108 or other triblock surfactants onto a variety of hydrophobic model surfaces, such as silica treated with trichlorovinylsilane. Gamma-irradiation of the surface under water causes the formation of surface-bound free radicals at the vinyl double bonds. These radicals attack neighboring atoms, and thus form covalent linkages between adsorbed polymers and the surface [14].

For surfaces that are not amenable to functionalization with vinyl groups, the radiation-activated double bonds can be moved to the adsorbed polymer by incorporating a polybutadiene centerblock into the triblock (PEO-PBD-PEO, Figure 1) [15]. The vinyl and backbone double bonds are susceptible to radical formation when irradiated in water, allowing the covalent attachment of adsorbed triblocks to underlying substrates. This is a particularly promising method for producing anti-fouling coatings on medical devices, as it does not require the use of potentially toxic cross-linking reagents, and can be applied to a wide variety of surfaces. Further, for devices that can be sterilized by gamma irradiation, a stable triblock-based PEO brush layer could be incorporated at little additional cost.

Model surfaces coated with PEO by γ -stabilization of triblocks have been shown to repel large proteins such as fibrinogen, a 340 kDa blood protein that is integral in platelet activation and the clotting cascade [14,16,17,18 19]. Although these and other studies have demonstrated substantial reduction in adsorption of fibrinogen and other proteins at triblock-coated surfaces, the protective effect was not absolute, and small amounts of protein remained detectable. Our previous work used ellipsometry [9], zeta potential, dye-labeled proteins and ELISA [17] on triblock-coated silica microspheres to demonstrate protein adsorption at PEO brush layers challenged with nisin and fibrinogen. In a related study, we used atomic force microscopy to examine the surface distribution of similar PEO-PBD-PEO triblocks on C₁₈-modified silicon wafers. Large (>10 nm) unprotected, triblock-free areas were evident even on these uniform surfaces [20; data not shown]. Additional layer defects would be expected due to the heterogeneous properties of the polyurethane surface.

However, it was not possible to determine unambiguously which of the two proteins were actually present on the surface. This is of particular interest for nisin-loaded brush layers, as

we speculate that the brush will stabilize the integrated nisin against competitive desorption, while providing protein-repellent functionality. The release rate of nisin could potentially be controlled by varying the properties (e.g. chain length and density) of the entrapping PEO layer [10,11,12]. Such applications potentially create new possibilities for drug release strategies from devices based on loading and storage of small therapeutic peptides or other biofunctional molecules in PEO layers.

Key questions left unanswered in our previous work are *a)* Do large blood proteins (e.g. fibrinogen) displace nisin from the brush layer; and *b)* Are negatively-charged fibrinogen molecules preferentially attracted to the cationic nisin trapped in the brush? Another important practical question is whether PEO-PBD-PEO triblocks can successfully produce functional coatings on real medical polymers. In order to approach these questions, we turned to time-of-flight secondary ion mass spectrometry (TOF-SIMS) to determine the chemical composition of medical-grade polymers coated with γ -stabilized PEO-PBD-PEO triblocks and challenged with nisin and fibrinogen.

Static TOF-SIMS is a sensitive and information-rich surface-analytical method that probes the top few nanometers of a sample [21,22]. Briefly, the sample is bombarded under ultra-high vacuum conditions with a stream of energetic ions, typically Ar^+ , Cs^+ , Ga^+ , or atomic clusters (e.g. Bi_3^+). The impact of these “primary” ions on the sample causes the partial decomposition and fragmentation of the molecules on the outer surface, in a manner analogous to a meteoric impact. The charged species in the ejected fragments (i.e. secondary ions) are analyzed by a time-of-flight mass spectrometer, which separates them on the ratio of their atomic mass to charge (m/z ratio). Because the secondary ions are composed of fragments of molecules at the very top surface, the surface chemical composition and even the orientation of biomolecules can be inferred from analysis of the mass spectra of the ejected species [23].

Despite considerable progress made in recent years, analysis of the large volume of data generated by the technique remains problematic, particularly for chemically complex samples such as proteins adsorbed on polymer-coated surfaces. Multivariate analysis techniques are often brought to bear to simplify the SIMS spectral data. One such technique is Principal Components Analysis (PCA), which finds orthogonal linear combinations of the variables that capture the maximum variance in the sample set. These principal components (PCs) thus describe most of the differences between each sample with only a few ordinals. Plots of sample data transformed by these PCs (i.e. scores) can be used to distinguish groupings and trends within the samples. [24,25,26,27,28]

The power of TOF-SIMS and PCA surface analysis of adsorbed protein films is demonstrated by a few examples from the literature. TOF-SIMS is extremely sensitive to adsorbed protein, with typical detection limits down to 0.1 ng/cm^2 for fibrinogen on mica. A strong correlation was also noted between the first principal component and adsorbed amount of protein, as determined by radiolabeling experiments [29]. Adsorption of proteins on Nb_2O_5 coated with PLL-*g*-PEO was analyzed with TOF-SIMS, and found to depend on chain density and length. Density-dependent integration of small proteins into the brush layer was also observed [7,8]. Multiple proteins adsorbed on silicon were differentiated by PCA of positive and negative spectra, based on differences in their relative amino acid composition [28,30]. Processing with a trained artificial neural network allowed robust classification of “unknown” proteins by analysis of the complete mass spectra [28]. Conformational changes and denaturation can also be inferred from changes in the exposure of hydrophobic amino acids at the surface [7,8,23].

Materials and Methods

Polymers and Reagents

Cylindrical extruded pellets of medical-grade Tygon® MPF-300, a proprietary polyether urethane (4 × 11 mm; Saint-Gobain, Valley Forge, PA), and Pellethane® 2363-80AE, a poly(tetramethylene glycol) urethane (5×10 mm; Scientific Commodities, Lake Havasu City, AZ) were a gift from Allvivo Vascular, Inc. (Lake Forest, CA). All aqueous solutions were made with HPLC-grade water and filtered (0.2 μm) to eliminate particulates immediately prior to use. All other reagents and solvents were of ACS reagent grade or better, and used as received.

Hydroxyl-terminated PEO-PBD-PEO triblock surfactants (Figure 1) were purchased from the University of Minnesota Polymer Synthesis Facility (Minneapolis, MN), stored desiccated at −20°C under argon, and used without further purification. According to the manufacturer, the triblocks have polybutadiene centerblocks ($M_n = 620$) with 73% vinyl side-groups (i.e. 1,2-addition product), and PEO side-chains of $M_n = 2,845$. The polydispersity index of the polymer (by size-exclusion chromatography) was approximately 1.11.

A commercial-grade purified preparation of nisin (3.4 kDa) was obtained from Prime Pharma (Gordons Bay, South Africa), and was determined to be substantially free of protein contaminants by MALDI-MS (data not shown). Plasminogen-free human fibrinogen (340 kDa) was purchased from Sigma-Aldrich (St. Louis, MO), and used without further purification.

Coating of Polymer Surfaces with PEO-PBD-PEO Triblocks

Thin (~1 mm) disks were cut from pellets of Tygon® and Pellethane®, and at the manufacturer's suggestion, washed to remove mold release, plasticizers and other soluble impurities. The disks were incubated with rotation for 24 hours in 20 mL of isopropyl alcohol (IPA). The disks were then rinsed with IPA, and the process repeated twice. The polymer samples did not swell to any appreciable degree during this treatment. The washed polymer disks were then dried at 35°C under vacuum for 2 h to remove residual solvent, and used without further characterization.

A 1% solution of PEO-PBD-PEO triblocks in water was prepared and filtered (0.2 μm) immediately before use, to guard against the presence of microbes or particulate matter. The washed and dried polymer disks were individually placed in 600 μL polypropylene microcentrifuge tubes, and covered with either triblock solution or water. The polymer disks were incubated for four hours at room temperature (23 °C) to allow self-assembly of the triblocks on the polymer surface. The bare or triblock-coated polyurethane disks were then irradiated by a ⁶⁰Co source over 8 days to a total dose of 80 kGy.

After irradiation, the polymer disks were transferred to clean 1.5 mL microcentrifuge tubes filled with water, and rinsed 4× with water to remove any loosely-bound triblocks. Care was taken at all times to avoid touching the flat surfaces of the polymer disks; the curved sides and conical bottoms of the microcentrifuge tubes kept the disks from resting flat against the tube surfaces.

Individual and Sequential Protein Adsorption

Nisin and fibrinogen were individually dissolved in phosphate-buffered saline (10 mM sodium phosphate with 150 mM NaCl; PBS). Because nisin is poorly soluble at neutral pH, the nisin was first dissolved in monobasic sodium phosphate (with 150 mM NaCl), then five

volumes of dibasic sodium phosphate/NaCl was added to raise the pH to 7.4, giving a final concentration of 0.5 mg/mL [9]. Fibrinogen solutions (1.0 mg/mL) were made in PBS at pH 7.4, with gentle shaking for four hours at 37°C to fully dissolve the protein [17]. All solutions appeared optically clear, but were filtered (0.2 µm) immediately prior to use to eliminate the possibility of any microorganisms or undissolved particulate matter.

Triblock-coated and control polymer disks were transferred to 0.6 mL microcentrifuge tubes, and covered with either PBS or freshly-prepared nisin solution. After incubation for four hours at 23°C, the disks were rinsed with PBS, transferred to new tubes, and rinsed 4× with PBS to remove loosely-bound nisin. The washed disks were then covered with either buffer or fibrinogen solution, and the above procedure was repeated. After the final PBS rinse, the disks were rinsed with water to remove the buffer salts. The polymer disks were then transferred to individual wells of a 24-well polystyrene plate, loosely covered, and dried under vacuum at ambient temperature overnight. All samples were prepared and analyzed in triplicate.

Time-of-Flight Secondary Ion Mass Spectrometry (TOF-SIMS)

Positive SIMS mass spectra were acquired for each sample using a TOF-SIMS IV instrument (Ion-TOF GmbH, Germany) equipped with a reflectron analyzer, a bismuth ion gun (25 keV, 10kHz) and a pulsed electron flood source for charge neutralization. The primary ion beam was comprised of Bi_3^+ at an ion current of 0.4 pA. The primary ion dose density was kept below 2×10^{12} ions/cm² to ensure that the static limit was not exceeded. At least five positive mass spectra were acquired from randomly chosen 100 µm × 100 µm areas on each sample, with a cycle time of 100 µs. The resolution of the $\text{C}_4\text{H}_8\text{N}^+$ peak at 70 Da was at least 5000 for each spectrum, and the spectra were calibrated to <10 ppm using three or more $\text{C}_n\text{H}_{2n-1}$ peaks (n from 2 to 5). Individual corrected intensities for peaks from 0 to 200 m/z (Table 1) corresponding to fragments from amino acids [28,29] and/or the underlying polyurethane substrates were extracted using the manufacturer's software (IonSpec v4.1). Considerable interference from the underlying polymer and triblock coating was evident in the spectra. Where possible, amino acid shoulder peaks were resolved from polymer background peaks by manual linear background removal; otherwise, the corrected intensity of the entire peak composite was used. Negative ion spectra were not collected, as they provide little peak information for most amino acids [21].

Principal Components Analysis

Principal components analysis [31] (PCA) of the corrected peak intensities was conducted using MATLAB v2009 (Mathworks, Inc., Natick, MA). Because of the chemical complexity of the surface being analyzed, and the use of manual discrimination of amino acid peaks from unrelated peaks, considerable variability was evident in the completed dataset. While commonly used for TOF-SIMS data (e.g. [29]), classical PCA techniques are very sensitive to outliers and atypical observations [31,32]. Thus, the datasets were analyzed using the robust PCA routines from the Library for Robust Analysis (LIBRA, 20-Oct-2009 version; [33]). The robustness parameter, α , was 0.80 (i.e. up to 20% of outliers resisted). Each spectrum was normalized to the sum of the intensities of the selected peaks and mean-centered prior to analysis [7,28]. Four or five principal components (PCs) were retained for each data set, depending on the position of the first knee in a cross-validated robust Predictive Residual Error Sum of Squares (PRESS) plot [34].

Results and Discussion

Polymer Coating and Protein Challenge

Thin disks of medical-grade Pellethane® and Tygon® were washed exhaustively with IPA to remove low-molecular weight impurities. Although both bulk polymers are classified as “medical grade” with very low extractables, processing agents such as ethylene-*bis*-stearamide are often present at the polymer surface. These surface contaminants can be removed by treatment with alcohols [35,36]. The alcohol-extracted polymer disks were used without further characterization.

The polymer samples were then coated with a PEO brush layer by incubation with an aqueous solution of PEO-PBD-PEO triblocks in water. The triblocks form a brush layer by adsorption of the hydrophobic polybutadiene centerblock, which is rich in vinylic and in-chain double bonds, to the hydrophobic polymer surface. Various coating conditions are reported in the literature. Previous workers [14,15,17,18,15,39] have immobilized PEO-PPO-PEO or PEO-PBD-PEO triblocks by adsorption from 0.1 to 10 mg/mL solutions for one to twelve hours, prior to covalent attachment to the surfaces through γ - or UV-irradiation.

It is well known that surface rearrangement occurs to various degrees in some polymers upon contact with water. This is especially true of polyurethanes, for which the amount of rearrangement is strongly dependent on the hard/soft block composition of the polymer [36,37,38]. We attempted to minimize inter-sample variation by incubation in buffer or aqueous triblock solution for several hours to allow the surface to relax, after which the adsorbed brush was stabilized by exposure to gamma radiation.

The vinyl groups (and to a lesser extent, the in-chain double bonds) in PBD are susceptible to formation of free radicals upon irradiation in water [15]. Radiolysis of water produces a variety of active radical species, including hydrogen, hydroxyl, and peroxy radicals that are transferred to the polyurethane and triblocks. These polymer-bound radicals attack neighboring groups on the triblocks and solid polyurethane surface, forming covalent linkages between the substrate and triblocks. Recombination of HO· radicals produces hydrogen peroxide, and polymer-bound hydroperoxides may also be formed. Cross-linking reactions between triblocks are also possible, further stabilizing the coating [15,39,40,41].

Pellethane® and Tygon® samples coated with triblocks prior to irradiation were designated as PTG and TTG, respectively; control samples irradiated in water (no triblocks) were designated PWG and TWG. According to manufacturer data, both polyurethanes may be sterilized by γ irradiation without damage. The coated and uncoated polymer disks were then challenged individually or sequentially with nisin and/or fibrinogen (N, F or NF). Protein adsorption times of four hours were consistent with our previous work [17].

TOF-SIMS Data Reduction

TOF-SIMS has been used to analyze protein adsorption on various model surfaces, including glass [21], silicon wafers [28], PTFE and mica [29,42], gold-alkanethiol monolayers [43], and PEO-*g*-PLL-coated niobium oxide [7,8]. In the current study, we attempted to analyze protein adsorption and displacement at PEO brushes formed by the radiation-induced covalent binding of PEO-PBD-PEO triblock copolymers to medical-grade polyurethane surfaces.

Analysis of the protein-PEO-polymer surface is confounded by many large signals from the underlying polymer substrate and the PEO coating, which create a multitude of fragments with masses similar to amino acid fragments. Representative spectra from 0–200 m/z and

tentative peak assignments are presented for each of the different surface treatments on Pellethane® (Figure 2) and Tygon® (Figure 3). The chemical complexity of the underlying polymer substrates is immediately obvious from the SIMS spectra. While the different spectra appear more-or-less identical at first glance, there are subtle changes in the relative intensity of the peaks between treatments and polymer substrates. The overall effect of such changes (rather than absolute peak areas) was expected to yield information about the surface composition following immobilization of PEO and adsorption of proteins to the polymers.

Some difficulty was encountered in obtaining the spectra, and considerable variation was noted in peak intensities between the individual spectra taken at different points on the sample surface, and between replicates of the same treatment. This was particularly evident in the comparatively noisy Tygon® samples, leading to the decision to use robust multivariate analysis to reduce the effect of this variation. In some cases, individual peaks were enhanced or attenuated, occasionally even nearly disappearing. For example, the substrate peak assigned $C_8H_9N_3$ is comparatively small in the PTGN and PWGNF representative Pellethane® spectra shown in Figure 2, while the $C_8H_9N_3$ peak is clearly present in other spectra from the same samples (not shown). These variations between measurement spots and samples may be related to differential charging, surface roughness, or other matrix effects, and warrant further investigation.

Polyurethane samples irradiated in water (PWG/TWG) exhibit a large number of C_mH_nO polyether peaks, as well as several nitrogen-containing species including CH_4N , C_2H_6N , and C_7H_8N . Following exposure to protein, the overall complexity of the SIMS spectra increased dramatically, and the relative intensity of nitrogen-containing species and other ions associated with amino acids generally increased. This is consistent with the adsorption of chemically complex protein molecules at the surface of the bare or PEO-coated polyurethanes.

Substrates irradiated in the presence of PEO-PBD-PEO triblocks (PTG/TTG) show increased hydrocarbon and polyether signals (particularly C_2H_5O), indicating the presence of immobilized PEO on the surface. The apparent intensity increase of the polyurethane C_7H_8N peak (m/z 106.066) was tentatively attributed to a diethylene glycol fragment or oxidized species ($C_4H_{10}O_3$, m/z 106.063) from the immobilized PEO chains. The total mass density of the PEO chains on the surface was not determined; they may also have been shortened by a chain scission mechanism during irradiation [41]. However, the ability of triblock-modified surfaces to repel large proteins is observed to be largely independent of the PEO chain length, with only a few repeat units required to effect repulsion [14,15].

In one typical study, Wagner excluded the two amino acid peaks that were overlapped by the underlying PTFE substrate [29]. In the present study, however, practically all of the peaks associated with amino acids were overlapped to some degree by signals from the underlying polyurethanes. These interferences are due to polyether, aromatic and nitrogen-containing groups in the polymer that are similar in structure to the amino acid side-chains. The substrate-derived signals were substantial in most cases, and it was usually impossible to completely separate the contribution from the substrate and amino acids. Figure 4 shows representative examples of interference by the underlying irradiated Pellethane® to the peaks associated with amino acid signals from adsorbed fibrinogen. Similar patterns of interference were observed for proteins adsorbed on Tygon® substrates (data not shown).

Interferences on the amino acid peaks from the underlying polyurethane were minimized by manually adjusting the peak integration limits or using linear background removal (e.g. Figure 4a,b). However, in many cases it was not possible to discriminate between signals

from amino acids and the polymer substrate (e.g. Figure 4c,d). In these cases, the intensity of the entire aggregate peak was used. These techniques were applied consistently to each sample, under the assumption that PCA would primarily resolve the relative variations in peak intensities caused by protein adsorption against a relatively consistent “fingerprint” from the underlying polymer.

Ideally, individual peaks corresponding to the unusual Lan, MeLan, Dha and Dhb amino acid residues in nisin [1] could be used to unambiguously distinguish it from fibrinogen on the surface. Unfortunately, in comparing spectra from films of the stock protein solutions, we were unable to identify well-separated peaks specifically attributable to fragments of these unusual amino acids (data not shown).

The observed changes in the SIMS spectra are consistent with protein adsorption on the triblock-coated and bare surfaces. They are also consistent with expectations based on our previous studies with nisin and/or fibrinogen adsorption at uncoated and triblock-coated surfaces, using *in situ* ellipsometry [9], zeta potential detection and ELISA [17], and measurements of antimicrobial activity retained by nisin at uncoated and triblock-coated polyurethane catheter segments and polystyrene [44].

Principal Components Analysis (PCA)

Initial attempts to use classical PCA produced somewhat scattered distributions of similar samples in the score plots (not shown), implying the presence of substantial amounts of noise in the data. To minimize the effects of the interferences, the ROBPCA robust principal components functions from the LIBRA package [33,45] were instead used to analyze the peak intensity data. The robust PCA algorithm implements a projection pursuit method that identifies two types of outliers, data points that lie in the PCA subspace but have a large score distance (SD outliers or good leverage points), and data points orthogonal to the PCA subspace (OD outliers). Classical PCA is very sensitive to orthogonal outliers because it tries to minimize all orthogonal distances. The LIBRA robust PCA algorithm reduces the influence of these outliers, providing a better overall fit to the data [33]. Error ellipses

representing the 97.5% confidence intervals (cut-off value of $\sqrt{\chi^2_{0.975}}$) were calculated for each sample group [31], before and after elimination of the orthogonal outliers identified by the robust PCA algorithm.

Protein Adsorption on Pellethane®

Figure 5 shows the score plots of the first two robust PCs for nisin and fibrinogen, adsorbed individually or sequentially on Pellethane® medical-grade polyurethane. The polymer was irradiated in water (PWG) prior to protein adsorption. The gray ellipse represents a 97.5% confidence interval computed for the complete dataset. The confidence ellipses were also computed (black), after eliminating the orthogonal outliers (OD outs, marked with an ‘X’) that were identified by the robust PCA algorithm [45]. No good leverage points (SD outliers) were identified in this dataset. All PC scores are normalized, so their magnitude does not directly reflect the total mass of protein at the surface. Later PCs did not provide any obvious additional information about any of the sample groups.

The first robust PC accounts for the majority of variance (84%) between the samples, and as expected, it clearly differentiates the protein-free irradiated polymer from the protein-challenged polymers. The major contribution to PC 1 (in this and all other cases) was $C_5H_7O^+$, an abundant fragment from the underlying polymers, but also associated with the amino acid valine (Table 1). Robust PC 2 (16%) further differentiated between the nisin- and fibrinogen-contacted bare Pellethane® surfaces. The PC 2 loadings were dominated by

$C_2H_6NO^+$ (Ser) and $C_3H_8NO^+$ (Thr), which loaded negatively. The major positive loadings were the $C_5H_8N_3^+/C_4H_5H_2^+$ ions, both associated with histidine. These and following PCA loadings are consistent with the composition of the tested proteins, as nisin contains only one each of serine, histidine and asparagine, has several Ala/Gly residues, and does not include any of the aromatic amino acids [1]. In contrast, fibrinogen has a substantial number of aromatic (Phe, Tyr, Trp), His, Ser and Asn residues [46]. However, because of substantial interference from the underlying polyurethane, these assignments cannot be unambiguously made to protein-derived fragments.

As anticipated, protein adsorption was evident on the uncoated hydrophobic polymer surfaces, with well-defined clusters corresponding to the nisin (PWGN) and fibrinogen-contacted samples (PWGF and PWGNF). The error ellipses of the samples contacted with fibrinogen, or with nisin followed by fibrinogen, are very close. This indicates that the surface chemistry is very similar between these samples, and is consistent with substantial coverage or displacement of the adsorbed nisin by the larger fibrinogen molecules (340 kDa vs. 3.4 kDa). Electrostatic interactions between the pre-adsorbed cationic nisin and the negatively charged fibrinogen could help to stabilize a fibrinogen overlayer, in a manner similar to layer-by-layer assembly of charged polymers [47]. The slight difference in the means of the two fibrinogen-contacted groups may be due to small amounts of adsorbed nisin remaining “visible” between the larger protein molecules.

Protein Adsorption on Triblock-Coated Pellethane®

Irradiation of Pellethane® in the presence of adsorbed PEO-PBD-PEO triblocks produces a stable coating of pendant PEO chains (see above). The score plot for PEO-coated Pellethane® with and without protein adsorption is shown in Figure 6. Some noise is present in the data, leading to large confidence intervals (gray ellipses). The robust PCA algorithm identified several good leverage points (SD outs) and orthogonal outliers (OD outs), and recalculation of the ellipses after excluding these points results in tighter 97.5% confidence intervals centered around the majority of data points.

The presence of triblocks (PTG) did not completely prevent the adsorption of either nisin or fibrinogen at the surface, as evidenced by the appearance of peaks corresponding to amino acids (see above). As before, the first PC clearly distinguishes between the polymer/triblock substrate with and without protein, while the second PC separates the nisin and fibrinogen-contacted surfaces. The major positive variables of PC 2 were $C_2H_6N^+$ (Ala) and $C_5H_8N_3^+$ (His), while $C_3H_7N_2O^+$ (Asn) and $C_8H_{10}N^+$ (Phe) were the most important negative variables.

Previous results from our laboratory [9,17] suggest that the inter-chain spacing formed by adsorbed triblocks is sufficiently large to allow integration of nisin peptides into the brush. Numerous studies indicate that large proteins (e.g. fibrinogen or fibrinectin) will measurably adsorb to PEO-coated surfaces, although the total adsorbed amount of protein is typically substantially lower than at the bare surfaces [3,8,9,14,16,17,15,29]. It is believed that, while the brush itself maintains its protein-repellent nature, the large proteins simply adsorb at “bare patches” in the brush layer. Such defects are thought to be unavoidable when the brush is formed by chains that do not easily rearrange or promote close, regular surface packing [10,11,12].

The brush chain spacing (and hence, protein rejection and peptide release properties) can be modulated by changing the coating conditions (temperature, concentration, ionic strength, etc.). For example, Kingshott, et al. formed high-density protein-repellent methoxy-PEO layers by grafting from a K_2SO_4 solution at the lower consolute solubility temperature (LCST). Under these conditions the polymers are only marginally soluble and begin to

aggregate [48,49], increasing the immobilized layer density. Extremely dense grafted PEO layers with excellent repulsion of proteins and DNA may also be prepared by grafting of PEO from a pure melt [50,51]. Clearly, modifications to the triblock coating process used in this work could improve the protein rejection and discrimination properties of the PEO layer. Such optimizations, however, are outside of the scope of this work.

In contrast to the bare Pellethane® in Figure 5, the protein adsorption patterns are reversed in the presence of triblocks (Figure 6). For Pellethane® coated with PEO triblocks, the 97.5% confidence interval ellipse of the fibrinogen-only cluster is distinguished from those of the nisin-contacted samples. Importantly, the samples contacted with nisin only (PTGN) are statistically identical to those challenged by fibrinogen (PTGNF). This result suggests that a nisin-loaded PEO brush layer retains its ability to repel large blood proteins such as fibrinogen. In addition, a challenge with fibrinogen does not appear to significantly alter the chemistry of the surface nisin/PEO brush. This implies that nisin is not substantially desorbed from the brush by contact with fibrinogen in the solution. Preliminary results from a separate study in our laboratory, using zeta potential and γ -stabilized triblock-coated microspheres, indicate that the elution of entrapped nisin from the microspheres upon repeated challenges with fibrinogen is not significantly different ($p < 0.05$) from that recorded with protein-free buffer (data not shown).

Protein Adsorption on Tygon®

Figure 7 shows robust PC score plots for nisin and/or fibrinogen adsorbed on Tygon® after gamma-irradiation in water (TWG). The 97.5% confidence ellipses before (gray) and after (black) elimination of orthogonal outliers are also shown. Substantially more noise appears in these samples, which may be partially due to difficulties with TOF-SIMS data acquisition. The cut surface of the Tygon® (and, to a much lesser extent, the Pellethane®) was rough on a sub-millimeter scale, and the secondary ion signal intensity varied substantially with location on the sample. In some cases, the ion yield was so low as to completely prevent acquisition of spectra, for reasons that remain somewhat unclear but may be partially related to differential charging of the rough polymer surface. Additionally, anomalous peaks from Cs^+ (m/z 132.906) were present to varying degree in most of the Tygon® spectra. We attribute these to contamination of the extractor cone resulting from an unrelated Cs-sputtered depth-profiling experiment performed immediately prior to analysis of the Tygon® samples, and note that they do not interfere with any peaks of interest in the present experiment.

Noise in the acquired data might be caused by patchy or incomplete PEO coverage of the surface, possibly related to hydrophilic domains exposed by surface rearrangement. Triblock adsorption is primarily due to hydrophobic association between the PBD centerblock and the substrate, and would be expected to be less favorable at such hydrophilic regions. It is also possible that processing aids and surface contaminants were incompletely removed, or were replenished by migration from the bulk. Despite the increased noise in the Tygon® spectra, however, the overall trends remain visible and consistent with the Pellethane® samples.

As with the bare Pellethane® samples (Figure 5), the Tygon® that was irradiated in water exhibited adsorption of both nisin (TWGN) and fibrinogen (TWGF), as evidenced in the spectra in Figure 3. Again, PC 1 (79%) distinguishes between the bare irradiated polymer and the protein-contacted samples, while PC 2 (18%) differentiates adsorbed nisin and fibrinogen. The samples exposed only to fibrinogen are statistically indistinguishable from those with nisin followed by fibrinogen. This is consistent with complete displacement or coverage of pre-adsorbed nisin from the Tygon® surface by contact with fibrinogen. The major variables contributing to PC 2 were $\text{C}_2\text{H}_6\text{NO}^+$ (Ser) and $\text{C}_3\text{H}_8\text{NO}^+$ (Thr), which loaded positively, and negative contributions from CH_4N^+ (Gly) and $\text{C}_5\text{H}_8\text{N}_3^+$ (His).

Activated groups such as hydroperoxides may be formed on polymers during irradiation [39], potentially forming covalent linkages between adsorbed protein and the polymer. However, nisin was substantially displaced by fibrinogen on uncoated, irradiated Pellethane® and Tygon®, indicating that such immobilization reactions are negligible in the current system. As the hydrated polyurethane surface is enriched with PEO-like soft segments but does not appear to covalently bind nisin, protein grafting onto the pendant PEO chains is also probably unimportant.

Protein Adsorption on Triblock-Coated Tygon®

For Tygon® surfaces coated with γ -stabilized PEO triblocks (Figure 8), the 97.5% confidence interval error ellipses for the protein-free polymer (TTG) and fibrinogen-contacted (TTGF) samples overlap in both principal components, although the variation of TTGF is larger. This suggests that, while fibrinogen is theoretically repelled by a PEO brush layer, the surface is not well protected from adsorbing fibrinogen. As mentioned previously, this may be attributable to adsorption at bare spots [10,11,12] or low overall PEO chain density (poor surface coating). The separation of nisin-contacted surfaces from bare and fibrinogen-only samples along PC 1 is not inconsistent with adsorption of nisin at the surface, although this is not immediately evident from the spectral data (Figure 3). Inconsistent final rinsing with water may also have been a factor, as suggested by the relatively large Na^+ peaks (22.989 m/z) in some of these spectra. Similarly, while the clusters for nisin-contacted samples (with and without fibrinogen challenge, TTGNF and TTGN) do overlap, the correlation is much poorer than for the other experiments. The PC 2 contributors for protein adsorbed on Tygon® with immobilized triblocks were dominated positively by $\text{C}_2\text{H}_6\text{NO}^+$ (Ser) and $\text{C}_4\text{H}_{10}\text{N}^+$ (Val), and negatively by $\text{C}_5\text{H}_8\text{N}_3^+$ (His) and $\text{C}_5\text{H}_7\text{O}^+$ (Thr and the underlying polyurethane). Because of the noise in these samples (compare Figure 5), however, it is difficult to make any definitive claims about the type or amount of protein on Tygon® surfaces.

The large error ellipses associated the Tygon® samples may be diagnostic of difficulty in achieving a uniformly dense triblock layer on the polymer surface, possibly due to residual surface contaminants, or segregation of hard/soft domains to produce hydrophilic patches [36]. Poor coatings could allow fibrinogen to adsorb into brush layer defects, displacing some of the unprotected nisin already adsorbed to the surface. Further testing and optimization of the triblock coating method is clearly indicated for devices based on Tygon® or related polyurethanes.

The PCA clustering trends are also in agreement with our previous studies [9,17], and indicate that while nisin is readily displaced on bare surfaces, it is integrated into and protected to some extent by a pendant PEO layer. We speculate that the pendant PEO stabilizes the adsorbed nisin, while retaining its ability to minimize adsorption of large proteins such as fibrinogen. We further speculate that any nisin that is initially lost upon contact with fibrinogen resides in the outermost region of the brush. This produces a surface coating that is, in effect, a short yet protein-repellent brush extending beyond a stabilized layer of nisin peptides (Figure 9). Although our previous results [17] are consistent with this hypothesis, further work is required to validate this proposed mechanism of partial nisin displacement and formation of a thin, protein-repellent PEO layer.

Conclusions

We have demonstrated successful modification of medical grade Pellethane® and Tygon® polymer surfaces by radiation-induced stabilization of adsorbed PEO-PBD-PEO triblocks. Difficulties were encountered in collecting TOF-SIMS spectra from the Tygon® samples, attributed to surface roughness and charging effects. The considerable variation in the

Tygon® spectra could also be indicative of incomplete or patchy surface modification. Because of these difficulties, a projection pursuit implementation of robust PCA was used to analyze the spectral data and to detect outliers. This analysis was able to distinguish the underlying polyurethane substrate from one with adsorbed proteins (PC 1), particularly on the Pellethane® samples. Further discrimination between adsorbed nisin and fibrinogen was possible, using differences in the relative yield of fragments associated with amino acids and the underlying substrate (PC 2). On bare polyurethane substrates, adsorbed nisin was almost completely displaced or covered by fibrinogen within a few hours. Nisin and fibrinogen also adsorbed to polyurethane coated with γ stabilized PEO-PBD-PEO triblocks. However, unlike the bare surface, the nisin was not substantially displaced when challenged with fibrinogen. Importantly, the nisin-loaded brush layer appeared to prevent the adsorption of fibrinogen on the coated surface, possibly because of short PEO chains projecting above the nisin layer.

The combination of protein repulsion and retention of small peptides or other therapeutic molecules by a readily adaptable, simple coating for implantable medical devices appears very promising. However, the efficacy of the coating will hinge on the retention of activity of the entrapped nisin or other peptides. Analytical studies of the adsorption, clustering, and desorption kinetics of nisin and similarly sized peptides at triblock-coated layers, along with the effects of sequential addition of fibrinogen, are currently under way in our laboratory. We are also evaluating the retention of antimicrobial activity of nisin-loaded PEO layers against *Staphylococcus epidermidis*. These studies will contribute to the subject of future reports.

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Figure 1. Approximate chemical structure of PEO-PBD-PEO triblock copolymer surfactant used to produce PEO brush layers. The structure is drawn for clarity; the PBD centerblock is actually a random copolymer of 1,2- and 1,4- subunits.

Figure 2. Representative positive ToF static SIMS spectra of Pellethane® polyurethane irradiated in water (PWG) or with PEO-PBD-PEO triblocks (PTG), then with individually or sequentially adsorbed nisin (N) or fibrinogen (F).

Figure 3. Representative positive ToF static SIMS spectra of Tygon® polyurethane irradiated in water (TWG) or with PEO-PBD-PEO triblocks (TTG), then with individually or sequentially adsorbed nisin (N) or fibrinogen (F).

Figure 4.

Representative interference of species derived from bare Pellethane® (PWG) and amino acid signals from adsorbed fibrinogen (PWGF). The gray areas illustrate typical integration boundaries and linear background removal used for each type of interference.

Figure 5.

Normalized robust PCs 1 and 2 for nisin and fibrinogen adsorbed individually (N, F) or sequentially (NF) on Pellethane® irradiated in water. 97.5% confidence ellipses for the entire dataset (gray) and after elimination of orthogonal outliers (black) are also shown.

Figure 6. Normalized robust PCs 1 and 2 for nisin and fibrinogen adsorbed individually (N, F) or sequentially (NF) on Pellethane® polyurethane coated with γ -stabilized PEO-PBD-PEO triblocks.

Figure 7. Normalized robust PCs 1 and 2 for nisin and fibrinogen adsorbed individually (N, F) or sequentially (NF) on Tygon® irradiated in water (TWG).

Figure 8. Normalized robust PCs 1 and 2 for nisin and fibrinogen adsorbed individually (N, F) or sequentially (NF) on Tygon® polyurethane coated with γ -stabilized PEO-PBD-PEO triblocks.

Figure 9. Speculated effect of integration and subsequent fibrinogen challenge of nisin in a PEO layer. Some nisin is eluted from the outer region of the brush, exposing the mobile PEO segments, which then reclaim their steric repulsive character.

Table 1

Positive ions characteristic of amino acids selected for analysis by robust PCA (based on [28]), and interfering ions from underlying polyurethane substrates.

<i>m/z</i>	Fragment	Peak Assignment [†]	<i>m/z</i>	Fragment	Peak Assignment [†]
18.03	NH ₄ ⁺	All	98.02	C ₄ H ₄ NO ₂ ⁺	Asn
30.03	CH ₄ N ⁺	Gly (C ₂ H ₆)	100.09	C ₄ H ₁₀ N ₃ ⁺	Arg (C ₆ H ₁₂ O)
44.04	C ₂ H ₆ N ⁺	Ala	101.10	C ₄ H ₁₁ N ₃ ⁺	Arg (C ₆ H ₁₃ O)
60.04	C ₂ H ₆ NO ⁺	Ser (C ₃ H ₈ O)	102.06	C ₄ H ₈ NO ₂ ⁺	Glu/Gln (C ₃ H ₆ N ₂ O ₂)
61.01	C ₂ H ₅ S ⁺	Met (C ₂ H ₅ O ₂)	107.05	C ₇ H ₇ O ⁺	Tyr (C ₇ H ₉ N)
68.05	C ₄ H ₆ N ⁺	Pro (C ₃ H ₈)	110.07	C ₅ H ₈ N ₃ ⁺	His
72.08	C ₄ H ₁₀ N ⁺	Val (C ₄ H ₈ O)	112.09	C ₅ H ₁₀ N ₃ ⁺	Arg (C ₅ H ₁₀ N ₃ /C ₅ H ₆ NO ₂)
74.06	C ₃ H ₈ NO ⁺	Thr (C ₄ H ₁₀ O)	120.08	C ₈ H ₁₀ N ⁺	Phe (C ₈ H ₁₀ N/C ₇ H ₆ NO)
76.02	C ₂ H ₆ SN ⁺	Cys (C ₆ H ₄)	127.10	C ₅ H ₁₁ N ₄ ⁺	Arg (C ₇ H ₁₅ N ₂)
81.05	C ₄ H ₅ N ₂ ⁺	His (C ₃ H ₅ O)	130.07	C ₉ H ₈ N ⁺	Trp (C ₉ H ₈ N)
82.05	C ₄ H ₆ N ₂ ⁺	His (C ₃ H ₆ O)	159.09	C ₁₀ H ₁₁ N ₂ ⁺	Trp (C ₁₁ H ₁₁ O)
83.05	C ₅ H ₇ O ⁺	Val (C ₃ H ₇ O)	170.06	C ₁₁ H ₈ NO ⁺	Trp (C ₈ H ₁₀ O ₄ /C ₉ H ₁₄ O ₃)
87.06	C ₃ H ₇ N ₂ O ⁺	Asn (C ₄ H ₇ O ₂ /C ₅ H ₁₁ O)			

[†] Peaks in parentheses are tentative assignments of major overlapping peak(s) from the polyurethane substrate.