A more quantitative understanding of peptide loading and release from polyethylene oxide (PEO) brush layers will provide direction for development of new strategies for drug storage and delivery. The antimicrobial peptide nisin shows potent activity against Gram-positive bacteria including the most prevalent implant-associated pathogens, its mechanism of action minimizes the opportunity for the rise of resistant bacteria and it does not appear to be toxic to
humans, suggesting good potential for its use in antibacterial coatings for selected medical devices. In this work, optical waveguide lightmode spectroscopy was used to record changes in adsorbed mass during cyclic adsorption-elution experiments with nisin, at uncoated and PEO-coated surfaces. PEO layers were prepared by radiolytic grafting of Pluronic® surfactant F108 or F68 to silanized silica surfaces, producing long- or short-chain PEO layers, respectively. Kinetic patterns were interpreted with reference to a model accounting for history-dependent adsorption, in order to evaluate rate constants for nisin adsorption and desorption, as well as the effect of pendant PEO on the lateral clustering behavior of nisin. Lateral rearrangement and clustering of adsorbed nisin was apparent on uncoated and F68-coated surfaces, but not on F108-coated surfaces. In addition, nisin showed greater resistance to elution by peptide-free buffer from uncoated and F68-coated surfaces. These results are consistent with shorter PEO chains allowing for peptide adsorption to the base substrate in the case of F68-coated surfaces, while adsorption to the F108-coated surfaces is apparently governed by the presence of a hydrophobic core within the brush layer itself. Further, these results suggest that while peptide location within the hydrophobic core provides stability against lateral rearrangement, the pendant PEO chains themselves provide no steric barrier to nisin rearrangement within the brush layer.
Quantifying Nisin Adsorption Behavior at Pendant Polyethylene Oxide Brush Layers

by

Justen K. Dill

A THESIS

submitted to

Oregon State University

in partial fulfillment of

the requirements for the

degree of

Master of Science

Presented June 1, 2012
Commencement June 2012
Master of Science thesis of Justen K. Dill presented on June 1, 2012

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Justen K. Dill, Author
ACKNOWLEDGEMENTS

First and foremost I would like to thank my major professor, mentor, and friend, Dr. Joe McGuire. He was the biggest influence in my decision to pursue a graduate degree and his help and guidance have been instrumental to my success in graduate school. I could not have done this without him. I would also like to thank my undergraduate advisor, Dr. Michelle Bothwell. While I never had the opportunity to work with her directly in my research, she has had a major impact on my higher education experience and has provided me with many invaluable lessons that cannot be learned in a laboratory. My sincere appreciation goes out to Dr. Karl Schilke who was absolutely essential to this work. His vast knowledge of all things science continually amazed me. I’d also like to thank Dr. Philip Harding for giving me the wonderful opportunity and experience of being his graduate teaching assistant for my first year in graduate school. The amount of practical knowledge I acquired from that position was incredible. A big thank you goes to my fellow McGuire lab mates Julie Auxier and Keely Heintz for always being there to bounce ideas off of and generally keep my head on straight. I also cannot forget my wonderful partner Melisa Lopez who has been with me since my very first day at Oregon State University. She keeps me balanced and never lets me forget what is truly important. Finally, I’d like to
thank all of my family and friends who have always supported me and been there for me during this extraordinary graduate school experience.
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**Introduction**

Infections associated with implantable devices are a major health concern. For example, over 450,000 people in the US require chronic hemodialysis in which AV grafts are commonly used. Unfortunately these have an infection rate between 11 and 20% with a mortality rate of 12 to 22% (Tai et al., 2008). Additionally, patients can be systemically heparinized to prevent clotting associated with blood damage during hemodialysis. The non-fouling characteristics of PEO coated surfaces have been extensively studied. The commonly held notion is that the highly hydrophilic, tethered PEO chains extend from the surface into an aqueous solution and provide a steric and hydration barrier that will prevent the adsorption of proteins (McPherson et al., 1998). Protein adsorption is often the first step in any biological response (Vogler, 2012). Controlling this interaction is an attractive option when attempting to impart biocompatibility to any foreign surface.

Recent findings also suggest the ability to entrap small peptides within a protein repellent PEO layer (Tai et al., 2008). This brings forth the idea of
imbuing the PEO layer with some sort of function (antimicrobial or anticoagulant) by entraining the proper peptides within the layer, providing a localized and not systemic effect. Drug eluting stents have currently been employed where a thin polymer layer containing the appropriate drug is implanted into the body (Williams, 2008). Further understanding of the mechanisms of peptide entrapment within a PEO layer will provide much needed direction for new research into biocompatible and functional surface coatings.

Nisin is a small (3510 Da) amphiphilic peptide that effectively inhibits Gram-positive bacteria. Its mode of action differs from traditional antibiotics in that it physically kills microbes by creating pores in their membranes. This negates the possibility of resistant strains of bacteria forming. More importantly for this work, nisin is known to penetrate PEO layers and become entrapped (Tai et al., 2008), making it an excellent candidate for investigation of the kinetics involved with its adsorption and sequestration as well as its retention of activity within a PEO layer.

Optical waveguide lightmode spectroscopy (OWLS) is an extremely sensitive technique for measuring the adsorption kinetics of peptides. It provides real-time, label free monitoring of adsorption events with sensitivity as low as 1 ng/cm² (Tie et al., 2003). OWLS waveguide sensors can also be grafted with pendant PEO chains providing an excellent simulation of peptide integration into
brush layers. Exposing uncoated and PEO coated surfaces to nisin rich solutions will provide a precise measure of adsorbed mass over time. This data can then be further analyzed to obtain kinetic rate constants for adsorption and desorption as well as the interfacial one-body cavity function of the surface. Together these parameters could provide a predictive capacity for future product designs around biocompatible and functionalized device surfaces.

In this work, surfaces were grafted with two different triblock copolymers which provided pendant PEO layers of different chain lengths and chain densities. These surfaces were then exposed to a nisin rich solution, allowing nisin to integrate into the PEO layers. OWLS instrumentation was used to precisely measure the adsorbed mass over time to obtain kinetics for adsorption and desorption. These findings were interpreted with reference to a model accounting for the history dependent nature of adsorption. Ultimately, the goal was to obtain further insight into the behavior of a peptide at a PEO layer so that new strategies for possible drug storage, delivery, and biocompatibility may be explored.
Literature Review

Protein Adsorption

Abundant research demonstrates that proteins and other macromolecules display the tendency to adsorb to an interface from a bulk solution. This phenomenon is driven by many natural forces such as hydrophobicity, ionic interactions, hydrogen bonding, and van der Waals forces to name a few (Tie et al., 2003). A protein’s structure can also be extremely complex and contain many positive and negatively charged regions, imparting attractive and repulsive characteristics while also allowing them to hydrogen bond. Because of this structure, hydrophobic and ionic interactions along with entropy gains from conformational changes during adsorption are often considered to be the most important driving forces behind it (Claesson et al., 1995). However, despite decades of research there is still strong contention on the very basic aspects of what actually occurs when proteins adsorb to a surface (Vogler, 2012). Fundamental issues that still appear open for debate include the application of complete thermodynamic and computational models, a protein’s capacity to adsorb in multilayer quantities, and the reversibility or irreversibility of the adsorption event. Adsorption can still be defined as “the partitioning of a chemical species (protein in the present case) between a bulk phase and an
interface” (Vogler, 2012). For the purposes of this research, adsorption is assumed to occur with an intrinsic “on” rate that is dependent on the concentrations present in solution exposed to an interface. It is also assumed that adsorbed proteins on the surface can change conformation and laterally cluster, giving rise to a history dependent model for adsorption with multiple desorbable states that will each have specific desorption rate constants (Tie et al., 2003).

**History Dependence of Adsorption**

Proteins and other macromolecules have previously been observed to have a history dependent adsorption behavior. This stems from the structural changes that can occur after a protein has initially contacted a surface (Joshi et al., 2006). Previous work with macromolecules as large as human serum albumin has clearly demonstrated this history dependent behavior of surfaces (Muragalelli et al., 1991). For a given amount of surface coverage, the rate of adsorption depends strongly on the formation history of the adsorbed layer. This is due to the fact that the rate of adsorption is not only sensitive to the amount of protein present in the adsorbed layer, but also to the layer’s structure. As more proteins are introduced to the surface, over time they may structurally
rearrange and form clusters of ordered domains (see Figure 1). This allows more free space for newly adsorbed molecules to bind (Calonder et al., 2001).

**Figure 1.** A schematic representation of two surfaces that have been exposed to the same protein solution is shown. The adsorbed layer on both surfaces is the same density however the surface on the right has had time to cluster and rearrange, providing more available space for a new adsorbing protein.

Multi-step adsorption-elution experiments can thus be performed in order to measure the change in the rate of adsorption from the first cycle to the second. This change can then be used to interpret how exactly the surface structure is changing, giving rise to the interfacial one-body cavity function for that surface. This cavity function describes the probability of any new adsorbing molecule finding an open area, or cavity, on the surface (Tie et al., 2003).
**PEO Layers**

Polyethylene oxide (PEO) coated surfaces have long been studied for their protein repellent nature (Lee et al., 2000). Triblock copolymers consisting of PEO-PPO-PEO chains (PPO refers to polyethylene oxide, a very hydrophobic polymer) have been used in previous work to render hydrophobic, protein attractive surfaces, hydrophilic (McPherson et al., 1998). In their work, PEO segments were grafted onto glass and radio-labeled lysozyme and fibrinogen were successfully repelled from the surface. Pluronic® surfactants such as F68 and F108 contain this same type of copolymer structure which is further illustrated in Figure 2.

![Figure 2](image)

**Figure 2.** The generic structure of the Pluronic® triblock copolymers is depicted here. F68 has PEO chains that are 80 units in length (n=80) and a PPO center block that is 27 units in length (m=27). F108 is a much larger molecule with 141 PEO units (n=141) and 44 PPO units (m=44) (Ryder et al., 2010).

Upon exposure to a hydrophobic surface, the center PPO block will associate at a hydrophobic interface, leaving the hydrophilic PEO chains extended into solution. Figure 3 illustrates this further.
Figure 3. Triblock copolymers are shown adsorbed to a hydrophobic surface. In aqueous solutions the hydrophilic PEO chains will be extended outward and can be considerably mobile (McPherson et al., 1997).

These associated layers can also be permanently tethered to the surface with the proper conjugation techniques. This was demonstrated by McPherson et al when they used trichlorovinylsilane to prepare a glass surface for adsorption of Pluronic® triblocks. The surfaces were γ-irradiated to covalently attach the PPO backbone through a form of radical chemistry. The tethered PEO chains were shown to successfully prevent adsorption of lysozyme and fibrinogen. The main mechanism for prevention of protein adsorption by PEO layers was thought to be steric repulsion (Amiji and Park, 1994). The PEO chains would form a brush layer and physically shield the surface. This protein repellent nature has been well documented in numerous research articles (Neff et al., 1999). However, adsorption-elution experiments involving smaller proteins have been shown to actually entrap the protein within the PEO layer (Tai et al., 2008). In their work, the small lantibiotic peptide Nisin was shown to penetrate the PEO layer and
become entrapped while still retaining its antimicrobial activity. Rigorous models that incorporated PEO chain length, thickness, and protein size have been created to try and further explain this entrapment phenomenon (Fang et al., 2005). They postulated that protein interactions with PEO brush layers are based on a generalized diffusion approach as illustrated in Figure 4.

![Figure 4](image)

**Figure 4.** A general model for quantifying protein entrapment in tethered polymer layers is shown. As the black polymer chains are placed closer together, the rate of integration by the large protein circles will decrease until they are too large to fit between chains. At this point, the time scale for protein adsorption is large enough that adsorption can be considered negligible (Reprinted from Biophysical Journal, 89/3, Fang, F. Satulovsky, J. Szleifer, I., “Kinetics of Protein Adsorption and Desorption on Surfaces with Grafted Polymers,” 1516-1533, 2005, with permission from Elevier.)

In their model, chain layer thickness that is greater than the size of the protein will cause a decrease in the adsorption and desorption kinetics as the chain
length increases. Kinetic and thermodynamic considerations can also be made when formulating a model for adsorption into a polymer brush layer. Fang et al. also postulated a potential energy well (see right side of Figure 4) within the PEO layer where proteins could become trapped (Fang et al., 2005). Two possible modes of adsorption can be considered: primary adsorption at the surface of the interface and secondary adsorption at the edge of the PEO layer (Halperin, 1999). Other findings suggest that there may exist a dilute, hydrophilic region at the distal end of the brush layer and a dense, hydrophobic region within the brush. Water molecules themselves may be physically excluded from this hydrophobic core. This would give rise to a very attractive inner region for amphiphilic peptides to become entrained (Sheth and Leckband, 1997). These models and results further confirm the entrapment theory and lead to the suggestion of designing strategies for the controlled release of therapeutic peptides from a PEO layer that has been engineered for a given peptide size.

**Biocompatibility, Drug Loading, and Release**

Adsorption of proteins to a surface is the initial step preceding any acute biological response (Vogler, 2012). Therefore, controlling this interaction with an implanted material is the primary goal of any design strategy for biocompatibility of an artificial device. Nosocomial and implant-associated infections can result in
significant harm and even death, adding increased and unnecessary health care costs to patients. For example, over 450,000 people in the US require chronic hemodialysis as a result of end stage renal failure. AV grafts are frequently used in these situations; however they have an infection rate between 11 and 20% with a mortality rate from these grafts of 12 to 22% (Tai et al., 2008). Patients are also systemically dosed with heparin to prevent clotting effects associated with blood damage during the hemodialysis process. Ideally, the antimicrobial and anticoagulant function should be localized to the foreign surface or device to prevent the dangers of systemic infections and clotting. This gives rise to the concept of a drug-eluting device or surface. Currently, drug eluting stents have been employed where a metal stent is coated with a thin polymer layer containing an appropriate drug (Williams, 2008). The drug is released into the body causing the down-regulation of an undesirable process such as thrombosis. The adsorption rate of fibrinogen to a surface has also been shown to decrease on surfaces that have been covalently modified with heparin (Joshi et al., 2006). In their work, heparin was bound to surface-activated silica wafers and exposed to varying levels of fibrinogen in solution. In all cases the adsorbed mass of fibrinogen on heparinized silica was less that on unheparinized silica. The initial rate of adsorption was also lower on heparinized silica. Other research has studied physically encapsulating therapeutic agents in a composite delivery
system to prevent an inflammatory response (Murua et al., 2011). Living cells were immobilized in a polymeric matrix surrounded by a semipermeable membrane. The therapeutic agent was continuously released by the inner cells while the outer membrane provides immunoprotection from the host and simultaneously allows free exchange of nutrients. While these methods seem attractive, complex biological systems can be hopelessly complex and difficult to engineer towards releasing specific amounts of peptide. Previous work with PEO brush layers has demonstrated a quantifiable rate of protein adsorption into the layer, and desorption from the layer (Tie et al., 2003). Precise kinetic data was easily obtained with relatively simple Optical Waveguide Lightmode Spectroscopy (OWLS) measurements. This provided a real time, quantitative analysis of the structural evolution of the loaded layer allowing one to obtain adsorption and desorption rate constants. These can ultimately be used in a predictive capacity to design optimal device coatings with known therapeutic concentrations and elution times.
**Optical Waveguide Lightmode Spectroscopy (OWLS) Theory**

The Optical Waveguide Lightmode Spectroscopy (OWLS) technique is an extremely precise method for measuring real time structural and kinetic events of biological thin films at solid-liquid interfaces. It can be applied *in situ* under approximate biological conditions and requires no labeling of adsorbed molecules (Ramsden, 1999). OWLS is an optical sensor technique that utilizes evanescent field characteristics of incoupled, polarized laser light (Voros et al., 2002). A helium-neon (He-Ne) laser shines polarized light (632.8 nm) onto a thin waveguide sensor (see Figure 5).

![Figure 5](image.png)

**Figure 5.** The technical specifications for OW 2400 waveguide sensors are shown. The dimensions are 0.5 mm (H) by 8 mm (W) by 12 mm (L). The refractive index of the glass support ($n_s$) is 1.53; The refractive index of the waveguide layer ($n_f$) is $1.7 \pm 0.3$ and the thickness of this layer ($d_f$) is 170-220 nm (Szekacs et al., 2009).
This waveguide sensor forms the base of a small flow cell (4.8 µL approximate volume) which is rotated about a central axis to vary the incident angle of the laser light. At precise resonance angles, the light is diffracted by the sensor grating, incoupled into the waveguide layer, and detected by photodiodes on edge of the flow cell. Figure 6 gives a schematic of this system.

**Figure 6.** A profile view of the OWLS waveguide system is shown. The He-Ne source is at a fixed vertical position and the entire flow cell is rotated to vary the incident angle (α). Any analyte interacting with the waveguide surface will cause changes in the optical refractive index of that layer and be detected by changes in the incident angle required for incoupling (Szekacs et al., 2009).

This incoupling of laser light occurs at four distinct incident angles (two positive and two negative), representing the two polarization modes (transverse electric, TE, and transverse magnetic, TM) of the laser as well as the symmetric nature of the planar waveguide (Horvath et al., 2001). Analytes adsorbing to the
waveguide surface will cause alterations in these incident angles (denoted by \( \alpha_{\text{TM}} \) and \( \alpha_{\text{TE}} \)). Once the incoupling angles are known, the effective refractive indices can be simultaneously calculated for both the electric (\( N_{\text{TE}} \)) and magnetic (\( N_{\text{TM}} \)) modes. \( N_{\text{TE}} \) and \( N_{\text{TM}} \) are used to calculate a baseline condition for the waveguide system. Once an analyte is injected, a linearized four-layer waveguide model is used to determine the new refractive index and thickness of the waveguide adlayer (\( n_a, d_a \)) and this is converted into adsorbed mass by applying the model that refractive index changes linearly with protein concentration (Ramsden, 1993). From this data, adsorbed mass versus time plots can be generated and further analyzed to determine adsorption and elution kinetics as well as surface coverage for different analytes.
Materials and Methods

Solution Preparation

Nisin (3510 Da) was obtained from Prime Pharma (Batch number 20050810, Gordons Bay, South Africa) and dissolved in 10 mM monobasic phosphate buffer (10 mM monobasic sodium phosphate, 150 mM sodium chloride). The pH was adjusted to 7.4 by dilution with the proper amount of dibasic phosphate buffer (10 mM dibasic sodium phosphate, 150 mM sodium chloride) to bring the final nisin concentration to 0.5 mg/mL. Solution was mixed using a magnetic stir bar overnight in a 37 °C incubator. Plasminogen-free human fibrinogen (340 kDa, 1 mg/mL, Sigma-Aldrich) was dissolved in 10 mM PBS (150 mM NaCl, pH 7.4) as needed. Prior to use, all protein solutions were drawn into sterile, disposable syringes and degassed for one hour at 700 torr vacuum. All protein free phosphate buffered saline (10 mM PBS, 150 mM NaCl) was degassed in a sterile, disposable syringe at 700 torr vacuum for four hours. The Pluronic® surfactants F108 and F68 were obtained from BASF (Mount Olive, NJ) and dissolved in PBS (10 mM sodium phosphate buffer, 150 mM NaCl), each at 5% (w/v) as needed. All water used was HPLC grade.
Surface Modification

OW 2400 waveguide sensors for use with OWLS instrumentation were purchased from MicroVacuum (Budapest, Hungary). A thin film of silica dioxide was applied by the manufacturer prior to purchase. Sensor cleaning consisted of submersion in chromosulfuric acid (Acros Organics, NJ) for 10 minutes, rinsing with HPLC grade water, and blow drying with dry nitrogen. Surface silanization was performed via vapor deposition with trichlorovinylsilane (TCVS, TCI America, Portland, OR). Silanization was carried out in a sealed vessel using dry Argon as the carrier gas (Popat et al., 2002). A simplified schematic of the deposition unit is shown in Figure 7. Cleaned OWLS sensors were arranged on the sample stage with the waveguides facing up. Dry argon was allowed to flow through the vapor deposition unit at an approximate flow rate of 30 SCFH for 20 minutes to equilibrate the internal environment and purge any atmospheric moisture. A 200 µL aliquot of TCVS was injected into the injection well and the T-valve was switched to direct Argon flow through the well to transport silane vapors into the main chamber. After one hour, another 200 µL aliquot of TCVS was injected into the well. Argon flow was continued for an additional hour before the T-valve was switched so that argon flow bypassed the injection well. Argon was passed
through the main chamber for an additional 20 minutes to purge any unreacted silane vapors from the system.

**Figure 7.** A schematic illustrating the vapor deposition system for silanizing OWLS waveguide sensors in a contained environment with TCVS is shown. This system was adapted from a similar procedure by Popat et al.

Silanized waveguides were cured at 150 °C for 20 minutes to stabilize the newly formed vinyl layer. Following a method proposed by McPherson et al. (McPherson et al., 1997), each silanized and cured waveguide was incubated for a minimum of 12 hours in a 1.5 mL eppendorf tube containing the correct
Pluronic® solution. After incubation, these tubes were exposed to γ-radiation from a ⁶⁰Co source (Oregon State University Radiation Center) for a total dose of 0.3 Mrad to achieve polymer grafting (McPherson et al., 1998). Typical exposure time was 6.5 hours. Sensors were then removed from incubation tubes, rinsed with 10 mM PBS (150 mM NaCl), dried with nitrogen, and stored in dry nitrogen filled eppendorf tubes until needed.

**Measurements with Optical Waveguide Lightmode Spectroscopy (OWLS)**

OWLS waveguides (with or without surface modifications) were immersed in 10 mM PBS (150 mM NaCl) overnight prior to use in order to equilibrate their surface with the buffer (Ramsden, 1994). The waveguide was then removed from solution and immediately installed in the OWLS flow cell (total volume of flow cell is approximately 4.8 µL). Experiments were carried out in an OWLS 210 electro-optic instrument controlled with BioSense 2.6 software (MicroVacuum, Budapest, Hungary). A Rheodyne manual sample injector (IDEX, Oak Harbor, WA) was used to inject protein samples through a flow loop (PEEK tubing, 2.3 mL approximate volume) to the OWLS flow cell. The flow rate was maintained at 50 µL/min in all experiments to ensure an injection residence time of at least 30 minutes in the flow cell. As refractive index measurements are highly sensitive to temperature variations, flow cell temperature was maintained
at 20 °C by an internal OWLS TC heater/cooler unit. Incident angle scans were performed from -5° to 5° at a step size of 0.01°. All four peaks were measured (the characteristic transverse electric (TE) and transverse magnetic (TM) peaks) to determine the relative refractive index of the surface adlayer. The OWLS instrument allows 4-10 peak scans per minute (depending on scanning speeds, peak ranges, and number of peaks used), which resulted in approximately 6 seconds being needed for determination of a single data point. This data acquisition speed provides rapid enough responses to be considered as real-time measurements of surface adsorption events (Szekacs et al., 2009).

Adsorbed mass versus time data was calculated from changes in the refractive index of the adlayer, applying the assumption that refractive index changes linearly with protein concentration (De Feijter et al., 1978).

**Fibrinogen Adsorption**

A 1 mg/mL fibrinogen solution was loaded into the injection loop. Approximately six mL of solution was passed through the loop to purge any remaining rinse buffer. Adsorption was performed for 10 minutes on coated and uncoated sensors to confirm the presence of a protein repellent PEO layer after grafting.
**Triblock Adsorption**

A 5% (w/v) Pluronic® F108 or F68 solution was loaded into the injection loop. Approximately six mL of solution was passed through the loop to purge any remaining rinse buffer. Adsorption was performed for 30 minutes, immediately followed by a 30 minute rinse with protein free buffer.

**Nisin Adsorption**

A 0.5 mg/mL nisin solution was loaded into the injection loop. Approximately six mL of solution was passed through the loop to purge any remaining rinse buffer. Adsorption was performed for 30 minutes, immediately followed by a 30 minute rinse with protein free buffer on uncoated, F108 coated, and F68 coated surfaces. This process was repeated two times during the course of one experiment. After the second rinse cycle, 1 mg/mL fibrinogen was loaded into the injection loop. Approximately six mL of solution was passed through the loop to purge any remaining rinse buffer. Fibrinogen was injected into the flow cell to confirm the presence of a protein repellent layer after nisin adsorption.
Results and Discussion

Triblock Adsorption

The Pluronic® surfactants F108 and F68 were adsorbed to silanized OWLS waveguides prior to covalent immobilization through γ-irradiation. Figure 8 shows OWLS adsorption experiments performed on these waveguide sensors to confirm triblock association with the hydrophobic surface.

Figure 8. Exposure of TCVS silanized OWLS waveguides to a 5% F108 or F68 solution (in PBS) and subsequent adsorption and desorption is shown. Apparent steady state adsorbed triblock mass was used in determination of PEO chain density.

Each triblock appeared to rapidly associate with the extremely hydrophobic surface of the silanized waveguide. This is likely due to the hydrophobic PPO
center block contained in each surfactant (McPherson et al., 1998). The adsorbed mass remained constant until surfactant free buffer was introduced to the flow cell, at which point most of the triblocks were removed from the surface. Each waveguide is irradiated in the same 5% triblock solution that was used in the triblock adsorption experiment. This is due to the rapid removal of adsorbed triblocks when the surface is exposed to triblock free solutions. It is therefore reasonable to assume the steady state amount of adsorbed triblock would remain covalently linked to the surface for further protein adsorption experiments after γ-irradiation. Surface PEO chain density was determined by the following conversion:

\[
(Triblock \ Surface \ Coverage)(Molecular \ Weight) \left( \frac{2 \ PEO \ Chains}{Molecule} \right) = (Surface \ Chain \ Density)
\]

This yielded a surface coverage of 0.25 PEO chains per nm\(^2\) for F108 and 0.34 chains per nm\(^2\) for F68. The higher molecular weight F108 (14600 Da) tends to form less dense but deeper brush layers. This is facilitated by the longer PEO chains of F108 (approximately 141 repeat units) as well as the longer PPO center block (approximately 44 repeat units). The smaller molecular weight F68 (8750 Da) tends to form a denser, more shallow layer. This is to be expected as F68 is smaller than F108 in each dimension. Its PEO chains are approximately 80 repeat
units and its PPO center block is approximately 27 repeat units. Figure 9 gives a schematic representation of these approximate coverages.

![Figure 9](image)

**Figure 9.** An approximate schematic representation of the different PEO layers created by F108 and F68 is shown. The much larger F108 tends to create a less dense but deeper brush layer while the smaller F68 tends to create a denser, shallow layer.

PEO brush layers have been known to form at surface coverages of approximately 0.2 chains per nm$^2$ (Unsworth et al., 2008) therefore it is likely that brush layers have been created on the waveguides used in this study.

**PEO Layer Stability and Protein Repulsion**

PEO layers are widely known to repel proteins from surfaces and prevent plasma protein adsorption (Lee et al., 2000). This phenomenon was used to confirm the presence of a repellent PEO layer on the waveguide surface.

Plasminogen-free human fibrinogen at 1 mg/mL in PBS was introduced to uncoated and PEO coated waveguide sensors. The results are shown in Figure 10.
Figure 10. Fibrinogen adsorption on uncoated and PEO coated surfaces is shown. When no PEO layer is present, Fibrinogen rapidly adsorbs to the hydrophobic surface of the waveguide.

As Figure 10 demonstrates, Fibrinogen will quickly associate with an uncoated, hydrophobic surface. However, when a PEO coated surface is exposed to the same fibrinogen solution, there is very little adsorption due to the repulsive nature of the grafted layer. This further validates the presence of a tethered PEO layer on the surface of OWLS waveguides after silanization, triblock adsorption, and subsequent γ-irradiation. Appendix A contains further experimental work regarding the repulsion of fibrinogen at pendant PEO layers.
Nisin Adsorption

Nisin adsorption was performed on uncoated, F108 coated, and F68 coated OWLS waveguide sensors. Representative data from these experiments is shown in Figure 11.

![Graph showing nisin adsorption](image)

**Figure 11.** Nisin adsorption on three different surfaces is shown. Nisin adsorbed rapidly to uncoated surfaces and was not easily removed through subsequent rinsing. PEO coated surfaces also showed nisin adsorption.

Adsorption of nisin on an uncoated, hydrophobic surface was consistent with amounts found in literature (Tai et al., 2008). Nisin adsorbing to a surface in an end on configuration would produce monolayer coverage of 150 ng/cm^2_. Rinsing
of the uncoated interface post adsorption appeared to remove little nisin suggesting a strong association between the peptide and the surface itself. Nisin also appeared to adsorb onto PEO coated surfaces as well. Prior research demonstrates that this observed nisin adsorption to protein repellent surfaces is actually nisin integration into the layer rather than nisin location at the PEO ends (Ryder et al., 2010; Tai et al., 2008). Figure 11 also demonstrates that nisin integrates more rapidly into an F108 layer than an F68 layer. The PEO chains of F108 are much longer than F68 (approximately 141 repeat units for F108 versus 80 for F68). These longer chains can be more mobile in solution and evidence has been reported that they may give rise to an inner hydrophobic core which would be very attractive for an amphiphilic peptide (Sheth and Leckband, 1997). This core may not be present in a PEO layer made up of smaller chains. The higher PEO chain density in an F68 layer may also be retarding nisin integration through steric effects. Nisin also appears to be more elutable from an F108 layer than an F68 layer. During the first and second rinse cycles, more nisin was removed from the F108 surface then the F68 surface. The lower chain density of the F108 layer may be responsible for this as the PEO chains will be more mobile in solution allowing nisin to exit the brush. The hydrophobic core initially present in an empty F108 layer may also be compromised as more and more nisin molecules fill the layer (see Figure 12).
Figure 12. A schematic proposed for the rapid nisin integration into an F108 layer is shown. Attractions between the hydrophilic domains on the peptide and the very mobile, hydrophilic PEO chains may result in a compromised hydrophobic core at higher adsorbed mass.

Figure 12 helps to further explain the rapid nisin integration into an F108 layer followed by large amounts of nisin elution during rinses. Attractions between the first nisin molecules to enter the brush and the PEO chains themselves may compromise the hydrophobic core initially present. More nisin would still be able to enter the brush layer but it would not be held as tightly and would easily elute during the rinse cycle. The shorter PEO chains in the F68 layer may not produce a hydrophobic core like F108 does. This would explain the slower adsorption of
nisin into the layer. Shorter PEO chains could also allow nisin to more closely associate with the underlying hydrophobic PPO center blocks as shown in Figure 13.

**Figure 13.** A schematic for proposed nisin integration into an F68 layer is shown. The shorter PEO chains may not produce a hydrophobic inner core resulting in slower nisin adsorption as well as allowing nisin to interact with the underlying hydrophobic PPO center blocks.

Figure 13 helps to further explain the slower integration of nisin into an F68 layer followed by less amounts of nisin eluting from the layer during rinses. The lack of a hydrophobic core may trap nisin more slowly and the shorter chains may allow nisin to closely associate with the underlying hydrophobic PPO layer facilitating a stronger attraction while becoming more resistant to elution during rinses.
Adsorption History Dependence

Further analysis of nisin adsorption experiments reveals a history dependent adsorption mechanism on some of the surfaces. Proteins have been shown to exhibit this history dependent adsorption behavior which is due to the slow relaxation of their structures as well as lateral clustering on the surface itself (Tai et al., 2008). This leads to the observation demonstrated in Figure 14.

**Figure 14.** Nisin adsorption on an uncoated waveguide is shown. The second adsorption step is shifted in time and overlaid on the first to facilitate comparison of adsorption rates at identical mass densities but different formation histories.

At a given surface loading, the rate of adsorption will depend on the formation history of that surface. In particular, Figure 14 shows that the initial adsorption...
rate on an uncoated surface is much slower than the adsorption rate after a rinse cycle. In both instances the adsorbed mass is the same but the formation history of the adsorbed layer is different. This is because adsorbed molecules can cluster and rearrange laterally on the surface to form more ordered domains which would open up more free space for newly adsorbing molecules to occupy (Tie et al., 2003). The free space is referred to as the cavity function, Φ, and is defined by Tie et al. (Tie et al., 2003) as the fraction of the surface where a newly adsorbing molecule can land without overlapping a previously adsorbed molecule.
Similar experiments were performed on F108 and F68 coated surfaces.

Figure 15 shows a nisin adsorption-elution experiment on an F108 coated waveguide.

**Figure 15.** Adsorption of nisin on an F108 coated waveguide is shown. The second adsorption step is time shifted and overlaid on the first to facilitate comparison at identical surface coverages but different formation histories.

Nisin rapidly integrates into the F108 layer and is more elutable than when it is just adsorbed to a bare surface. In Figure 15 the initial adsorption rate is almost identical to the second adsorption rate at the same surface coverage. This lack of history dependence suggests the PEO chains in the F108 layer inhibiting the nisin
from clustering on the surface. As mentioned previously, this could be due to the presence of a hydrophobic core within the brush and/or steric effects of the chains themselves. Nisin adsorption on an F68 coated surface is shown in Figure 16.

**Figure 16.** Adsorption of nisin on an F68 coated waveguide is shown. The second adsorption step is time shifted and overlaid on the first to facilitate comparison at identical surface coverages but different formation histories.

Nisin integrates into an F68 layer as well but apparently not as rapidly as with an F108 layer. This may be due to the higher PEO chain density present in an F68 layer. Less nisin was eluted from an F68 layer than an F108 layer which could be due to the shorter PEO chains of F68 allowing nisin to more closely associate
with the underlying surface facilitating a stronger association. Figure 16 also shows an apparent history dependence of adsorption with an F68 coated surface that is not seen on an F108 surface. This may be due to the lack of a hydrophobic core within the shorter F68 brush layer. If this core is the dominant force preventing nisin clustering within the brush then it would stand to reason that an F68 layer may not be able to prevent lateral mobility. The steric effects of the PEO chains in an F68 layer may not be strong enough to prevent nisin mobility on the surface.

**Determination of Adsorption Kinetics and the Cavity Function**

When an adsorbing surface is exposed to a protein solution, the initial adsorption rate is usually limited by transport of the adsorbing molecule from the solution to the interface (Dijt et al., 1994). Eventually the surface will fill to an extent where the rate limiting step will be the adsorption step itself. At this point the rate of adsorption can be described by

$$\frac{d\Gamma^*}{dt} = k_a C_b \Phi - \sum_i k_{d,i} \Gamma_i$$  

(1)

where $\Gamma^*$ is the adsorbed mass, $t$ is time, $k_a$ is the intrinsic rate of adsorption, $C_b$ is the bulk concentration of the adsorbing molecules in solution, and $k_{d,i}$ and $\Gamma_i$ are the desorption rate constant and the density of adsorbed molecules in the
ith adsorption state (Tie et al., 2003). As stated previously, $\Phi$ is the cavity function and it is a function of many different factors including the adsorbed density, adsorption mechanism, and the system’s initial condition. Since the adsorption to the surface will be influenced by any currently adsorbed molecules, we add an exponential term describing this interaction, $e^{-\frac{-\underline{U}_s(\Gamma)}{kT}}$, where $-\underline{U}_s(\Gamma)$ is an average energy for the first adsorbates, $k$ is the Boltzmann constant, and $T$ is the temperature. Inserting this new term into Equation 1 gives

$$\frac{d\Gamma}{dt} = k_a e^{-\frac{-\underline{U}_s(\Gamma)}{kT}} C_b \Phi - \sum_i k_{d,i} \Gamma_i$$

(2)

We can also define an apparent adsorption rate, $k'_a = k_a e^{-\frac{-\underline{U}_s(0)}{kT}}$, which can be experimentally determined when adsorption initially occurs on an empty surface (at $\Gamma=0$). To fit $k'_a$ into equation 2, we first multiply through by $\left(\frac{e^{-\underline{U}_s(\Gamma)}}{e^{-\underline{U}_s(0)}}\right)$ as shown in equation 3.

$$\left(\frac{e^{-\underline{U}_s(0)}}{e^{-\underline{U}_s(\Gamma)}}\right) \frac{d\Gamma}{dt} = \left(\frac{e^{-\underline{U}_s(0)}}{e^{-\underline{U}_s(\Gamma)}}\right) k_a e^{-\frac{-\underline{U}_s(\Gamma)}{kT}} C_b \Phi - \left(\frac{e^{-\underline{U}_s(0)}}{e^{-\underline{U}_s(\Gamma)}}\right) \sum_i k_{d,i} \Gamma_i$$

(3)

Rearranging some terms gives equation 4.

$$\frac{d\Gamma}{dt} = k_a e^{-\frac{-\underline{U}_s(0)}{kT}} C_b \Phi \left(\frac{e^{-\underline{U}_s(\Gamma)}}{e^{-\underline{U}_s(0)}}\right) - \sum_i k_{d,i} \Gamma_i$$

(4)
We define the modified cavity function as $\Phi' = \Phi \left( \frac{e^{-U_f(r')}}{e^{-U_f(0)}} \right)$. We can also simplify the apparent adsorption rate constant, $k'_a$, by combining it with the bulk concentration, $C_b$. This gives a “$kC$” term describing the apparent rate of adsorption as a constant, provided the bulk solution concentration remains unchanged throughout the experiment (this “$kC$” term is analogous to the combined “$k_i a$” term commonly used in mass transfer). Equation 4 can now be rewritten as

$$\frac{d\Gamma'}{dt} = kC\Phi' - \sum_i k_{d,i}\Gamma'_i$$  \hspace{1cm} (5)

Plots generated from OWLS measurements of adsorbed mass versus time can now be analyzed to determine the kinetic parameters in Equation 5 and calculate the modified cavity function for each surface. Figure 17 a-c demonstrates how the intrinsic adsorption constant, $kC$, is obtained.
Figure 17. The rate of adsorption versus the adsorbed density for the first adsorption cycle of nisin on an uncoated surface (a), an F108 coated surface (b), and an F68 coated surface (c) is shown. The solid line is a best fit to data points in the linear, surface limited adsorption regime.
Figure 17 a-c is a plot of the rate of adsorption versus the concentration of adsorbed mass on the surface. The y-intercept of the best fit line in Figure 17 a-c is used to determine $k_C$ for each surface. At zero surface coverage ($\Gamma = 0$), the modified cavity function will be equal to 1 and Equation 5 can be reduced to

$$\frac{d\Gamma}{dt} = k_C(1)$$

The y-intercept will be equal to $k_C$ at this point. Since $k_C$ is a function of the apparent adsorption rate constant as well as the bulk protein concentration, it should remain constant because neither of these parameters will be altered.

Figure 18 a-c shows how the desorption rate constants were obtained for each surface.
Figure 18. The negative of the desorption rate versus adsorbed density for the first desorption cycle of nisin on an uncoated surface (a), an F108 coated surface (b), and an F68 coated surface (c) is shown. The solid lines are best fits to two linear regions. They are used to determine desorption rate constants as well as adsorbed states and populations.
Figure 18 a-c is a plot of the desorption rate versus the concentration of adsorbed mass on the surface. The presence of two distinct linear regions of data indicates three adsorbed states: one easily removed (state 1), one slowly removed (state 2), and one irreversibly bound to the surface (state 3). Equation 7 describes this desorption model.

\[- \frac{d\Gamma}{dt} = (k_{d,1}\Gamma_1' + k_{d,2}\Gamma_2' + k_{d,3}\Gamma_3')\]  

(7)

The slope of the linear fit lines are the two desorption constants, \(k_{d,1}\) and \(k_{d,2}\). Any protein adsorbed in state 3 will be irreversibly bound, meaning \(k_{d,3}\) will be zero. The populations of protein in states 1 and 2 for each surface can also be determined from Figure 18 a-c. A simple mass balance can be constructed by looking at the adsorbed amount at the onset of desorption, at the intersection of the two linear fit lines where all of state 1 was removed, and at the x-intercept where all of state 2 was removed. This is further illustrated in Figure 19.
Figure 19 shows desorption rate versus concentration of adsorbed mass data for an uncoated surface. The presence of three adsorbed states expands Equation 5 into

\[
\frac{d\Gamma}{dt} = k_c \Phi' - \left( k_{d,1} \Gamma_1 + k_{d,2} \Gamma_2 + k_{d,3} \Gamma_3 \right) \tag{8}
\]

Once all rate constants have been obtained, Equation 5 can be solved for the modified cavity function for each surface using the adsorption rate versus concentration of adsorbed mass data. We employ the assumption that after the first desorption cycle, all remaining surface bound protein is in state 3 and will
not be removed. We also make a similar assumption during the initial adsorption step. The first proteins to reach an uncoated surface will likely adopt a more tightly bound state as there is ample free surface available for further attachment. These initial molecules may also change conformation, increasing noncovalent contacts with the energetically dissimilar surface. Similarly, the first proteins to reach a PEO coated surface will optimally be entrained in the brush, and be more resistant to elution. Table 1 shows the adsorption rates, adsorbed populations, desorption rates, the modified cavity function at the onset of the second adsorption cycle, and the modified cavity function at that same surface coverage during the first adsorption cycle.

**Table 1.** Calculated kinetic parameters representative of all three experimental surfaces is shown. Φ′₁A and Φ′₂A refer to the modified cavity function at identical surface coverages during the first and second adsorption cycle, respectfully. The acronym H.D. refers to the surface demonstrating history dependence in multi-step adsorption experiments.

<table>
<thead>
<tr>
<th></th>
<th>Uncoated Surface</th>
<th>F108 Coated Surface</th>
<th>F68 Coated Surface</th>
</tr>
</thead>
<tbody>
<tr>
<td>kC (ng/cm² min)</td>
<td>0.96</td>
<td>2.2</td>
<td>1.1</td>
</tr>
<tr>
<td>k₄,1 (min⁻¹)</td>
<td>0.0054</td>
<td>0.0071</td>
<td>0.0086</td>
</tr>
<tr>
<td>k₅,2 (min⁻¹)</td>
<td>0.001</td>
<td>0.003</td>
<td>0.002</td>
</tr>
<tr>
<td>Γ₁ (ng/cm²)</td>
<td>10.5</td>
<td>85.6</td>
<td>50.9</td>
</tr>
<tr>
<td>Γ₂ (ng/cm²)</td>
<td>17.4</td>
<td>29.3</td>
<td>28.4</td>
</tr>
<tr>
<td>Γ₃ (ng/cm²)</td>
<td>184.1</td>
<td>40.4</td>
<td>82.1</td>
</tr>
<tr>
<td>Φ′₁A</td>
<td>0.10</td>
<td>0.52</td>
<td>0.12</td>
</tr>
<tr>
<td>Φ′₂A</td>
<td>0.27</td>
<td>0.59</td>
<td>0.52</td>
</tr>
<tr>
<td>H.D.</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>
Both an uncoated surface and an F68 coated surface showed increases in the modified cavity function between the first and second adsorption cycle. As stated previously, this suggests some sort of structural surface rearrangement of the adsorbed molecules, most likely involving clustering into more ordered domains. This suggests that surfaces coated with the shorter F68 PEO chains behave more like an uncoated surface, possibly due to the chains inability to prevent nisin from interacting with the underlying surface. The F108 surface demonstrated little change in the cavity function between adsorption cycles indicating a lack of peptide mobility provided by the longer PEO chains. One may argue that nisin entrained in an F108 PEO layer still interact with the underlying surface, however the lack of history dependence for these surfaces further suggests some other means of lateral stabilization, perhaps a hydrophobic core within the brush layer.

**Mass Transfer Considerations**

The adsorption of proteins to a surface usually involves both transport to the surface and binding on the surface. These two events are important considerations when analyzing adsorption kinetics. If mass transfer to the surface is not sufficiently rapid enough, there will be no way to accurately
calculate the kinetics of adsorption because any adsorption data gathered will be a function of both the transport and adsorption events. Krisdhasima et al. applied the a one-dimensional mass balance model to estimate mass transfer effects (Krisdhasima et al., 1992). From this work, the adsorbed mass at any time can be modeled by

$$\Gamma = 2M_r C (Dt/\pi)^{1/2}$$  \hfill (9)

Equation 9 gives the adsorbed mass at any time that would be predicted by mass transfer of protein to the surface where $M_r$ is the molecular weight of the protein, $C$ is the bulk protein concentration, and $D$ is the diffusion coefficient which is estimated to be $10^{-6}$ and $10^{-7} \text{ cm}^2/\text{s}$ (Krisdhasima et al., 1992) for most proteins. The largest diffusion coefficient of $10^{-6}$ was chosen for this simulation because of nisin’s relatively small size. Figure 20 compares the experimentally obtained adsorbed mass versus time data on the most hydrophobic surface with what mass transfer would predict for adsorption. This surface was chosen for comparison because it should have the fastest rate of protein binding to the surface. In transport-controlled adsorption, the rate of protein transport to the surface is slower than the rate of protein binding at the surface. For this simulation, we have chosen the slowest rate of protein transport and the fastest rate of protein binding to give mass transfer every opportunity for limiting the surface adsorption.
Figure 20. A comparison of experimentally obtained data on the most hydrophobic surface with data predicted by pure mass transfer is shown. The mass transfer data is difficult to see as it lies almost entirely on the y-axis.

Figure 20 clearly demonstrates an adsorption limited regime for these experiments. Mass transfer events occur much faster than surface adsorption under these experimental conditions. If adsorption on the most hydrophobic surface is not transport controlled then adsorption events on the hydrophilic PEO coated surfaces are unlikely to be transport controlled because these surfaces will have slower adsorption binding kinetics. Therefore the adsorption and desorption rates determined for all surfaces should be indicative of kinetically controlled events.
Conclusion

In this work, the Pluronic® surfactants F108 and F68 were successfully grafted to a TCVS silanized surface through radiolysis. These layers were shown to repel adsorption of fibrinogen. The much smaller peptide, nisin, was also adsorbed to uncoated and PEO coated surfaces. Nisin adsorption to these layers has been shown to actually be nisin integration and entrapment in the layer. Multiple nisin adsorption-elution cycles on uncoated surfaces demonstrated a history dependence of adsorption. In this case, adsorbed layers of identical surface coverage showed much different adsorption rates. With reference to analysis by the history dependent adsorption model, this indicates that the surface structures present in each case are different, due most likely to lateral clustering of adsorbed nisin to form more ordered domains. Similar experiments were performed on F108 and F68 coated surfaces with varying results. F108 surfaces showed rapid nisin integration into the layer and large amounts of elution from the layer, possibly due to a lower PEO chain density. Adsorption history dependence was not observed in F108 layers. We believe this is due to the presence of a hydrophobic core within the brush layer that may provide lateral stability for the nisin molecules and still allow them to be exchanged with the bulk solvent during rinse cycles. F68 surfaces showed slower nisin integration
possibly due to a higher PEO chain density. Less nisin elution during rinse cycles was also observed with F68 brush layers. This may be a result of the shorter PEO chains of F68 allowing nisin greater access for association with the underlying hydrophilic PPO center blocks. Cyclic nisin adsorption-elution experiments on F68 surfaces also showed history dependence. We believe this is due to the shorter PEO chains being unable to provide a sufficient hydrophobic core for lateral stabilization. The steric forces of the chains may not provide enough force to prevent rearrangement of the nisin within the brush layer on their own.

Determination of the modified cavity function for each surface provided further insight to the surface structure. The cavity function for the uncoated and F68 coated surfaces increased between the first and second adsorption cycle. This further suggests lateral rearrangement and clustering of adsorbed nisin. Conversely, the cavity function for the F108 coated surface did not change between adsorption cycles indicating little or no surface rearrangement.
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Appendix A: Relevant Manuscript (in Review)

SHORT COMMUNICATION

Nisin integration into PEO layers does not compromise their ability to repel fibrinogen

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Abstract

Nisin, an amphiphilic, antimicrobial peptide, has been shown to integrate into polyethylene oxide (PEO) brush layers; however, the presence of integrated nisin may compromise the protein repulsive character of the PEO layer. The introduction of fibrinogen to nisin-loaded brush layers has been observed to cause changes consistent with partial elution of nisin and/or location of fibrinogen at the interface. Preferential location of fibrinogen on modified surfaces was investigated via FITC-labeling and optical waveguide lightmode spectroscopy (OWLS). Results from each technique indicate that fibrinogen does not adsorb substantially to PEO-coated surfaces, even when the layer is loaded with nisin.

Keywords: nisin adsorption; fibrinogen repulsion; Pluronic® F108; PEO brush layer; optical waveguide lightmode spectroscopy; sequential adsorption
Introduction

The adsorption of nisin (a small, cationic, antimicrobial peptide [1]) and its resistance to elution by fibrinogen at PEO-coated surfaces have been examined through ellipsometry [2], zeta potential [3], and TOF-SIMS [4]. These studies indicate that introduction of fibrinogen to nisin-loaded PEO layers causes changes consistent with partial elution of nisin and/or location of fibrinogen at the interface. Questions surrounding the possibility of fibrinogen adsorption warrant further investigation, as location of procoagulant proteins at a peptide-loaded PEO layer would significantly reduce the viability of a medical device coating based on such an approach.

Blood protein repulsion is largely independent of PEO chain length, when the chain density is sufficiently high (> 0.5/nm²) to produce a brush configuration [5,6,7,8]. Despite their generally protein-repulsive character, theory predicts that sufficiently small proteins may instead be integrated into PEO brushes [9]. Thus, it is fair to hypothesize that, with multilayer adsorption of nisin in a brush, after the outermost peptides are eluted, the PEO segments extending beyond the level of entrapped nisin will again be mobile and provide a steric repulsive barrier to blood protein adsorption (Figure 21). Based on zeta potential and an anti-fibrinogen ELISA assay, Ryder et al. tentatively concluded that nisin is partially eluted from PEO layers in the presence of fibrinogen, but the steric repulsion of the layer was retained [3]. A later study of nisin and fibrinogen adsorption using TOF-SIMS was in qualitative agreement with this conclusion [4]. Here direct and quantitative evidence is provided, from two complementary methods, that sequential introduction of nisin and fibrinogen at a PEO brush layer does not result in substantial fibrinogen adsorption.
Figure 21: Nisin has been shown to integrate into an immobilized PEO brush. It is expected that upon contact with blood proteins, the outermost nisin peptides will elute from the brush, allowing the exposed PEO to regain a protein-repulsive character.

Materials and Methods

Surface modifications and PEO coatings

Silica microspheres (1 µm, Fiber Optic Center, New Bedford, MA) were cleaned and silanized with trichlorovinylsilane (TCVS) in anhydrous CHCl₃, as described by Ryder et al [3]. SiO₂-coated sensors for optical waveguide lightmode spectroscopy (OWLS 210, MicroVacuum, Budapest, Hungary) were cleaned with chromosulphuric acid and silanized at 25 °C with TCVS vapor. TCVS-modified surfaces were coated by adsorption of 10 mg/mL Pluronic® F108 triblocks (PEO₁₄₁-PPO₄₄-PEO₁₄₁, BASF, Mount Olive, NJ) in 10 mM sodium phosphate buffer with 150 mM NaCl, pH 7.4 (PBS), and then γ-irradiated (⁶⁰Co, 0.3 Mrad) to covalently stabilize the PEO brush layers [3,6]. The process is summarized in Figure 22a.

Adsorption of nisin and fibrinogen

Solutions of nisin (3.4 kDa, 0.5 mg/mL, Prime Pharma, Gordons Bay, South Africa), plasminogen-free human fibrinogen (340 kDa, 1 mg/mL, Sigma-Aldrich) and fluorescein isothiocyanate (FITC)-labeled human fibrinogen (1 mg/mL, Molecular Innovations, Novi, MI) in PBS were made and filtered (0.2 µm) immediately before use. TCVS-modified or PEO triblock-coated surfaces were rinsed thrice with PBS, and then individually or sequentially contacted with nisin and/or fibrinogen solutions (Figure 22b).
Quantification of FITC-labeled fibrinogen adsorbed on microspheres

Silica microspheres, with and without F108 coatings, were incubated with nisin (0.05 mg/mL) in PBS or protein-free buffer for 4 h, then rinsed thrice with PBS. The microspheres were then incubated with FITC-labeled fibrinogen solution (or buffer) overnight and rinsed thrice with PBS. The microspheres were dissolved in hot (80-100 °C) 1N NaOH, and the absorbance at 490 nm used to calculate the mass of adsorbed FITC-labeled fibrinogen [10].

Optical waveguide lightmode spectroscopy (OWLS)

OWLS provides real time, label-free detection of protein adsorption on waveguide sensor chips [11]. TCVS-treated OWLS sensors, with and without γ-stabilized F108 coatings, were equilibrated in PBS overnight. After a steady baseline was achieved, 0.5 mg/mL nisin in PBS was injected into the flow loop and flowed over the sensor for 30 min (50 µL/min). The sensor was then rinsed with PBS for 30 min, after which fibrinogen (1 mg/mL) was injected into the flow loop for 30 min. The sensors were then rinsed with flowing PBS for 30 min. Protein-free PBS was substituted for the protein solutions as appropriate.
Results and Discussion

Quantification of FITC-labeled fibrinogen on microspheres

The surface loading of adsorbed FITC-fibrinogen was estimated from the measured $A_{490}$ and the manufacturer-specified $F/P$ ratio of the fluorescein-labeled protein in the dissolved silica solution. The results, normalized to the negative control (hydrophobic TCVS silica), are presented in Figure 3. Qualitatively, the presence of FITC-labeled fibrinogen is clearly visible as a yellow tint on the microspheres without PEO coatings, while the PEO coated surfaces exhibit much less color. Quantitatively, significantly less FITC-fibrinogen adsorbed on surfaces coated with PEO by immobilization of Pluronic® F108. Further, the presence of nisin did not significantly affect the adsorption of FITC-fibrinogen on either PEO-coated or uncoated microspheres. The protein-repellent nature of the PEO coating is apparently unaffected by the presence of nisin in the brush.

Figure 23: Relative adsorption of FITC-labeled fibrinogen onto hydrophobic TCVS and PEO-coated 1-µm silica microspheres (top). Visible yellow tint (bottom) indicates adsorption of FITC-fibrinogen onto microsphere surfaces.
Optical Waveguide Lightmode Spectroscopy (OWLS)

In a representative experiment, cyclic adsorption of nisin reached ~230 ng/cm² on a hydrophobic, TCVS-treated sensor then desorbed to approximately 215 ng/cm² upon rinsing with PBS (Figure 24, top). In contrast, adsorption of nisin was decreased (~125 ng/cm²) on an F108-coated sensor, and desorbed to ~70 ng/cm² after rinsing with PBS. Non-zero adsorption and resistance to elution of nisin is attributed to entrapment of the small peptide in the PEO brush.

Fibrinogen also adsorbed substantially onto uncoated, hydrophobic TCVS-treated sensors. Adsorption of fibrinogen on a sensor previously exposed to nisin was less than a protein-free surface (60 vs. 260 ng/cm², respectively). This difference is likely due to decreased available surface area and competitive displacement or coverage of the adsorbed nisin by the much-larger fibrinogen, and is consistent with previous results of nisin/fibrinogen adsorption on uncoated polymer surfaces. Fibrinogen was not substantially desorbed from the uncoated sensors during rinsing. The slight increase in adsorbed mass during rinsing of the TCVS without nisin sensor was attributed to residual protein in the flow loop.

In contrast, OWLS sensors coated with a γ-stabilized F108 PEO brush layer exhibited markedly lower adsorption of fibrinogen (<25 ng/cm²), clearly demonstrating the repulsion of large proteins by the PEO brush (Figure 4, bottom). Importantly, the presence of pre-adsorbed nisin in the brush had no effect on the adsorption of fibrinogen onto F108-coated sensors. The PEO brush retained its steric repulsive activity against fibrinogen when loaded with substantial quantities of nisin, despite the potential for electrostatic attraction between the two proteins.
Figure 24: Adsorption and desorption of nisin (top), and subsequent adsorption and desorption of fibrinogen (bottom) onto hydrophobic TCVS or PEO-coated OWLS sensors.

Acknowledgments

This work was supported in part by the National Institute of Biomedical Imaging and Bioengineering (Grant No. R01EB011567). The content is solely the responsibility of the authors and does not necessarily represent the official views of NIBIB or the National Institutes of Health. The authors thank Allvivo Vascular Inc. for invaluable assistance on this project.
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