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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.

1. 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 uncommon amino acids lanthionine (Lan) and 3-methyllanthionine (MeLan), and the presence of the post-translationally modified amino acids didehydroalanine (Dha) and didehydrobutyric 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 copolymers 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 [10–12]. 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 vinylic 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

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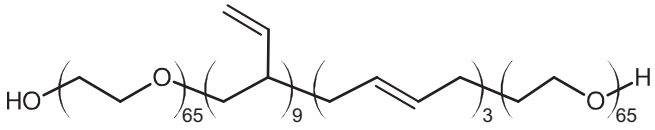

Fig. 1. Approximate chemical structure of PEO–PBD–PEO triblock copolymer surfactant used to produce PEO brush layers. The structure is drawn for clarity; the PBD centerblock is actually a random copolymer of 1,2- and 1,4-subunits.

centerblock into the triblock (PEO–PBD–PEO, Fig. 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 [14,16–19]. 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 C_{18}-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 [10–12]. 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 [24–28].

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 [26]. 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].

2. Materials and methods

2.1. 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 (Fig. 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 = 2845. 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