AN ABSTRACT OF THE DISSERTATION OF

Yuan-Ching Tai for the degree of Doctor of Philosophy in Chemical Engineering presented on April 19, 2007.
Title: Nisin Adsorption and Function at Hydrophobic Surfaces Coated with the Poly[ethylene oxide]-Poly[propylene oxide]-Poly[ethylene oxide] Triblock Surfactant Pluronic® F108.

Abstract approved:

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Joseph McGuire

The adsorption and elution of the antimicrobial peptide nisin at hydrophobic, silanized silica surfaces coated with the poly[ethylene oxide]-poly[propylene oxide]-poly[ethylene oxide] surfactant Pluronic® F108 was measured *in situ*, with ellipsometry. While such layers are known to inhibit protein adsorption, nisin was observed to adsorb in multilayer quantities, to an extent similar to its adsorption at uncoated, hydrophobic surfaces. The rates of nisin adsorption and elution were generally slower at F108-coated surfaces. And, the sequential adsorption of nisin, including two adsorption-elution cycles at each surface, showed greater differences in adsorption rates between the first and second adsorption cycles, when evaluated at identical mass density, for uncoated relative to F108-coated surfaces. These results indicate that nisin adsorption occurs via "entrapment" within the PEO brush layer at F108-coated surfaces, in this way slowing adsorption and spontaneous elution, and inhibiting post-adsorptive molecular rearrangements by reducing the lateral mobility of nisin. While F108-coated layers rejected adsorption of serum albumin, sequential adsorption experiments carried out with

nisin and albumin showed a low level of albumin adsorption when nisin was present at the interface.

The activity of these nisin-loaded layers was evaluated in the presence and absence of blood proteins, after contact periods up to one week. While an increase in serum protein concentration reduced the activity of nisin retained on both the bare hydrophobic and F108-coated surfaces of these materials, the F108-coated surfaces retained more antimicrobial activity than the bare surfaces. These results support the notion that the pendant PEO chains of the F108 coating inhibited the exchange of nisin by blood proteins. F108-coated microspheres and polyurethane catheter segments were observed to retain more antimicrobial activity than their uncoated counterparts in the absence of blood proteins as well, but the difference in function between bare and F108-coated substrates was most pronounced in the presence of blood proteins. Circular dichroism spectroscopy studies conducted with nisin in the presence of F108-coated and uncoated, silanized silica nanoparticles suggested that nisin experienced conformational rearrangement at a greater rate and to a greater extent on bare hydrophobic relative to F108-coated surfaces.

Nisin Adsorption and Function at Hydrophobic Surfaces Coated with the Poly[ethylene oxide]-Poly[propylene oxide]-Poly[ethylene oxide] Triblock Surfactant Pluronic® F108

by

Yuan-Ching Tai

A DISSERTATION

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Doctor of Philosophy dissertation of Yuan-Ching Tai presented on April 19, 2007

APPROVED:

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Major Professor, representing Chemical Engineering

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Chair of the Department of Chemical Engineering

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Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

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Yuan-Ching Tai, Author

ACKNOWLEDGMENTS

In spring 2003, I quit after six years of a stable job in south Texas and moved to Corvallis, Oregon to pursue my Ph.D. degree. It was not an easy decision to make then, especially for a married man at my age. But I felt that it was about time to make a constructive move, a change, in my life, and I believed that would be the right decision to make. Turned out that was indeed a good decision, and I have never regretted it ever since - - this has been such a great time in my life. During my four years at Oregon State University, Dr. Joseph McGuire, my major professor, accepted me, supported me and guided me in my research and personal life too. There were ups and downs during the years. No matter what happened, Joe always had this nearly blind confidence in me and encouraged me. I would not be where I am today, if it were not for Joe.

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CONTRIBUTION OF AUTHORS

Dr. Jennifer A. Neff synthesized some of the materials used in experiments described in Chapter 2, and was involved in the work related to Chapters 2 and 3. Dr. Pranav Joshi performed experiments with end-group activated triblock polymers summarized in Chapter 2, and providing some of the motivation for the ellipsometric investigation.

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DEDICATION

For our beloved daughter Helen. We love you so much.

NISIN ADSORPTION AND FUNCTION AT HYDROPHOBIC SURFACES COATED WITH THE POLY[ETHYLENE OXIDE]-POLY[PROPYLENE OXIDE]-POLY[ETHYLENE OXIDE] TRIBLOCK SURFACTANT PLURONIC® F108

CHAPTER 1

INTRODUCTION

Controlling biological interactions with materials is of great importance to the design of permanent as well as resorbable implants, cell and tissue scaffolds, and diagnostic probes, among others. Implant-associated infections can result in systemic infections that in the worst-case scenario can lead to multiple organ failure and death. Most materials used in medical devices are susceptible to bacterial adhesion. Once bacteria adhere to a solid surface, they generate and become embedded within a polysaccharide matrix to form a biofilm. Both the host immune system and antimicrobials become less effective against the bacteria due in part to difficulty in penetrating the biofilm matrix. To reduce morbidity and mortality due to device related infections, it is critical to prevent biofilm formation. Controlling biological interactions with materials is therefore of great importance to the design of permanent and resorbable medical devices.

A number of antimicrobial coatings have been evaluated for their ability to reduce the incidence of implant-related sepsis. But in general, the prophylactic use of antibiotic-coated implants increases the risk of producing resistant strains of bacteria, while the use of other kinds of antibacterial compounds (antiseptics) provides inferior results in comparison to the use of clinical antibiotics. Lantibiotics are antibiotic compounds that include one or more lanthionine rings. The unique physical structure of lantibiotics makes them different in mode-of-action from traditional antibiotics, suggesting that they offer a

means for preventing the rise of resistant microorganisms. Nisin, a lantibiotic, kills Gram-positive bacteria through a multi-step process that destabilizes the phospholipid bilayer of the cell and creates transient pores.

Nisin can adsorb to surfaces, maintain activity, and kill cells that have adhered, *in vitro*. Application of nisin-coated implants *in vivo* was reported by Bower et al. (2002), but the duration of coating activity was short. The nisin was bound by non-specific adsorption to hydrophobic catheter materials in that work, and the loss in activity could be attributed to nisin exchange with blood proteins at the surface. Since that time considerable effort in our laboratory had been placed on "tethering" nisin to solid surfaces for long-term antibacterial activity, in a manner allowing the level of solvent accessibility and molecular mobility needed for membrane binding, insertion, and pore formation to be preserved (Joshi et al., 2006). This was carried out by introduction of thiolated nisin to polystyrene microsphere surfaces to which pyridyl disulfide-activated, PEO-PPO-PEO triblock polymers had been attached. The disulfide-linked, nisin-containing block copolymers were evaluated for activity against the Gram positive indicator strain *Pediococcus pentosaceus*. Thiol-modified nisin was introduced to (unactivated) PEO-PPO-PEO triblocks in similar experiments for controlled comparison of antimicrobial activity, in all cases. It was routinely determined, however, that antibacterial activity with the unactivated samples, even through repeat washing, was not significantly different than that recorded with tethered nisin. These findings suggested that some amount of nisin was apparently entrapped in the PEO chains of the triblocks, possibly involving specific physical interactions between nisin and the PEO chains that stabilize the entrapment.

We investigated that possibility, by study of the adsorption and elution behavior of nisin at uncoated and PEO-PPO-PEO triblock-coated hydrophobic surfaces using *in situ* ellipsometry. That work is described in Chapter 2. In Chapter 3 we summarize an evaluation of the antimicrobial activity of polystyrene microsphere and polyurethane catheter segment surfaces, coated with nisin-loaded, PEO-PPO-PEO triblock layers. Bioactivity measurements were made in the presence and absence of blood proteins. Chapter 3 also includes an investigation of adsorbed nisin structure, by application of circular dichroism spectroscopy of nisin in the presence of F108-coated and uncoated, silanized silica nanoparticles.

NISIN ADSORPTION TO HYDROPHOBIC SURFACES COATED WITH THE PEO-PPO-PEO TRIBLOCK SURFACTANT PLURONIC® F108

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CHAPTER 2

NISIN ADSORPTION TO HYDROPHOBIC SURFACES COATED WITH THE PEO-PPO-PEO TRIBLOCK SURFACTANT PLURONIC® F108

Abstract

The adsorption and elution of the antimicrobial peptide nisin at hydrophobic, silanized silica surfaces coated with the poly[ethylene oxide]-poly[propylene oxide]-poly[ethylene oxide] surfactant Pluronic® F108 was measured *in situ*, with ellipsometry. While such layers are known to inhibit protein adsorption, nisin was observed to adsorb in multilayer quantities, to an extent similar to its adsorption at uncoated, hydrophobic surfaces. The rates of nisin adsorption and elution were generally slower at F108-coated surfaces. And, the sequential adsorption of nisin, including two adsorption-elution cycles at each surface, showed greater differences in adsorption rates between the first and second adsorption cycles, when evaluated at identical mass density, for uncoated relative to F108-coated surfaces. These results indicate that nisin adsorption occurs via "entrapment" within the PEO brush layer at F108-coated surfaces, in this way slowing adsorption and spontaneous elution, and inhibiting post-adsorptive molecular rearrangements by reducing the lateral mobility of nisin. While F108-coated layers rejected adsorption of serum albumin, sequential adsorption experiments carried out with nisin and albumin showed a low level of albumin adsorption when nisin was present at the interface.

Key Words: nisin adsorption kinetics, *in situ* ellipsometry, Pluronic® F108, PEO-PPO-PEO triblock surfactants

2.1 Introduction

Controlling biological interactions with materials is of great importance to the design of permanent as well as resorbable implants, cell and tissue scaffolds, and diagnostic probes, among others. Most materials used in medical devices are susceptible to bacterial adhesion. Once bacteria adhere to a solid surface, they generate and become embedded within a polysaccharide matrix to form a biofilm. Both the host immune system and antimicrobials become less effective against the bacteria due in part to difficulty in penetrating the biofilm matrix. In addition, bacteria in biofilms have more capacity to develop resistance to antimicrobials. To reduce morbidity and mortality due to device related infections, it is critical to prevent biofilm formation.

Implant-associated infections can result in systemic infections that in the worst-case scenario can lead to multiple organ failure and death, despite successful resolution of the original medical condition. The cost of such infections is high. For example, a single episode of central venous catheter-related bacteremia has been estimated to cost up to $50,000 with an attributable mortality rate between 4 and 35% (Beekman et al., 2005). Infection is also a major problem for dialysis patients. Over 450,000 people in the US alone have end stage kidney failure and require chronic hemodialysis. For these patients, vascular access procedures are a major cause of morbidity and mortality. AV grafts are used in about 42% of patients with an infection rate between 11 and 20%. The mortality rate due to infection of these grafts is between 12 and 22%.

A number of antimicrobial coatings have been evaluated for their ability to reduce the incidence of implant-related sepsis. But in general, the prophylactic use of antibiotic-coated implants increases the risk of producing resistant strains of bacteria, while the use

of other kinds of antibacterial compounds (antiseptics) provides inferior results in comparison to the use of clinical antibiotics. Lantibiotics are antibiotic compounds that include one or more lanthionine rings. The unique physical structure of lantibiotics makes them different in mode-of-action from traditional antibiotics, suggesting that they offer a means for preventing the rise of resistant microorganisms (van den Hooven et al., 1996; Wiedemann et al., 2001; van Heusden et al., 2002; Hsu et al., 2004). Lantibiotics such as nisin can adsorb to surfaces, maintain activity, and kill cells that have adhered *in vitro* (Bower et al., 1995; 2001; 2002), The structure of nisin, by far the most extensively investigated lantibiotic with reference to biomaterials applications, is shown schematically in Fig. 1. *Staphylococcus aureus* and *Staphylococcus epidermidis* are the most frequently encountered biomaterial-associated pathogens (Dugdale, 1990; Raad, 1992), and both are Gram-positive bacteria. Nisin kills Gram-positive bacteria through a multi-step process that destabilizes the phospholipid bilayer of the cell and creates transient pores.

Application of nisin-coated implants *in vivo* was reported by Bower et al. (2002), but the duration of coating activity was short. The nisin was bound by non-specific adsorption to hydrophobic catheter materials in that work, and the loss in activity could be attributed to nisin exchange with blood proteins at the surface. Since that time considerable effort in our laboratory has been placed on “tethering” nisin to solid surfaces for long-term antibacterial activity, in a manner allowing the level of solvent accessibility and molecular mobility needed for membrane binding, insertion, and pore formation to be preserved (Joshi et al., 2006). In particular we have synthesized thiol-modified nisin derivatives, by chemical modification of the primary amine group at the N-terminal residue. These were then chemically coupled to end-group activated poly[ethylene oxide]-poly[propylene oxide]-poly[ethylene oxide] (PEO-PPO-PEO) triblock polymers to form nisin-containing

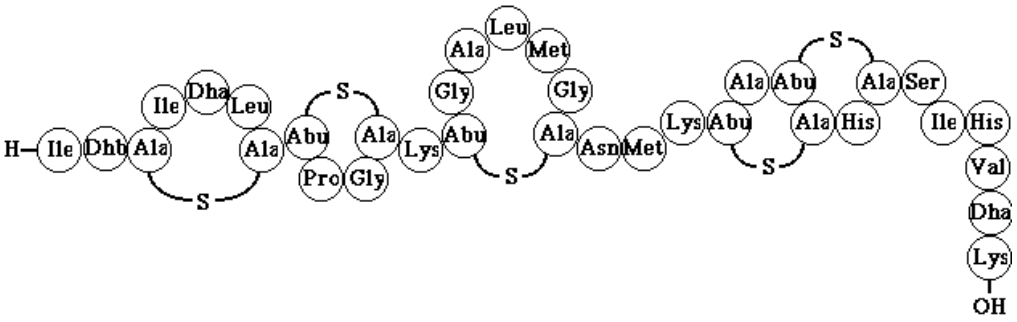

Figure 2.1 Schematic of nisin A. Abu: 2-aminobutyric acid; Dha: Dehydroalanine; Dhb: Dehydrobutyrine.

block copolymers. The end-group activated triblocks were produced from the Pluronic® surfactant F108 (MW 14,600, with two end blocks of PEO that are 129 monomer units in length and a center block of PPO that is 56 monomer units in length, i.e., HO-$(CH_2$-CH_2-$O)_{129}$-$(CHCH_3$-CH_2-$O)_{56}$-$(CH_2$-CH_2-$O)_{129}$-H). End-group activation involved replacing the terminal hydroxyl groups of the PEO chains with pyridyl disulfide moieties. Nisin was secured to the PEO chains in an "end-on" orientation, through a disulfide linkage.

This reaction was carried out by introduction of thiolated nisin to polystyrene microsphere surfaces to which the end-activated triblocks had been attached (via hydrophobic association of the PPO block with the polystyrene surface), and was also carried out in homogeneous liquid phase. The disulfide-linked, nisin-containing block copolymers were separated from unreacted nisin by dialysis, and evaluated for activity against the Gram positive indicator strain *Pediococcus pentosaceus*. Thiol-modified nisin was introduced to (unactivated) Pluronic® F108 in similar experiments for controlled comparison of antimicrobial activity, in all cases. We routinely determined, however, that antibacterial activity with the unactivated F108 samples (i.e., the "control" samples), even through repeat washing, was not significantly different than that recorded with disulfide-linked nisin. These findings suggested that some amount of nisin was apparently entrapped in the PEO chains of the F108 triblocks, possibly involving specific physical interactions between nisin and the PEO chains that stabilize the entrapment.

It is well known that PEO resists protein interactions and F108 coated surfaces in particular have been shown to prevent protein adsorption (Li and Caldwell, 1991; Green et al., 1998; Neff et al., 1999). It was therefore believed that nisin would not interact with the PEO-PPO-PEO triblocks, other than at the reactive site on the end-activated samples. The

ability of the Pluronic® surfactant to entrap nisin was thus unexpected. Exploring the interaction between nisin and PEO layers is important, as better understanding in this context may provide direction for improved anti-infective function in coated, implantable devices, and improved strategies for drug loading and release from such devices, among others. In addition, the protein rejection properties of the F108 layer, if retained after nisin adsorption, would inhibit exchange of the peptide by blood proteins and in this way improve the potency of a coating prepared through non-specific adsorption of the biologically active agent.

In this paper we describe the adsorption and elution of nisin at silanized silica surfaces coated with the Pluronic® F108. Pendent PEO chains were immobilized on the surface through hydrophobic interaction between the apolar PPO block and the hydrophobic silica. Nisin adsorption and elution kinetics were measured *in situ*, with ellipsometry.

2.2 Materials and Methods

Protein and surfactant solutions

Nisin was obtained from Prime Pharma (Batch number 20050116-1, Gordons Bay, South Africa) and was dissolved in filtered, 10 mM monobasic sodium phosphate solution to ensure complete solubilization. Filtered, 10 mM dibasic sodium phosphate was then added to bring the pH to 7.0. Bovine serum albumin (BSA; Sigma-Aldrich) was dissolved in filtered, 10 mM sodium phosphate buffer at pH 7.0. Solutions of nisin and BSA (each at 5.0 mg/mL) were aliquoted into 0.5 mL samples and stored at -80 °C; they were thawed immediately before use. Pluronic® F108 was purchased from BASF (Mount Olive, NJ) and was dissolved in 10 mM phosphate buffer (pH 7) as needed.

Surface modification

Silica wafers (Type P, DOP B, thickness 525 ± 18 μm, resistivity 0.01 - 0.02 ohm·cm; WaferNet Inc., San Jose, CA) were cut into 1×5 cm strips using a tungsten pen. The silica strips were rinsed with acetone to remove dust and organic residues, washed in a solution of NH_4OH:H_2O_2:H_2O (1:1:5 volume ratio) at 80 °C for 10 min, then washed in a solution of HCl:H_2O_2:H_2O (1:1:5 volume ratio) at 80 °C for 10 min. They were then rinsed with deionized water and pure ethanol, and blown dry with a nitrogen stream. From this point, two methods were used to render the silica surfaces sufficiently hydrophobic for F108 coating. In one method, the dried silica strips were placed in a glass dish and transferred to a vacuum oven (200 °C) housing an uncovered, 2 mL volume of hexamethyldisilzane (HMDS, Product number H7300, United Chemical Technologies, Bristol, PA). HMDS vapor deposition was allowed to occur for 90 min, at 20-25 in. Hg vacuum and 200 °C. In another method, the cleaned, dried silica strips were transferred to a solution of 5% (V/V) PS200 ("Glassclad 18", Product number PS200, United Chemical Technologies, Bristol, PA) in water and agitated for 15-20 s. The samples were then rinsed thoroughly with deionized water and cured at 100 °C for 1 h. The hydrophobicities of surfaces coated in these ways were measured by contact angle analysis. The contact angle was typically between 85° and 95° following HMDS treatment, and between 65° and 75° following PS200 treatment. Silanized silica surfaces were stored in an electronic desiccator until needed.

Surface coating with Pluronic® F108

HMDS- or PS200-treated samples were coated with F108 triblocks by incubation with 0.50% F108 in 10 mM sodium phosphate buffer. Incubation took place overnight (at least

16 h), in the fused quartz trapezoid cuvette used in the ellipsometry experiments. The coated sample was rinsed, *in situ*, with multiple cuvette volumes of phosphate buffer prior to the introduction of protein.

PEO coatings were also prepared by covalent attachment of the F108 triblocks. McPherson et al. (1997) and Park et al. (2000) described pretreatment with a silane and subsequent γ-irradiation to covalently bind PEO-PPO-PEO triblocks (via the PPO block) to glass, metal and pyrolytic carbon surfaces. In short, through absorption of radiation or interaction with water-derived radicals, surface-bound free radicals are formed. These free radicals attack the adsorbed PPO block, forming new covalent bonds between the surface and polymer (McPherson et al., 1997). In this work, the F108 triblocks were covalently attached according to methods similar to those cited above. In brief, the silica samples were silanized with octadecyltrimethoxysilane, and the surface-polymer covalent attachment induced by uv radiation. After uv treatment, samples were washed with PBS.

In situ ellipsometry

A silica sample (coated with Pluronic® F108 or uncoated) was placed into a fused quartz, trapezoid cuvette (Hellma Cells, Germany) which was secured on the sample stage of an automatic *in situ* ellipsometer (Model L-104 SA, Gaertner Scientific Corp., Chicago, IL) modified to allow for stirring and flow. After 4.5 mL of 10 mM phosphate buffer (pH 7.0) was injected into the cuvette, the ellipsometer stage was adjusted to obtain a maximum in reflected light intensity and steady optical properties. Surface optical properties were recorded every 15 s for 30 min before 0.5 mL of protein or Pluronic F108 solution was injected into the cuvette to yield a final protein concentration of 0.50 mg/mL, or final F108 concentration of 0.5 % (w/v). Adsorption was allowed to occur for a desired period

of time, after which the surface was rinsed *in situ* with 10 mM sodium phosphate buffer at a flow rate of 31.6 mL/min for 5 min. Film properties were then monitored for a desired "incubation" period. Any additional protein adsorption and rinsing-incubation steps carried out in a given experiment were performed in the same manner described above.

A one-film-model ellipsometry program (Krisdhasima et al., 1992) was used to determine the adsorbed mass of protein from the ellipsometrically determined values of film thickness and refractive index according to Cuypers et al. (1983). A partial specific volume of 3.837 mL/g and a molecular weight to molar refractivity ratio of 0.729 g/mL. Each experiment was performed at least twice on each type of surface, with an average deviation from the mean of about 0.005 $\mu g/cm^2$.

2.3 Results and Discussion

F108 adsorption at hydrophobic silica

Figure 2.2 provides representative results for adsorption of F108 on a (PS200) silanized silica surface, rinsed sequentially at 60 and 90 min. The surface concentration of F108 reached a plateau immediately after its introduction to the cuvette, indicative of a high affinity adsorption at the hydrophobic surface, and consistent with adsorption patterns recorded for a number of small-molecule surfactants (Arnebrant and Wahlgren, 1995). The adsorbed mass decreased somewhat after the first rinse, and this can be attributed to the elution of loosely bound triblocks. No such decrease in adsorbed mass was recorded after the second rinse step. (We note here that the PS200-treated silica samples supported a more stable F108 coating for monitoring protein adsorption and elution kinetics. Unless otherwise indicated, all F108-coatings represented by the data presented here were prepared from PS200-treated substrates.)

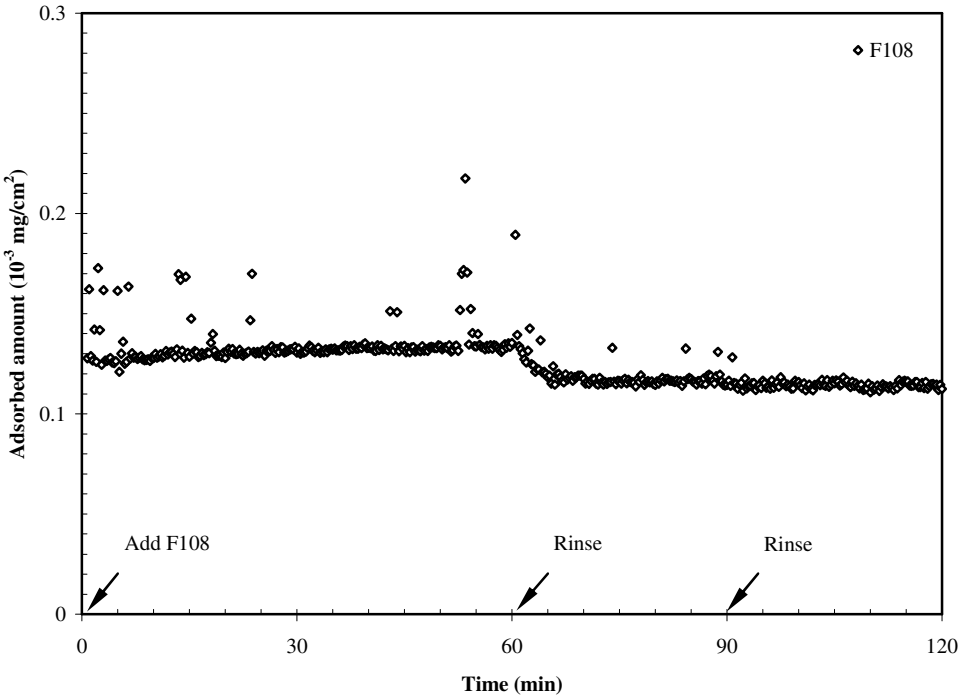

Figure 2.2 Pluronic F108 adsorption and elution on a hydrophobic surface.

Nisin adsorption and elution kinetics

Figure 2.3 provides a representative comparison of nisin adsorption and elution kinetics at bare hydrophobic and F108-coated surfaces. Nisin adsorption did not reach a plateau in either case, and based on dimensions of nisin in solution (if modeled as a cylinder, about 2 x 5 nm) a monolayer of molecules adsorbed "side-on" (i.e., in an area equal to 2 x 5 = 10 nm^2) and "end-on" (i.e., in an area equal to 2 x 2 = 4 nm^2) would result in an adsorbed mass of 0.058 and 0.145 $\mu g/cm^2$, respectively. Thus the patterns shown in Fig. 2.3 are probably consistent with multi-layer adsorption in each case. Nisin adsorption to the F108-coated surface was generally slower than adsorption to the bare hydrophobic surface, likely owing to steric inhibition by the pendant PEO chains. Nisin elution in protein-free buffer was similar at each surface initially, but elution was observed to continue only at the bare hydrophobic surface. Presumably, this is due to greater solvent accessibility to nisin that is loosely held in the outer layers in the case of adsorption to the uncoated surface, while nisin integrated into the PEO "brush" would show greater resistance to elution.

The effect of the PEO chains in adsorption and elution can be further revealed by analysis of nisin adsorption-elution data with reference to a "history dependent" adsorption mechanism (Calonder et al., 2001; Tie et al., 2003). A number of macromolecular species, including proteins, exhibit history dependent adsorption behavior owing to the slow relaxation of non-equilibrium structures at the interface. That is, for a given protein at a given surface loading, the rate of adsorption depends on the formation history of the adsorbed layer. This is particularly relevant near monolayer surface coverage when protein-protein interactions can influence the availability of surface area suitable for

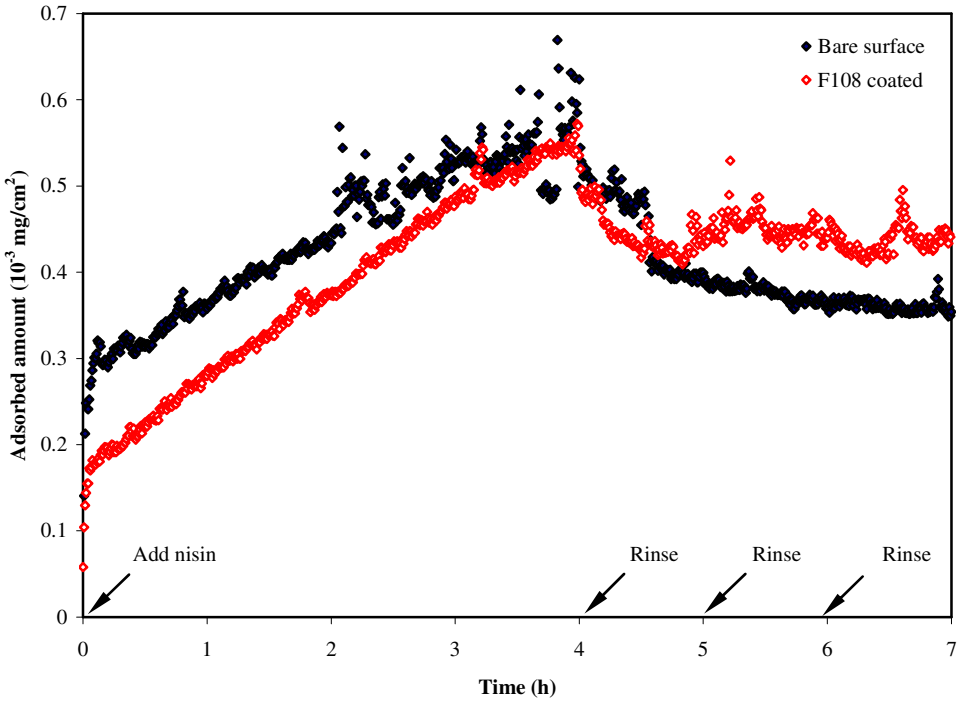

Figure 2.3 Comparison of nisin adsorption and elution kinetics at bare hydrophobic and F108-coated surfaces.

adsorption. Tie et al (2003) studied the adsorption of fibronectin, cytochrome c and lysozyme using optical waveguide lightmode spectroscopy in multi-step mode, where an adsorbing surface is alternately exposed to a protein solution and a solution free of protein. In general, they observed the initial adsorption rate during the second step exceeded that observed at the same surface coverage during the first step. They postulated that, for a given mass density at an interface, if proteins were arranged in "clusters" or aggregates, more "cleared" surface area would be available for further adsorption relative to proteins being randomly distributed. On the other hand, if the adsorbed protein films were at equilibrium, we would expect the same adsorption rates during each cycle, since the proteins would have identical structural characteristics.

Adsorption rate data can thus provide important information relevant to adsorbed layer structure, and we used the kind of sequential adsorption kinetic data presented in Fig. 2.4 for this purpose. Figure 2.4 shows results of a two, nisin adsorption-elution steps performed in sequence at a bare hydrophobic surface and an F108-coated surface. For each surface, the initial slope of the second adsorption step was compared to the slope at the point in the first adsorption cycle with the same initial mass density. At both the coated and uncoated surface, the initial adsorption rate during the second step exceeded that observed at the same surface coverage during the first step. But the increase in slope was substantial in the case of uncoated silica as compared to F108-coated silica. That is, the first and second step adsorption rates were not as different for the F108-coated surface, indicating that less post-adsorptive rearrangement (or less "clustering") of nisin occurred in this case. We suggest the lateral mobility needed for cluster formation and the generation of unoccupied surface area was inhibited by the PEO chains.

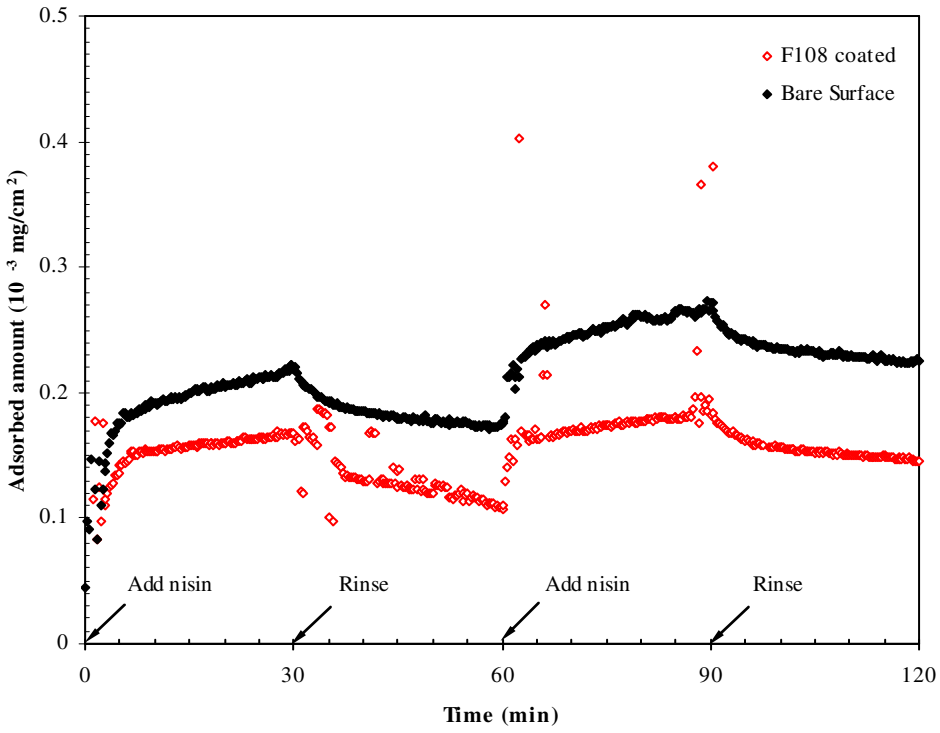

Figure 2.4 Sequential adsorption and elution kinetics exhibited by nisin at a bare hydrophobic and F108-coated surface.

Functional evaluation of the F108 layer

Figure 2.5 shows two representative plots of nisin adsorption and elution kinetics recorded on F108-coated surfaces. In one case the F108 triblocks were coated as described here (i.e., incubation of the silanized hydrophobic surface overnight with 0.5% F108, followed by buffer rinse to remove loosely bound F108 from the surface and replace the F108 solution with buffer), and in the other case the F108 triblocks were coated by covalent attachment via the PPO chains. As shown in Fig. 2.5, the nisin adsorption and elution kinetics were quite similar at each surface. In regard to nisin adsorption, this indicates that the F108 layers prepared by hydrophobic association between the PPO block and the silanized surface functioned in a manner substantially similar to the F108 layers prepared by covalent attachment between the PPO block and silanized surface.

While nisin was clearly observed to adsorb in multi-layer quantities at the F108-coated surfaces, these surfaces retained their "large" protein rejecting properties as shown in the case of BSA adsorption in Figs. 2.6 and 2.7. Figure 2.6 shows no change in adsorbed mass recorded upon introduction of BSA (MW 66 kDa) to an F108 layer prepared by 22 h incubation of the surface with F108 solution, and no change in adsorbed mass recorded upon rinsing and incubation in protein-free buffer. This indicates that BSA was rejected by the F108 coated surface. Figure 2.7 shows results of a similar experiment, but where F108 was incubated with the surface for only 30 min prior to rinsing. Again, no change in adsorbed mass was recorded upon introduction of BSA to the F108 layer, suggesting that the triblocks adopted their energetically favorable molecular orientation, with PPO block associating with the surface and pendant PEO chains extending away from the surface, in a fairly short period of time.

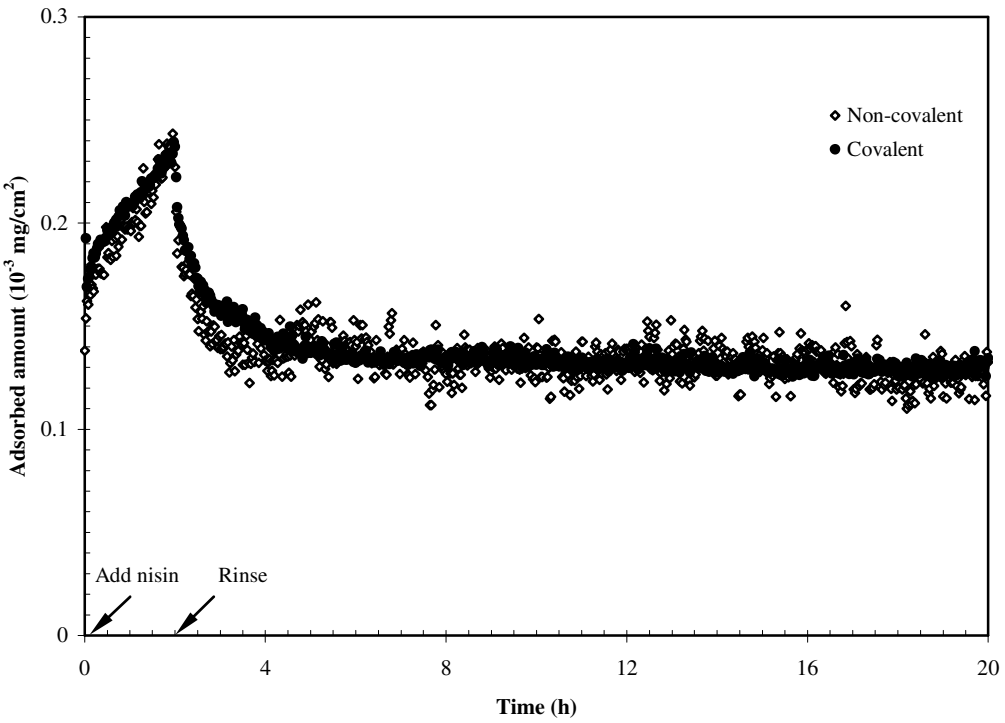

Figure 2.5 Nisin adsorption and elution kinetics recorded at hydrophobic surfaces coated with F108 triblocks by hydrophobic association, and by covalent attachment.

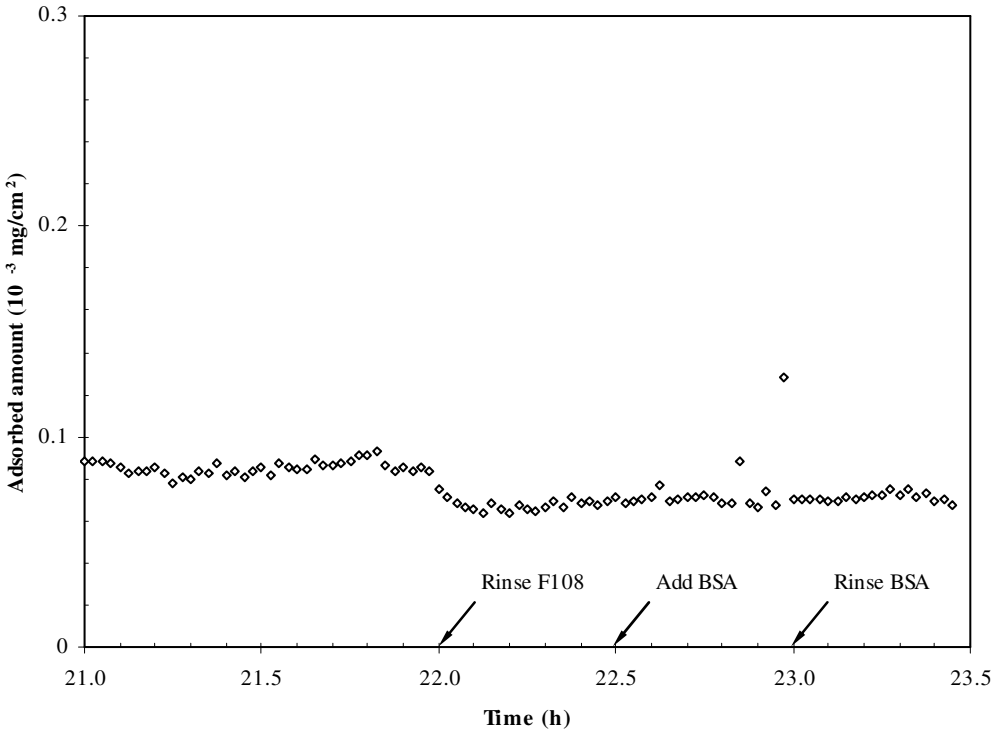

Figure 2.6 BSA adsorption and elution on an F108-coated surface prepared by incubation for 22 h followed by 30 min incubation in phosphate buffer.

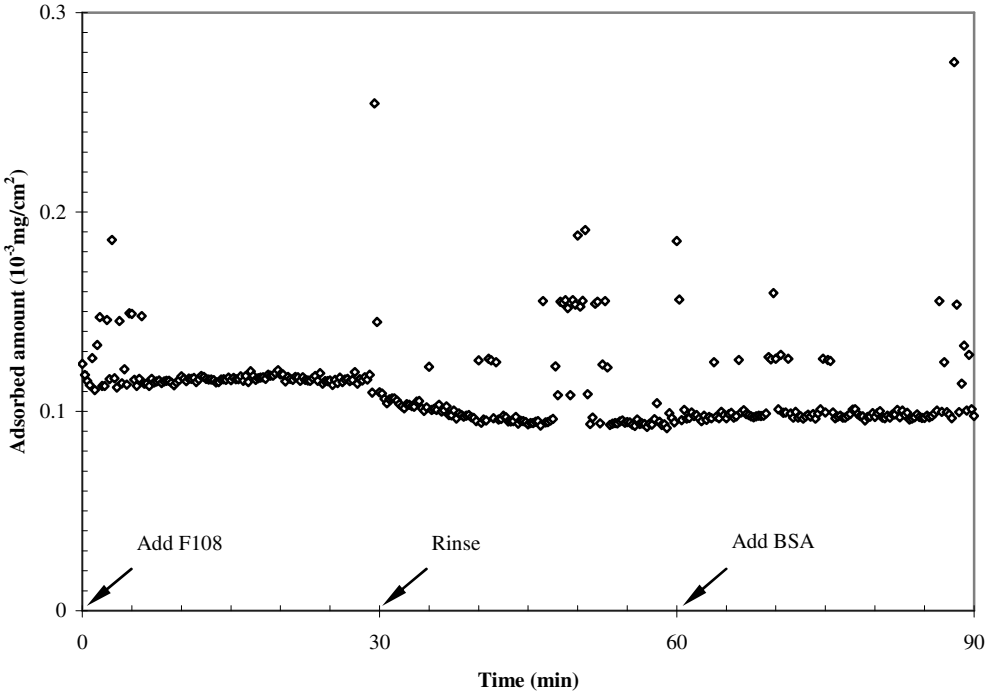

Figure 2.7 BSA adsorption and elution at an F108-coated surface prepared by incubation for 30 min followed by 30 min incubation in phosphate buffer.

Figures 2.8 and 2.9 show representative results of sequential adsorption experiments performed with nisin and BSA at F108-coated surfaces. As shown in Fig. 2.8, when BSA is introduced first, scattered data show adsorption of BSA to be of very low affinity. (Adsorption data recorded for BSA at F108-coated surfaces, interpreted with reference to optical constants of the coated surface - - as opposed to optical constants of the bare, silanized surface as done in Figs. 2.6 and 2.7 - - were not easily reproducible, due to what we conclude are very low values of adsorbed mass. In particular, when the change in optical properties of a film (relative to the reference substrate optical properties) is below a certain threshold, the mathematical routine used here to determine film thickness and refractive index does not yield reliable results, instead often reporting negative values accompanied by noise. The data shown in Fig. 2.8 for BSA adsorption should therefore be taken only as an indication that the affinity of BSA for the F108-coated surface is very low.) Following a rinse and 30 min incubation of the surface in protein-free buffer, adsorbed mass increased immediately upon introduction of nisin. Also as shown in Figure 2.8, BSA adsorption on bare hydrophobic surface showed a relatively higher adsorbed mass and stability. Figure 2.9 shows that when nisin is introduced first, an increase in adsorbed mass is evident upon introduction of BSA. This may be attributed to electrostatic attraction between the cationic peptide nisin and the negatively-charged BSA. This effect must be further explored in terms of extent of adsorption and tightness of serum protein binding. But it is important to note that these tests were conducted at a low ionic strength relative to human blood, where electrostatic charges are more effectively masked, reducing electrostatic attractions like that suggested in Fig. 2.9.

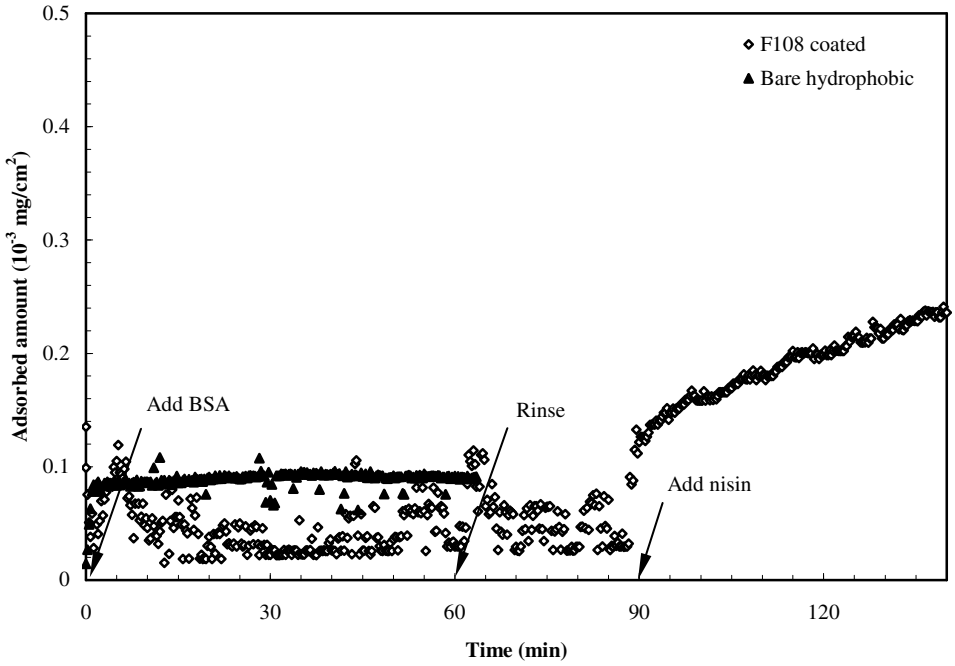

Figure 2.8 Sequential adsorption of BSA and nisin on F108-coated surface and BSA adsorption on bare hydrophobic surface for 60 minutes

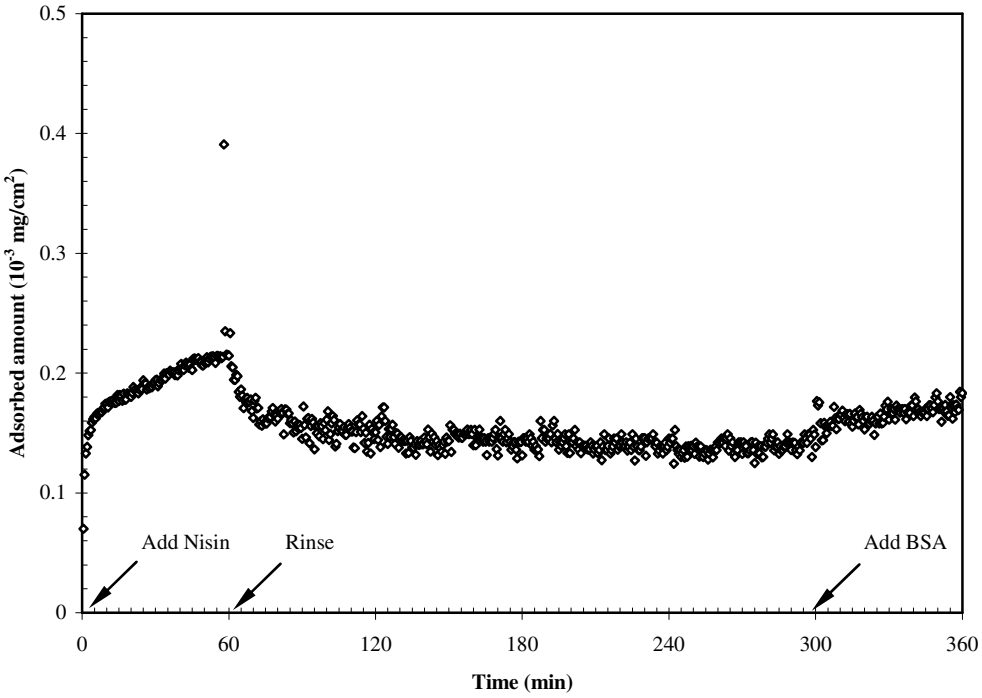

Figure 2.9 Sequential adsorption of nisin and BSA on an F108-coated surface

2.4 Conclusions

The adsorption of the antimicrobial peptide nisin to stable, adsorbed layers of the PEO-PPO-PEO triblock surfactant Pluronic® F108 was quantified by *in situ* ellipsometry. While it is well understood that such layers inhibit protein adsorption, nisin, presumably due to its relatively small size, was able to adsorb in multilayer quantities. Comparison of nisin adsorption and elution kinetics at uncoated and F108-coated surfaces suggested that the rate of nisin adsorption and elution, and its lateral mobility at the interface, were generally greater at uncoated surfaces. This would be consistent with nisin adsorption or "entrapment" within the PEO brush layer, as opposed to adsorption to the PEO chains in a non-penetrating manner. F108-coated layers rejected adsorption of the protein BSA. However, sequential adsorption experiments carried out with nisin and BSA showed measurable adsorption of BSA if nisin was present at the interface. These phenomena, along with evaluation of the structure and biological activity of nisin in such layers, are currently under study and will contribute to the subject of a future report.

Acknowledgement

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NISIN ANTIMICROBIAL ACTIVITY AT HYDROPHOBIC SURFACES COATED WITH THE PEO-PPO-PEO TRIBLOCK SURFACTANT PLURONIC® F108

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CHAPTER 3

NISIN ANTIMICROBIAL ACTIVITY AT HYDROPHOBIC SURFACES COATED WITH THE PEO-PPO-PEO TRIBLOCK SURFACTANT PLURONIC® F108

Abstract

The antimicrobial peptide nisin has been observed to adsorb at hydrophobic surfaces coated with the poly[ethylene oxide]-poly[propylene oxide]-poly[ethylene oxide] (PEO-PPO-PEO) surfactant Pluronic® F108, to an extent similar to its adsorption at uncoated, hydrophobic surfaces. While pendant PEO chains are known to inhibit protein adsorption, nisin adsorption presumably occurs via "entrapment" within the PEO brush layer at such surfaces. In order to evaluate nisin function following its adsorption to PEO layers, the antimicrobial activity of nisin-loaded, F108-coated polystyrene microspheres and F108-coated polyurethane catheter segments was evaluated against the Gram-positive indicator strain, *Pediococcus pentosaceous*. The activity of these nisin-loaded layers was evaluated in the presence and absence of blood proteins, after contact periods up to one week. While an increase in serum protein concentration reduced the activity of nisin retained on both the bare hydrophobic and F108-coated surfaces of these materials, the F108-coated surfaces retained more antimicrobial activity than the uncoated surfaces. Circular dichroism spectroscopy studies conducted with nisin in the presence of F108-coated and uncoated, silanized silica nanoparticles suggested that nisin experienced conformational rearrangement at a greater rate and to a greater extent on bare hydrophobic relative to F108-coated surfaces. These results support the notion that the pendant PEO chains of the

F108 coating confer some degree of conformational stability to nisin while also inhibiting its exchange by blood proteins.

Keywords: PEO-PPO-PEO triblock surfactant, Pluronic F108, lantibiotics, nisin, activity and conformation, circular dichroism.

3.1 Introduction

In a previous paper we described the adsorption and elution of nisin (Figure 1) at silanized silica surfaces coated with the PEO-PPO-PEO triblock copolymer Pluronic® F108 (Tai et al., 2007). Nisin adsorption and elution kinetics were measured *in situ*, with ellipsometry. While it is well understood that F108 layers inhibit protein adsorption, nisin, presumably due to its relatively small size, was able to adsorb in multilayer quantities. Comparison of nisin adsorption and elution kinetics at uncoated and F108-coated surfaces suggested that the rate of nisin adsorption and elution, and its lateral mobility at the interface, were generally greater at uncoated surfaces. This was considered consistent with nisin adsorption or "entrapment" within the PEO brush layer, as opposed to adsorption to the PEO chains in a non-penetrating manner. We also observed that F108-coated layers rejected adsorption of bovine serum albumin.

Medical device-related infections are a major problem and antibacterial coatings are needed to prevent illness, morbidity and excess cost. However, current antibacterial coatings can support the rise of drug resistant bacteria. Nisin is a lantibiotic, i.e., of a class of antibiotic compounds that includes one or more lanthionine rings. The unique physical structure of lantibiotics makes them different in mode-of-action from traditional antibiotics, suggesting that they offer a means for preventing the rise of resistant microorganisms (van den Hooven et al., 1996; Wiedemann et al., 2001; van Heusden et al., 2002; Hsu et al., 2004). In this regard a better understanding of the interaction between nisin and PEO layers may provide direction for improved anti-infective function in coated, implantable devices, and improved strategies for drug loading and release from such devices, among others. In addition, the protein rejection properties of the F108 layer,

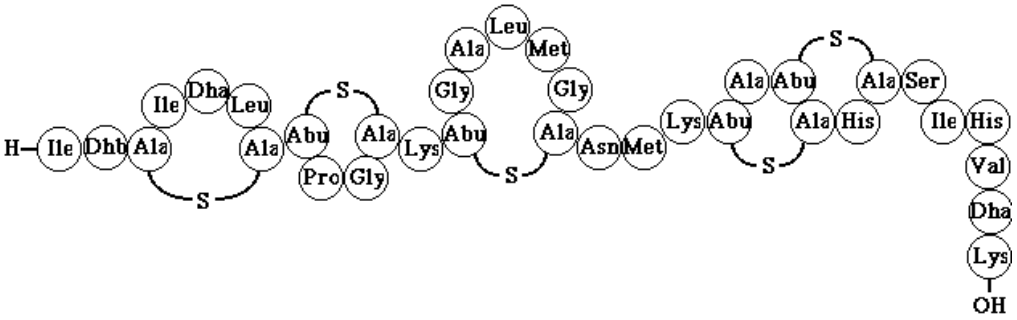

Figure 3.1 Schematic of nisin A. Abu: 2-aminobutyric acid; Dha: Dehydroalanine; Dhb: Dehydrobutyrine.

if retained after nisin adsorption, would inhibit exchange of the peptide by blood proteins and in this way improve the potency of such a coating.

In this paper we describe the antimicrobial activity of nisin-loaded, F108-coated polystyrene microspheres and F108-coated polyurethane catheter segments in the presence and absence of blood proteins. In addition, circular dichroism spectra were recorded for nisin in the presence of F108-coated and uncoated, silanized silica nanoparticles in order to gain information on conformational rearrangement on bare hydrophobic relative to F108-coated surfaces.

3.2 Materials and Methods

Nisin was obtained from Prime Pharma (Batch number 20050116-1, Gordons Bay, South Africa) and was dissolved in filtered, 10 mM monobasic sodium phosphate solution to ensure complete solubilization. Filtered, 10 mM dibasic sodium phosphate was then added to bring the pH to 7.0. Solution of nisin was aliquoted into 0.5 ml in vials, frozen at -80 °C and thawed just before use. Fresh horse blood was obtained from College of Veterinary Medicine, Oregon State University. Immediately upon receipt of blood, blood cells were separated from serum by centrifuging horse blood at 8000 rpm for 10 minutes. Serum was removed and frozen at -80 °C for storage. Pluronic® F108 was purchased from BASF (Mount Olive, NJ) and was dissolved in 10 mM phosphate buffer as needed. Polyethylene glycol (PEG, M.W. 6000, Product # 81253, Fluka, Steinheim, Germany) was obtained from Sigma Aldrich and dissolved in 10 mM sodium phosphate buffer.

Nisin activity at F108-coated and uncoated polystyrene microsphere surfaces

Surface coating with Pluronic® F108. Polystyrene microspheres (Part 81002497100290, 1.247 μm diameter, Seradyn, Ramsey, MN) were mixed and incubated in 5 mg/ml Pluronic F108 in phosphate buffer overnight on a rotator. The hydrophobic PPO block of the Pluronic F108 molecule adsorbs on the polystyrene surface such that the hydrophilic PEO chains extend into the solution phase. Unbound F108 was removed by repeated microsphere washing, vortexing and sonication, centrifugation and re-suspension in phosphate buffer to ensure the minimum residual amount of Pluronic F108 in solution. Nisin was also incubated in microsphere-free phosphate buffer (10 mM) and F108 containing solution as control samples. Concentrations used for these samples were 8×10^{-3} mg/ml for nisin and 5 mg/ml for Pluronic F108.

Incubation of microsphere samples with nisin. The F108-coated and bare microsphere samples were independently mixed and incubated with 8×10^{-3} mg/ml nisin in phosphate buffer for 1 h at RT on a tube rotator. After 1 h of incubation, unbound nisin was removed by repeated washing, sonication, centrifugation and re-suspension to ensure the minimum residual amount of nisin in solution. The nisin coated microsphere beads were suspended again in phosphate buffer for 1 h, another repeated washing steps were performed to remove the eluted/desorbed nisin in the solution. Supernatant was assayed by agar diffusion test on a *Pediococcus* seeded plate immediately to ensure no detectable amount of nisin left in the solution of the final wash step. The nisin treated microspheres were then incubated in phosphate buffer or equine serum of desired concentration (0, 10, 50 and 100 % v/v) for desired period of time (0, 1, 4, 7 days) at 37 °C.

Cultivation of *P. pentosaceus*. MRS broth was used for cultivation of a nisin sensitive *Pediococcus pentosaceus* strain FBB 61-2. 52.2 g of MRS (Cat. No. 1.10661, EMD Chemicals, Inc. Gibbstown, NJ) was dissolved in 1 liter of DI water and autoclaved at 121 °C for 30 min. *P. pentosaceus* was incubated overnight (20 h) at 37 °C and placed on an orbital shaker at 220 rpm. The optical density OD_{600} of overnight culture, and 100-fold dilution of the overnight culture was measured to ensure consistency of cell density.

Antibacterial activity assay. Nisin treated, bare and F108 coated microspheres were incubated in 10 mM sodium phosphate buffer and horse serum containing buffer at 37 °C for desired period of time (1, 4, 7 days) in order to record decay of activity on surfaces. The incubated microspheres were then washed twice to remove nisin residue in the supernatant. Washed microspheres were then allowed to contact with 100-fold dilution of overnight *P. pentosaceus* culture by mixing at 37 °C for 4 h. The microsphere-bacteria mixture was then 100-fold diluted sampled. 0.5 ml culture samples were evenly dispersed with 44 °C, melt MRS agar in Petri dishes. The dishes were incubated at a 37 °C incubator for 48 h until the bacteria colonies became visible. The number of colonies indicates the potency of nisin released from the surface of microspheres.

Nisin activity at F108-coated and uncoated polyurethane catheter segments

Surface coating with Pluronic® F108. 22 GA, 1.0" I.V. catheter (BD, REF 381423, Sandy, Utah) was incubated with 0.5% F108 in 10 mM sodium phosphate buffer for at least 24 hour in disposable glass test tubes. After incubation, catheters were rinsed by multiple test tube volumes of phosphate buffer to remove uncoated F108 in solution. This was done by continuously added and removed buffer with two Masterflex L/S pumps connected in the test tube containing the catheter.

Incubation of catheter samples with nisin. The bare and F108-coated catheters were incubated in 0.5 mg/ml nisin for 1 h at RT. After 1 h, the catheter segments were rinsed with multiple test tube volumes of sodium phosphate buffer to remove unbound nisin on the surface. Nisin treated catheters were then incubated in 50% (v/v) horse serum in phosphate buffer, or in 10 mM phosphate buffer for desired period of time.

Antibacterial activity assay. Nisin sensitive *P. pentosaceus* were used to seed MRS agar dishes. After desired period of incubation time, nisin treated catheter segments were rinsed with copious amount of phosphate buffer. The inner wall of catheter segments was rinsed by pushing phosphate buffer through using a syringe. The nisin-treated segments were then inserted onto the gel of *P. pentosaceus* seeded plates. Plates were incubated at 37 °C for 48 h until halos of the kill zone showed. The area of kill zone was recorded as an indication of nisin activity released from the surface of nisin treated catheter segments. Figure 3.2 represents a typical example of such assay.

Circular dichroism. CD spectra were recorded between 300 and 180 nm on a Jasco J-720 spectropolarimeter with a 0.2-mm path length cylindrical cuvette at 25 °C. Six scans were recorded and averaged in order to increase the signal-to-noise ratio. Hexadecylsilane hydrophobised silica nanoparticles (Product R816, 190 m^2/g of specific surface area, Degussa, Frankfurt, Germany) were used as sorbent for nisin and Pluronic F108 adsorption. Nanoparticles were first coated with Pluronic F108 by mixing with F108 in phosphate buffer overnight on a rotator. The amount (1.35 mg/ml) of Pluronic F108 used was just enough to cover all surface area of the nanoparticles in solution. It is estimated by specific coating density of Pluronic F108, 3.3 mg/m^2 (Neff et al, 1999). F108-coated and bare hydrophobic silica

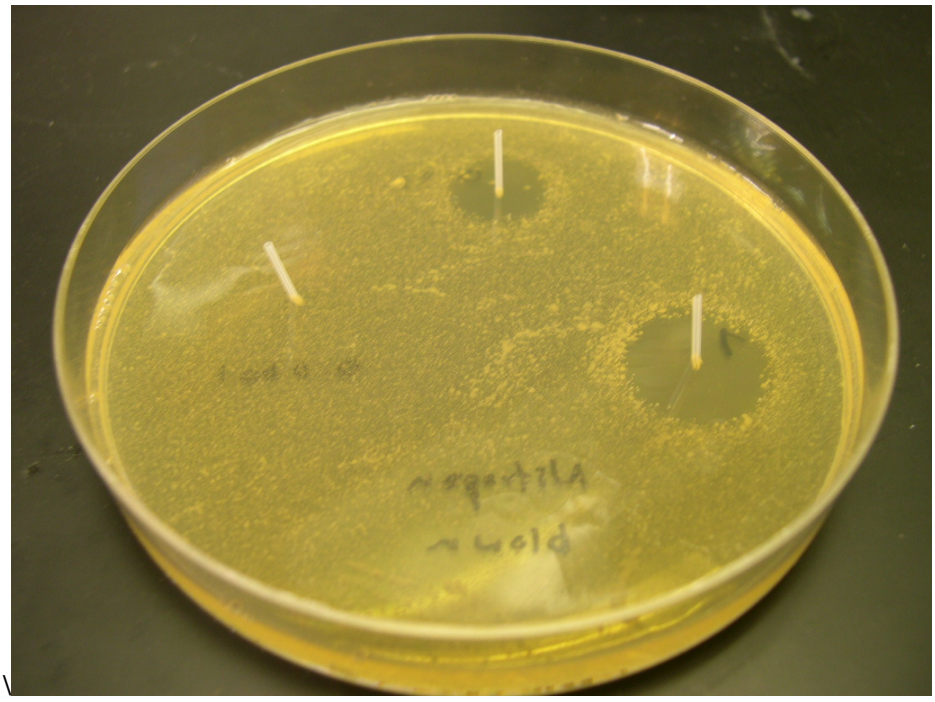

Figure 3.2 Example of a modified agar diffusion assay for nisin treated segments on nisin sensitive *Pediococcus* seeded plates. Area of kill zone halo around the segments indicates the activity strength of nisin released from surfaces.

nanoparticles were then incubated with nisin (0.5 mg/ml) for desired period of time (4 h to 1 week) at room temperature. 1.25 fold of calculated coating area of nanoparticles was used to contact nisin (2.19 mg/ml silica nanoparticle). In earlier work we determined that the adsorbed mass of nisin reached about 0.15 μg/cm^2 on hydrophobic surface after 1 hour of adsorption (Tai et al., 2007). All nisin in the solution was estimated to be adsorbed on hydrophobised silica surface in 2 h. CD spectra of both nisin treated samples and blank samples (nanoparticles+buffer, nanoparticles+F108+buffer, F108+buffer, and buffer only) were measured and recorded in order to subtract background signals to obtain nisin-only spectra. For 1X nanoparticle-to-nisin ratio CD samples, the concentrations used are 10 mM sodium phosphate buffer, 0.5 mg/ml nisin, 2.19 mg/ml silica nanoparticle and 1.35 mg/ml Pluronic F108. Nisin was also incubated with nanoparticle-free solution with PEG and F018 at various concentrations (1X and 5X, PEG-to-nisin or F108-to-nisin ratio). For 1X ratio PEG-to-nisin or F108-to-nisin CD samples, the PEG concentration used was 0.85 mg/ml; Pluronic F108 was 2.08 mg/ml, and nisin was 0.5 mg/ml.

3.3 Results and Discussion

Nisin activity at F108-coated and uncoated polystyrene microsphere surfaces

Figure 3.3 shows antimicrobial activity on surfaces of bare and F108 coated microspheres incubated with solution of various horse serum % at 37 °C for 7 days. Results show that activity of nisin on surfaces decreased with increase concentration of serum protein. Also, antimicrobial activity of nisin on F108 coated surface was always higher than that on the bare hydrophobic surface of PS microspheres, when challenged with blood proteins. It suggests that F108 layer can prevent “loaded” nisin from being exchanged by blood proteins on the surface.

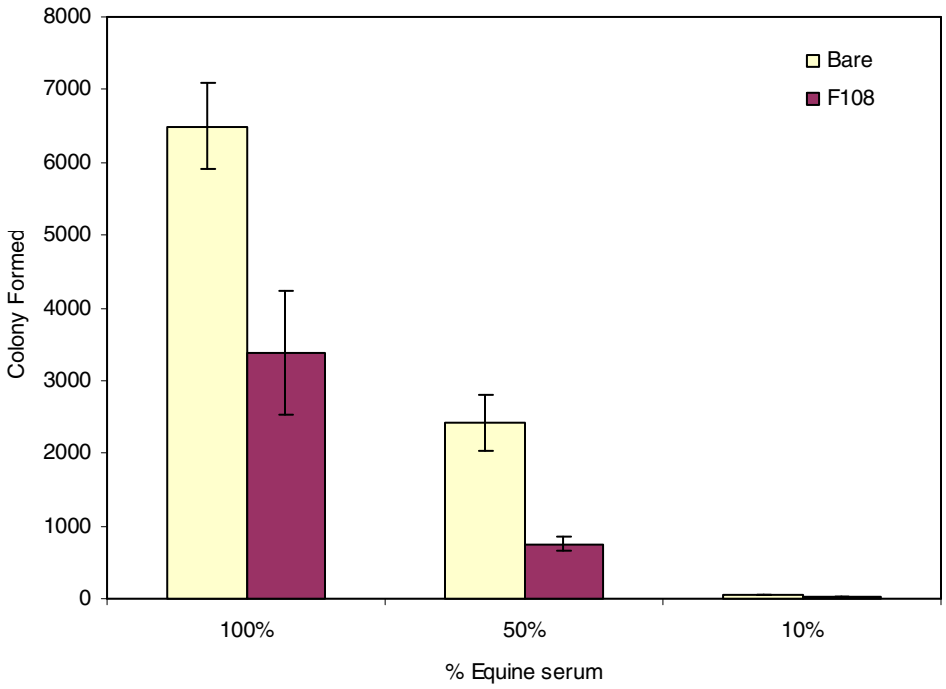

Figure 3.3 Result of colony count activity assay of nisin on microsphere in horse serum protein of various concentrations, 7-days incubation.

Figure 3.4 shows the decay of nisin activity on bare microspheres and microsphere coated with F108 in 10 mM phosphate buffer at 37 °C. Results show that antimicrobial activity of nisin on F108-coated microspheres was slightly higher than that observed on bare hydrophobic polystyrene surfaces. However, nisin on both F108 coated surface and bare surface didn't lose much activity with time in phosphate buffer. It is also observed that nisin in phosphate buffer and in F108-containing buffer lost activity relative quicker then nisin on the F108-coated and bare surfaces, implying that high concentration, multiple layers of adsorbed nisin on surface has some sort self-stabilizing mechanism to prevent its activity loss.

Figure 3.5 shows antimicrobial activity of nisin on microspheres when challenged with 50% serum proteins in phosphate buffer. As shown in the figure, the lost of nisin activity on both bare microspheres and F108 coated microspheres was substantial upon introduction of serum proteins, when compared to non-protein challenged experiment group shown in Figure 3.4. In addition, after 4 days of incubation with serum proteins, activity retained on the F108 coated microspheres was over twice as much as what retained on the bare surface of microspheres. This suggests that the protein repelling function of the F108 coating prevented nisin from been exchanged by blood proteins, hence maintained its antimicrobial activity over time. Also as indicated by 1-day and 4-day activity of nisin in solution without microspheres, F108 seems to have certain stabilizing function for nisin activity. Presumably this is due to F108 inhibiting the formation of nisin-serum protein aggregation, hence retained nisin's cell-wall binding and penetration ability. However the mechanism is not clear and needs to be further investigated.

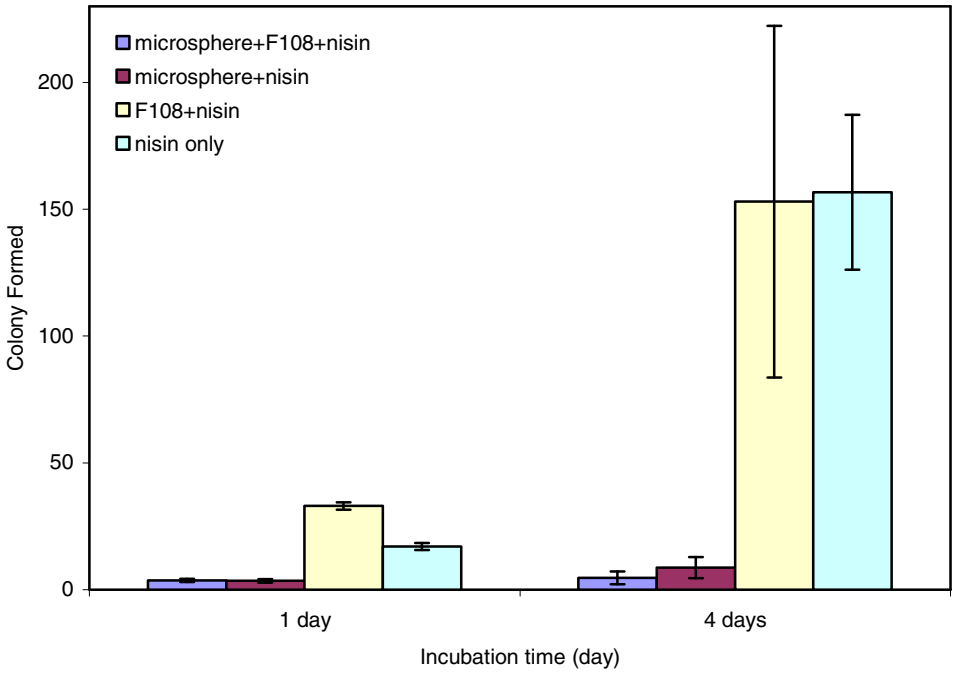

Figure 3.4 Activity of nisin-treated microspheres in 10 mM sodium phosphate buffer.

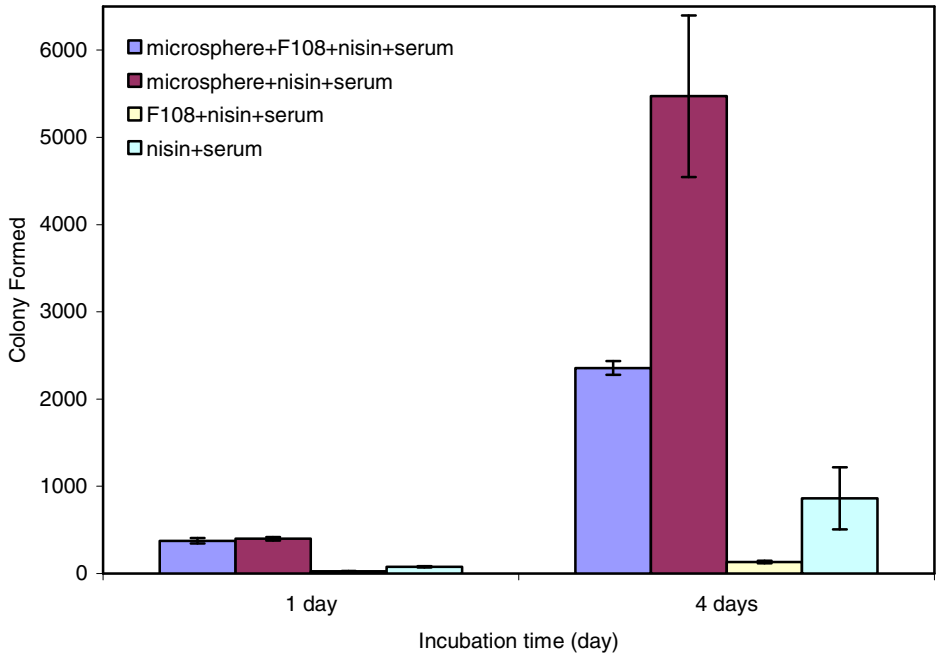

Figure 3.5 Activity of nisin treated microspheres, 50% of blood proteins challenged.

Nisin activity at F108-coated and uncoated polyurethane catheter segment surfaces

Figure 3.6 shows the activity of nisin on the bare and the F108 coated catheter segments incubated in 10 mM sodium phosphate buffer. As suggested by the results, nisin activity decayed with time on both bare surface and F108 coated surface. In general, nisin shows higher activity on the F108 coated samples than on the bare samples on the samples of the longer incubation periods (72 and 144 h). In another set of experiments as results shown in Figure 3.7, nisin treated catheters segments were challenged by 50% blood proteins. It shows relatively larger contrast of antimicrobial activity between the F108-coated and bare catheter segments when they were incubated with serum proteins. As indicated by the results, activity of nisin on the bare hydrophobic decayed relatively quicker with time, while activity maintained at almost the same level over time on F108 coated surface, from 24 hours to 144 hours. These supplement results again suggest that F108 coating can preserve nisin in its PEO brush layer and prevent it from being exchanged by blood proteins, therefore retain and improve efficacy of its antimicrobial activity on surfaces.

Circular dichroism

Figure 3.8 shows CD spectra of nisin incubated in a nanoparticle-free solutions containing F108 and polyethylene glycol (PEG, M.W. 6000, Product # 81253, Fluka, Steinheim, Germany) of various concentrations for desired length of time (2 h and 1 week). Molecular weight of PEG selected here to use is to approach the molecular weight of PEO chain on the Pluronic F108 triblock copolymer (M.W.≈5400). As shown in Figure 3.8, the spectra of nisin of all groups are quite similar, implying neither Pluronic F108 nor PEG in the solutions can alter nisin conformation.

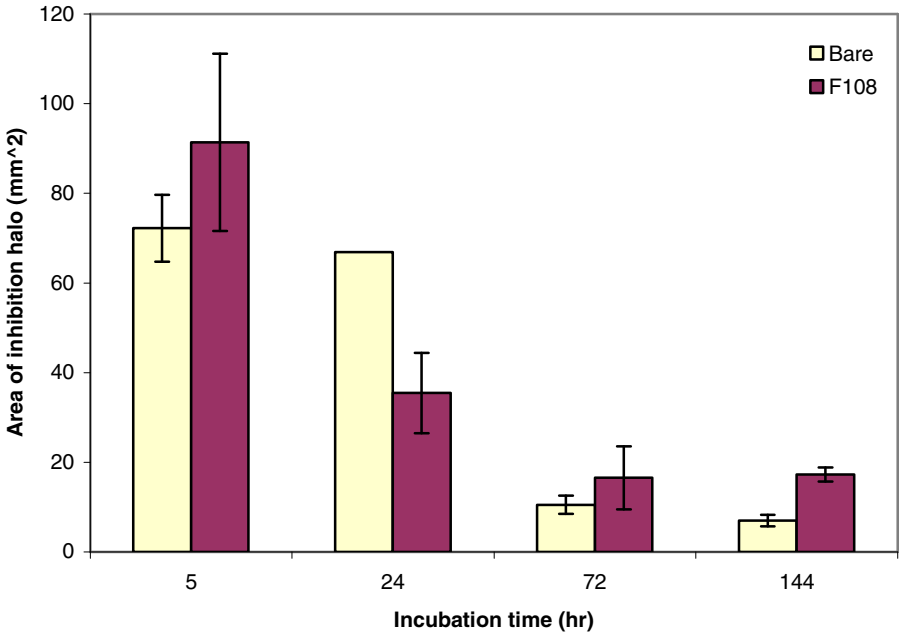

Figure 3.6 Activity of nisin on PU catheter in 10 mM sodium phosphate buffer

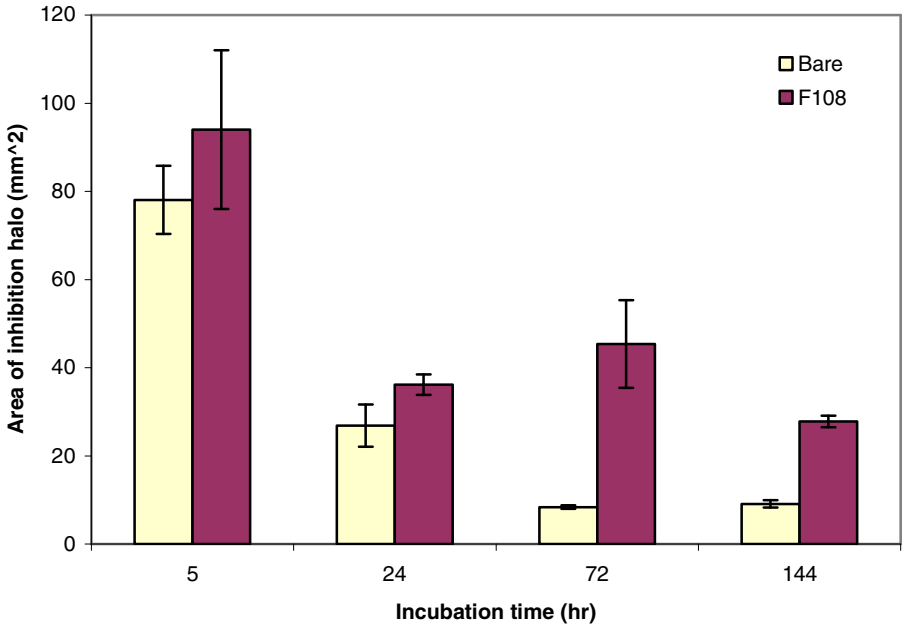

Figure 3.7 Nisin activity on PU catheter in 25% horse serum proteins

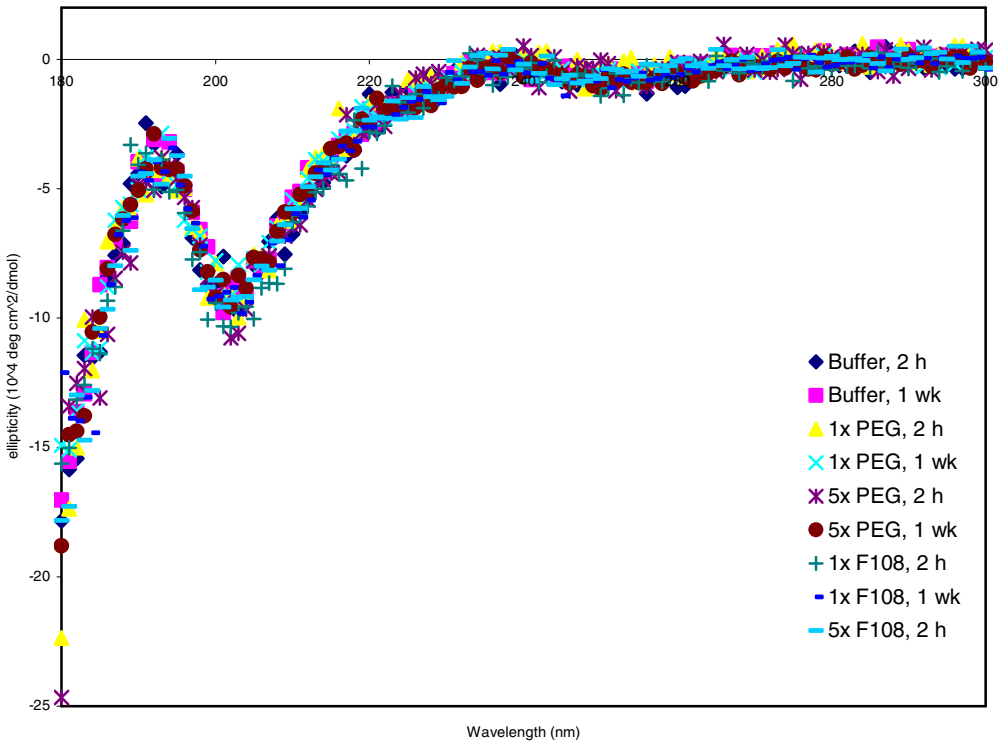

Figure 3.8 CD spectra of nisin incubated in a nanoparticles-free solutions containing F108 and polyethylene glycol (PEG, M.W. 6000) of various concentrations for desired length of time (2 h and 1 week)

Figures 3.9 and 3.10 shows CD spectra of nisin contacting silica particles in 10mM sodium phosphate buffer (pH 7) at 25 °C, for 2 hours and 1 week respectively. As shown in Figure 3.9, when nisin contacted hydrophobic silica nanoparticles, the ellipticity shifted upward between 190 nm and 220 nm, while spectra of nisin on F108-coated nanoparticles stayed relatively closer to spectra of native nisin in phosphate buffer. In another experiment, nisin was incubated with nanoparticles for extended period of time (1 week) and CD spectra were recorded. As shown in Figure 3.10, CD spectra of nisin in phosphate buffer and on F108 coated surface in between 210 nm and 260 nm are nearly identical, while nisin on the bare hydrophobic nanoparticles are relatively different from the other two groups. Ellipticity shifted upward with time when contacted with nanoparticles on both bare surface and F108 coated surface, however, the shape and altitude of the spectra of nisin on F108 coated surface are relatively closer to nisin in phosphate buffer. Nisin in phosphate did not change much in conformation as indicated by CD spectra of 2 hours and 1 week of incubation. These results suggest that nisin underwent relatively larger conformation rearrangements on bare hydrophobic surface of silica nanoparticles, than that on the F108-coated surface.

It needs to note here that current available CD spectra analysis programs such as CDSSTR (Manavalan et al, 1987), K2D (Böhm et al, 1992) or CONTIN (Andrade et al, 1993; Merolo et al, 1994) are not designed for small proteins or polypeptides, while convex constrain analysis programs such as CCA+ of Perczel et al. (1992), are recommended (Greenfield, 1996). The reason it is hard to fit the spectra of small polypeptides using CDSSTR, CONTIN or other programs which use protein data sets is when proteins have equal amounts of beta and random conformations, there spectra contributions tend to cancel out. If the protein has a low content of alpha helices as well, the ellipticity is

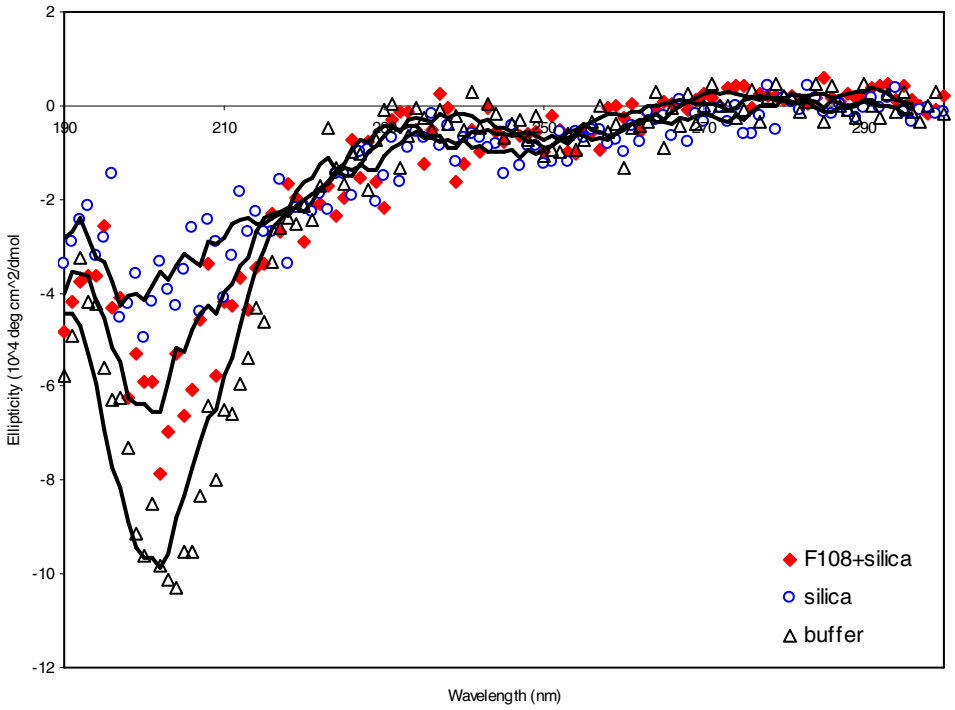

Figure 3.9 CD spectra of nisin contacting silica particles in 10mM sodium phosphate buffer (pH 7) at 25 °C, for 2 h.

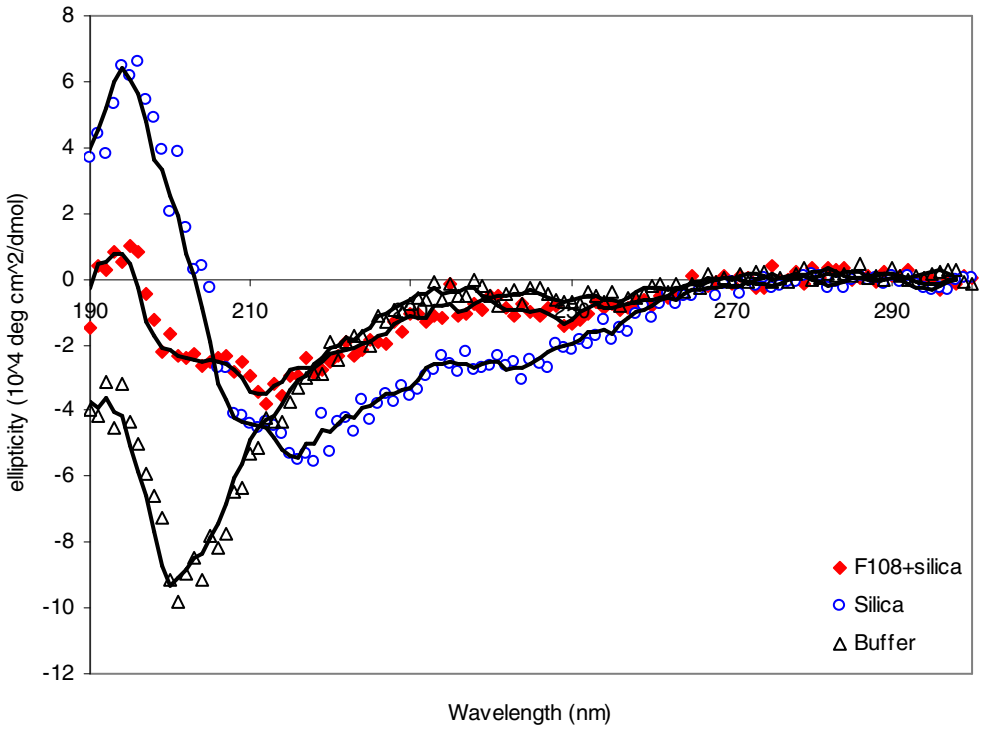

Figure 3.10 CD spectra of nisin contacting silica particles in 10mM sodium phosphate buffer (pH 7) at 25 °C, for 1 week.

dominated by the contributions of aromatic groups. When these programs are used to analyze peptide, which may have very low ellipticity and be basically disordered, the program will find the best fit to be a mixture of beta and extended (e.g. poly-L-proline II) like conformations. In addition, these programs do a poor job of analyzing the spectra of pure beta polypeptides or beta amyloids, because there aren't high resolution X-ray structures of these peptide and therefore their conformations never get into the data sets used for decovoluting protein spectra. However, the CCA+ or similar programs give results only in the categories of the typical protein secondary structures such as α-helix or β-turns. Nisin is an unusual polypeptide with a structure that contains many unusual amino acids and thioether bonds (Figure 3.1). As nisin has only 34 amino acid residues, it does not contain any typical known secondary structures, such as helices and β-conformations. Therefore, a quantitative breakdown in the percentage of typical secondary structures is not suitable. Nonetheless, conformational changes in nisin structure are still detectable using circular dichroism, since nisin is an asymmetric biomolecule.

In another set of experiments, same amount of nisin was contacted with 5 fold of silica nanoparticles. It is indicated in the CD spectra that increasing surface area shifted the ellipticity upward between 215 nm and 190 nm both on bare surface (Figure 3.11) and F108-coated surface (Figure 12), however, variation of spectra of the F108 coated groups from (native) nisin in phosphate buffer, are relatively smaller than the bare-surface group. In addition, ellipticity of bare surface groups of both 5-fold and 1-fold silica nanoparticles shifted downward between 260 nm and 215 nm, while spectra of the F-108 coated groups remained similar with spectrum of native nisin in buffer. These results indicate that the degree of conformational rearrangement of nisin was dependent on contacting surface area

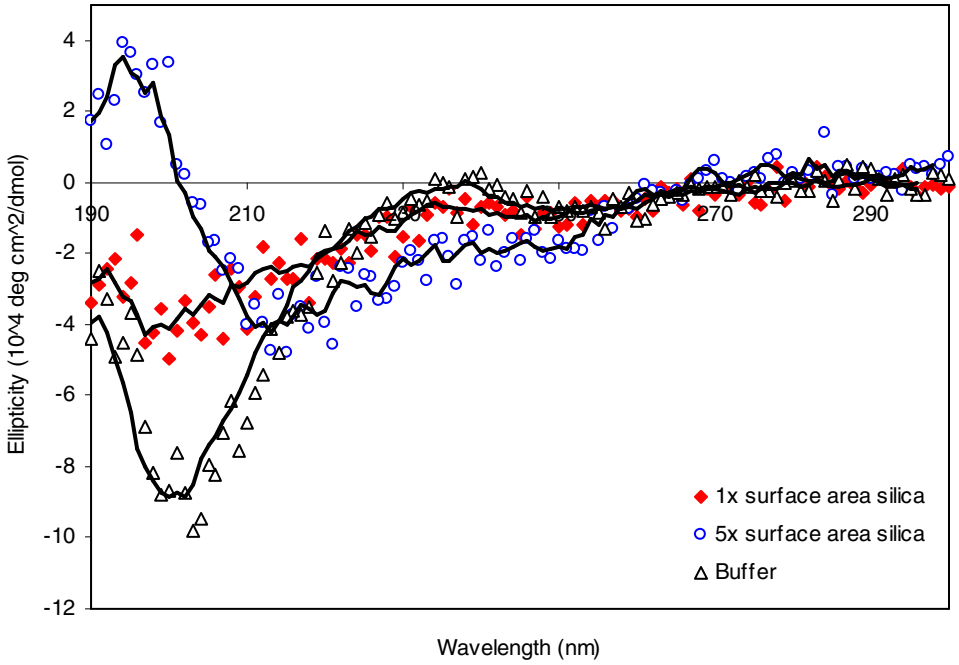

Figure 3.11 CD spectra of nisin contacting 1-fold and 5-fold of bare hydrophobic silica nanoparticles.

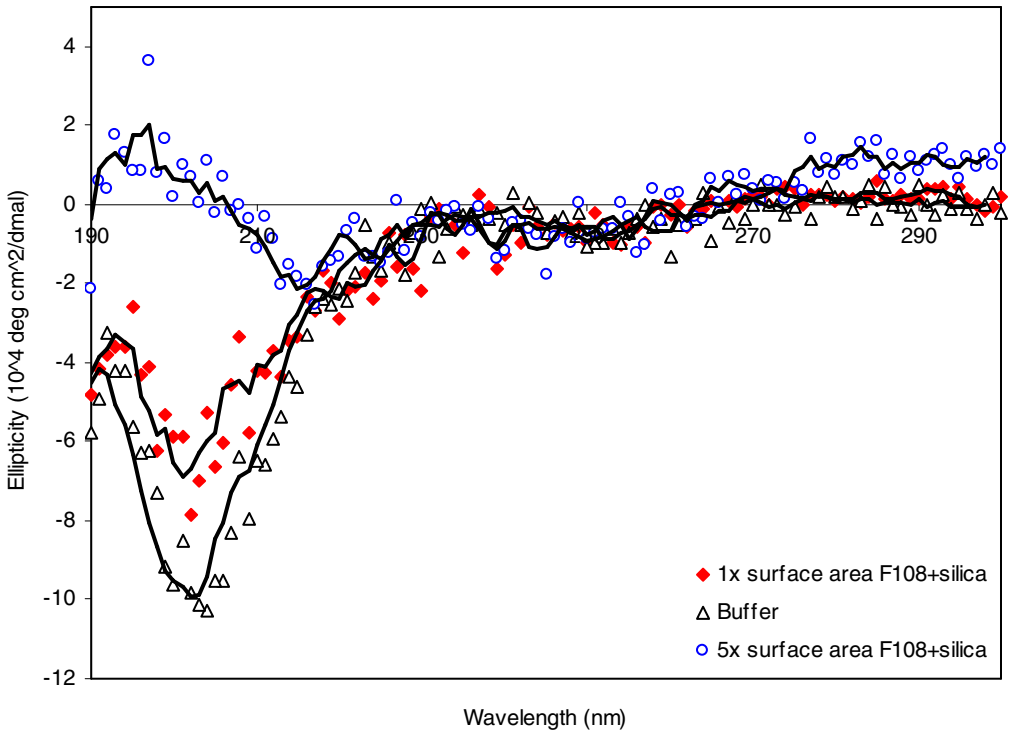

Figure 3.12 CD spectra of nisin contacting 1-fold and 5-fold of F108 coated silica nanoparticle

because increase number of nanoparticles increases the possibility of released or exchanged nisin from surface to contact surfaces of other nanoparticles. Results also suggest that F108 coating can prevent alteration of nisin structure on hydrophobic surfaces to some extent.

3.4 Conclusions

From the bioassays and CD conformation studies of nisin on surface, we conclude that protein-exchange played a major role in the decay of nisin activity on the surface, while surface-induced structural change and irreversible adsorption of nisin may have contributed partly to the activity loss. Pluronic F108 rejects blood proteins and stabilizes loaded nisin in its PEO brush layer, making it a promising controlled-release media for nisin on the surface of blood-contacting biomaterials. The mechanism of any nisin structure stabilization function of Pluronic F108 is unknown at this time, and future investigation is needed.

Acknowledgments

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CHAPTER 4

GENERAL CONCLUSION

The adsorption of the antimicrobial peptide nisin to stable, adsorbed layers of the PEO-PPO-PEO triblock surfactant Pluronic® F108 was quantified by *in situ* ellipsometry. While it is well understood that such layers inhibit protein adsorption, nisin, presumably due to its relatively small size, was able to adsorb in multilayer quantities. Comparison of nisin adsorption and elution kinetics at uncoated and F108-coated surfaces suggested that the rate of nisin adsorption and elution, and its lateral mobility at the interface, were generally greater at uncoated surfaces. This would be consistent with nisin adsorption or "entrapment" within the PEO brush layer, as opposed to adsorption to the PEO chains in a non-penetrating manner. F108-coated layers rejected adsorption of the protein BSA. However, sequential adsorption experiments carried out with nisin and BSA showed measurable adsorption of BSA if nisin was present at the interface. Enhanced serum protein adsorption at nisin-loaded F108 layers is an issue that warrants further investigation.

Nisin antimicrobial activity at F108-coated polystyrene microspheres and F108-coated polyurethane catheter segments was also evaluated, against the Gram-positive indicator strain, *Pediococcus pentosaceous*. While an increase in serum protein concentration reduced the activity of nisin retained on both the bare hydrophobic and F108-coated surfaces of these materials, the F108-coated surfaces retained more antimicrobial activity than the bare surfaces. F108-coated microspheres and polyurethane catheter segments were observed to retain more antimicrobial activity than their uncoated counterparts in the absence of blood proteins as well, but the difference in function between bare and F108-

coated substrates was most pronounced in the presence of blood proteins. These results support the notion that the pendant PEO chains of the F108 coating inhibited the exchange of nisin by blood proteins. Circular dichroism spectroscopy studies conducted with nisin in the presence of F108-coated and uncoated, silanized silica nanoparticles suggested that nisin experienced conformational rearrangement at a greater rate and to a greater extent on bare hydrophobic relative to F108-coated surfaces.

Taken together, these results show feasibility for using the PEO-PPO-PEO copolymer coating to improve nisin retention and prolonged activity in the presence of blood. As nisin is a lantibiotic that offers a means for preventing the rise of resistant bacteria, this kind of coating strategy would also improve patient care by decreasing their risk for developing infections that are difficult to treat.

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APPENDICES

ADSORPTION OF RECOMBINANT FACTOR VIII AT THE CHARGED SURFACES AND HYDROPHOBIC SURFACES COATED WITH PLURONIC® F108

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APPENDIX A

ADSORPTION OF RECOMBINANT FACTOR VIII AT THE CHARGED SURFACES AND HYDROPHOBIC SURFACES COATED WITH PLURONIC® F108

Abstract

Interactions of rFVIII with Pluronic® F108 layer and hydrophobic silanized surfaces, hydrophilic, neutral and positively-charged surfaces were investigated, using *in situ* ellipsometer. Hydrophilic, positively-charged nickel oxide and neutral zinc sulfide coatings on silica surfaces were synthesized and characterized via zeta potential and contact angle measurements. rFVIII adsorption was recorded by an *in situ* ellipsometer on each of these materials. Results show that the surfactant Pluronic® F108 coating significantly reduces rFVIII protein adsorption on surfaces, and therefore promotes rFVIII stability and activity retention in the presence of an interface. Results also indicate that the positively-charged surface shows strong affinity for rFVIII. The importance of electrostatic attraction in adsorption to nickel oxide was evident as significantly reduced rFVIII adsorption was observed with an increase in salt concentration. Adsorption to zinc sulfide showed a lower affinity than adsorption to the charged surfaces, but was appreciably higher than rFVIII adsorption to F108 layers. This study provides a good indication of the importance of steric rejection as a requirement for rFVIII repulsion.

Introduction

Factor VIII is a 280 kDa plasma protein which plays an important role in the coagulation system. The severe bleeding disorder haemophilia A is associated with the functional absence of Factor VIII, demonstrating its essential role in the cascade of blood coagulation. Factor VIII functions as a cofactor in the Ca^{2+}-phospholipid dependent conversion of Factor X and Xa by activated Factor IX (Kane et al., 1988; Mann et al., 1990). The amino-acid sequence of Factor VIII consists of several discrete domains, denoted A1-A2-B-A3-C1-C2 (Vehar, 1984; Toole, 1984), where an 80 kDa light chain comprises domains A3, C1, C2 and a 180 kDa heavy chain comprises domains A1, A2 and B (Eaton et al., 1986).

Factor VIII used to be derived from human plasma, historically. The risk of pathogen transmission and the limited availability of plasma have led to the development of a recombinant Factor VIII (Jiang 2002). rFVIII is the largest molecule ever successfully genetic engineered and is the most complex protein manufactured (Boedeker 2001). The rFVIII molecule can lost its native conformation and activity due to both chemical and physical degradation. The degradation involves changes in higher order structure and may be caused by a number of pathways including aggregation, precipitation or adsorption onto surfaces (Wang 2003). It is well accepted that proteins can exist in multiple adsorbed "states" on a surface. Surface-induced conformation changes undergone by adsorbed protein, result in multiple non-covalent bonds with the surface (McGuire et al, 1999). Once a protein has adsorbed to a surface, it is often thermodynamically favorable for the molecule to undergo some degree of rearrangement. As conformational changes proceeds, the likelihood of desorption decreased. Therefore, proteins can lose their structure or functions on the surfaces due to irreversible adsorption or surface-induced denaturization.

Surface adsorption of rFVIII is rapid (DiMichele 1996). Osterberg et al. (1997) showed that approximately 50% of rFVIII product may be lost due to adsorption just during sterile filtering. Therefore, a solution to improve the loss of productivity is imperative.

Previously in our group, structure changes and activity of the rFVIII were evaluated on negatively and positively-charged microspheres. In addition, rFVIII adsorption had been investigated on negatively-charged hydrophobic and hydrophilic surfaces using *in situ* ellipsometer. Study of rFVIII adsorbed mass from solutions at different ionic strengths suggests that electrostatic forces may be relevant in determining the amounts of rFVIII adsorbed. The rFVIII tertiary structure was changed upon contacting hydrophobic nanoparticle surfaces, but the structure remained largely comparable to native rFVIII upon exposure to hydrophilic surfaces. The biological activity of rFVIII was reduced when the protein was exposed to hydrophobic surfaces. In this regard, we applied a non-ionic surfactant Pluronic F108 on the surfaces and evaluated its interaction with rFVIII. Pluronic® F108 is a $(PEO)_{129}$-$(PPO)_{56}$-$(PEO)_{129}$ triblock copolymer, where the PEO arms of F108 are hydrophilic and PPO domain is hydrophobic. It is well known that PEO resists protein interactions and F108 coated surfaces in particular have been shown to prevent protein adsorption (Li and Caldwell, 1991; Green et al., 1998; Neff et al., 1999).

In this work, we further investigated behaviors of rFVIII at neutral, hydrophilic and positively-charged surfaces, for comparison to our previous results. In particular, rFVIII adsorption and elution on the Pluronic F108 coated surfaces was evaluated with *in situ* ellipsometer.

Materials and Methods

Protein, buffer and surfactant

rFVIII (0.791 mg/ml in KG-2 buffer with 22 ppm Tween 80) was obtained from Bayer HealthCare. rFVIII was aliquoted into 0.5 ml's and stored in -80 °C freezer upon receipt, and thawed prior to use. KG-2 buffer used for ellipsometer study were prepared according to procedure provided by Bayer HealthCare Inc. (30 mM NaCl, 2.5 mM $CaCl_2$, 22 g/l glycine, 3.1 g/l L-histidine and 10 g/l of sucrose, solution adjusted to pH 6.8). All chemicals for the KG-2 buffer preparation were obtained from Sigma-Aldrich. KG-2 buffer was 0.2 μm filtered and stored in -80 °C freezer if not used immediately. Pluronic® F108 surfactant was obtained from BASF (Mount Olive, New Jersey, Product 583062). 2.5 g Pluronic® F108 powder was dissolved in 50 ml of KG-2 buffer to obtain a 5 % (w/v) concentration. The 5 % F108 solution in KG-2 buffer was aliquoted into 1 ml's and stored in -80 °C freezer, and thawed prior to use. Additional salt was added to achieve desired ionic strength in experiments.

Silanization of silica surfaces

Silica wafers obtained from WaferNet, Inc. (Thickness 507-543 μm, surface resistance 0.01 – 0.02 Ω-cm) were cut into 4 × 1 cm strips using a tungsten pen and then silanized. Silanized silica strips were used as substrate for protein adsorption/desorption experiments. Hexamethyldisilazane, (HMDS) obtained from United Chemical Technologies, Inc. was used for silica surface modification. Briefly silica surfaces were rinsed with pure acetone, then cleaned with basic (1:1:5 of NH_4OH:H_2O_2:H_2O) and acidic (1:1:5 of HCl:H_2O_2:H_2O) solution in 80 °C water bath for 10 mins. Cleaned silica stripes were rinsed with copious amount of pure water and pure ethanol, and blown dried by

nitrogen stream. Dried silica stripes were transferred into a 200 °C preheated vacuum oven (Sheldon Manufacturing Inc, Model 1415M) with 2 ml of HDMS in a separate 5-ml beaker and maintained at 20-25 in-Hg for 1.5 hours for silanization. Hydrophobicility of silanized surfaces was obtained by measuring contact angle using static contact angle and surface tension analyzer (First Ten Angstroms, Portsmouth, VA, Model FTA135). Contact angle of HDMS surface-modified silica is usually around 90-96°. Silanized silica surfaces will be stored in an electronic desiccator (BEI Products, Pequannock, New Jersey, Product 420741115) for further use.

Surface coating with Pluronic® F108

HMDS-treated samples were coated with Pluronic F108 by incubating surfaces with 0.5% F108 in 10 mM sodium phosphate buffer in the fused quartz trapezoid cuvette on ellipsometer overnight (16 h or longer). Before ellipsometry experiments, loosely attached F108 on the surface and free F108 in solution was rinsed away with multiple cuvette volumes of phosphate buffer.

PEO coatings were also prepared by covalent attachment of the F108 triblocks. McPherson et al. (1997) and Park et al. (2000) described pretreatment with a silane and subsequent γ-irradiation to covalently bind PEO-PPO-PEO triblocks (via the PPO block) to glass, metal and pyrolytic carbon surfaces. In short, through absorption of radiation or interaction with water-derived radicals, surface-bound free radicals are formed. These free radicals attack the adsorbed PPO block, forming new covalent bonds between the surface and polymer (McPherson et al., 1997). In this work, the F108 triblocks were covalently attached according to methods similar to those cited above. In brief, the silica samples were silanized with octadecyltrimethoxysilane, and the surface-polymer covalent

attachment induced by uv radiation (Neff et al., 2004). After uv treatment, samples were washed with PBS.

Nickel oxide and zinc sulfide coated surfaces

Nickel oxide ($NiO_{1-x} \cdot zH_2O$) nano-layer coatings were prepared on silica using resources and expertise in Dr. Chih-hung Chang's laboratory (OSU), based on a protocol published by Tolstobrov et al (1999). In brief, the silica samples were cleaned with nitric acid at 90-100 °C and with NaOH. They were then immersed in solution of NH_4OH, $(NH_4)_2SO_4$ and $NiSO_4$ for the surface reaction for 40 s. The chemically modified silica samples were then rinsed with deionized water and placed in a H_2O_2 solution for 40 s, then rinsed with DI water again. This reaction cycle was repeated 15 times. The treated silica plates were then transferred to a 240 °C oven for complete ion deposition and annealing. The zinc sulfide coating was made by a similar protocol, except that $ZnCl_2$ and $Na_2S \cdot 9H_2O$ were used rather than $NiSO_4$ and H_2O_2 solutions.

Surface characterization: Contact angle and zeta potential measurements

Hydrophobicity of the surfaces was quantified by contact angel measurement. The contact angle between each surface and water was measured (First Ten Angstroms, Model FTA135). Zeta potential measurement was carried out with a "clamping cell" unit on an Anton Paar EKA (Electro Kinetic Analyzer) equipped with a Ag/AgCl electrode. Briefly, the coated silica samples were secured to a plate with rectangular channels that allowed an electrolyte (1 mM KCl) to circulate through the system. The cell chamber was then closed, and the clamping cell unit was secured and connected to the EKA. Before the measurement, the pH electrode was calibrated with pH 4, 7 and 10 standards and conductivity was calibrated with 1 mM KCl standard solution as recommended by the

EKA instruction menu. The flow system and electrode were rinsed three times automatically in each direction, and zeta potentials of the coated silica plates (and the PMMA reference plate) were then measured three times and averaged. The surface zeta potential was +12.76 mV on the nickel oxide surface and +1.84 mV on the zinc sulfide surface.

In situ ellipsometry study of rFVIII adsorption-desorption kinetics

Silanized silica strips were used as substrate for rFVIII and F108 adsorption experiments. Amount of protein or F108 adsorbed on surfaces was measured by an automatic *in situ* ellipsometer (Gaertner Scientific Corp., Skokie, IL) equipped with a 1mW He-Ne laser light source, a trapezoidal fused quartz cuvette and a magnetic stir plate. At the beginning of each experiment, 4.5 ml of filtered KG-2 buffer was introduced into the quartz cuvette. A silica strip was mounted on the sample holder and lowered into the cuvette to be immersed into KG-2 buffer. Optical properties of the bare hydrophobic or F108-coated surfaces in solution were then measured and recoded for 30 minutes. After the bare-surface measurement, 0.5 ml protein or Pluronic F108 was introduced into the cuvette to reach a 10-fold dilution. The solution in cuvette was gently stirred for 3 mins before and after the actions of adding or rinsing to obtain a more homogeneous state. After a desired period of adsorption time, 10 cuvette volumes of filtered KG-2 buffer were continuously added and removed by two MasterFlex peristaltic pumps connected at the cuvette to rinse away unadsorbed protein in the solution. A program by Krisdhasima was used to determine the adsorbed amount on surfaces according to ψ and Δ values collected by automatic ellipsometer.

Results and Discussion

Surface characterization: Contact angle and zeta potential

Contact angle quantifies hydrophobicity on surfaces. The contact angle between water and HDMS silanized surface was 90-95º, $NiO_{1-x}{\cdot}zH_2O$ was 54.2º, while that for zinc sulfide was 46.5º. The surface zeta potential was +12.76 mV on the nickel oxide surface and +1.84 mV on the zinc sulfide surface. This may be compared to -37.95 mV for hydrophilic (bare) silica and -29.47 mV for hydrophobic silica (Omkar Joshi, 2006). The sign of the zeta potential identifies whether the charge is positive or negative, and its magnitude corresponds to the magnitude of charge density. We thus consider nickel oxide to impart a positive charge density, while zinc sulfide renders the surface nearly neutral.

F108-rFVIII sequential adsorption

Figure 1 shows the adsorbed mass recorded as a function of time when F108 is introduced to silanized silica for 30 min, the surface is rinsed and incubated with (F108-free) buffer for 30 min, and then rFVIII is introduced and allowed to contact the surface for 60 min. The surface is then rinsed and incubated with buffer. The plot indicates very little adsorption of rFVIII following coating with F108. In particular, the amount of rFVIII retained on the surface at the end of the experiment is less than 0.02 $\mu g/cm^2$, which is less than 4% of the amount retained on the same surface in the absence of F108 (i.e., 0.50 $\mu g/cm^2$; see Figure 2). We believe this suggests total repulsion of rFVIII by F108. While the reduction seen is 96% (as opposed to 100%), it is likely this is due to incomplete or inefficient coating. An HDMS-silanized surface is not only suboptimal for F108 coating, but in general, 12-24 h contact is recommended to ensure a uniform F108 coating on a hydrophobic surface. The coating here was prepared in 30 min, then aged for an additional 30 min. In this regard we believe the non-zero retained amount of rFVIII at the

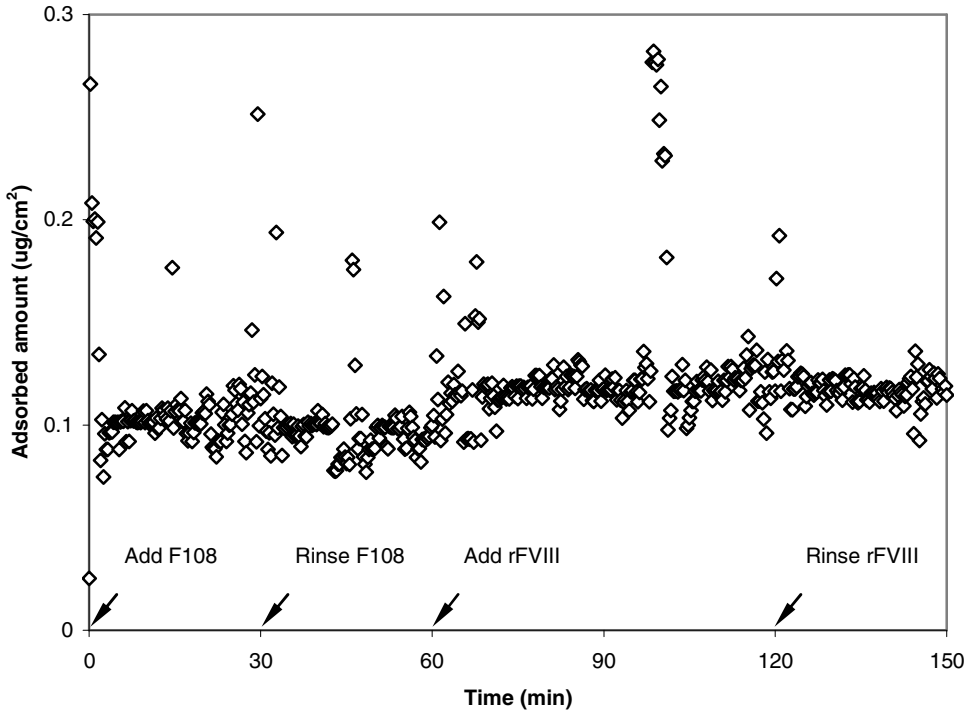

Figure A.1. F108-rFVIII sequential adsorption on HDMS silanized hydrophobic surface.

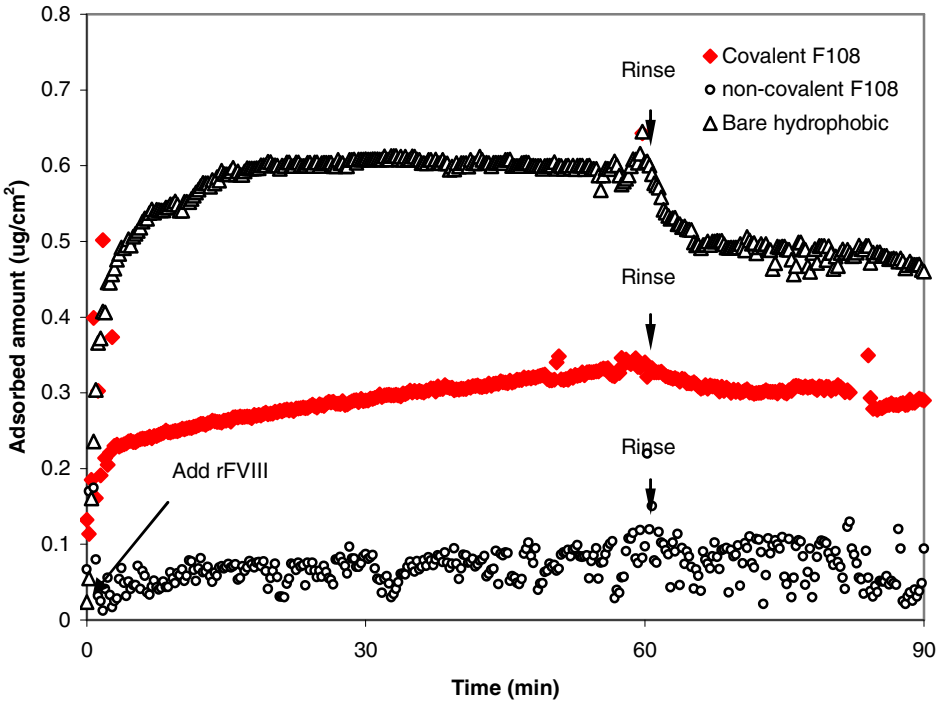

Figure A.2 rFVIII adsorption on F108 covalently attached surface and non-covalent surface, compared to adsorption and elution on bare hydrophobic surface.

surface is a result of rFVIII adsorption to “bare spots” on the surface, or exchange with loosely held F108.

rFVIII adsorption to silanized silica, and to F108 pre-coated surfaces

Figure 2 shows representative results obtained when rFVIII is allowed to adsorb directly to silanized silica, and when rFVIII is allowed to adsorb to an F108 precoat, covalently and non-covalently. In these cases adsorption was allowed to occur for 1 h prior to rinsing and incubation in buffer. In order to measure adsorption on an F108 layer, HDMS-treated silica strips were incubated with 0.5 % F108 for at least 16 h (in the quartz cuvette, housed on the ellipsometer sample stage) to allow for proper molecular orientation, rinsed with 10 cuvette volumes of filtered KG-2 buffer, then held under static conditions for 1 h prior to determination of substrate optical constants. Figure 2 shows adsorption of rFVIII on the F108-coated surface is quite low in comparison to the bare hydrophobic surface. After 60 min of adsorption, data recorded for the F108 layer show considerable scatter, but otherwise suggest an amount of rFVIII adsorption similar to that observed in Figure A1. As above, we attribute any rFVIII adsorption to incomplete or inefficient coating on the HDMS-silanized surface (coating time was probably not an issue, but HDMS-silanized surfaces are suboptimal substrates for F108 coating). As shown in Figure A2, the adsorption mass of rFVIII on the F108 covalently attached surface is much lower than it on the bare hydrophobic surface, but higher then the non-covalent F108 coated surface. This observation indicates that the F108 covalently attached surface only partially repelled rFVIII, while we expected to see less adsorption of rFVIII on the surface. Presumably we think that that there is relatively a large potion of “bold spots” on the F108 attached surface to adsorb rFVIII, and it is probably due to preparation of the F108 grafting.

rFVIII on charged surfaces

rFVIII adsorption on (positively-charged) nickel oxide

As shown in Figure A3 the adsorbed mass of rFVIII attained a value of about 0.75 μg/cm^2 after 1 h. The rFVIII layer was not much affected by the rinsing step, indicating high affinity and irreversible adsorption (with respect to buffer elution). Upon increasing NaCl concentration in the formulation by a factor of five (from 30 to 150 mM), the adsorbed amount of rFVIII was reduced to about 0.35 μg/cm^2. This can be attributed to shielding of charges involved in electrostatic interaction between the protein and surface, indicating attractive forces between opposite charges play an important role in rFVIII adsorption to nickel oxide. This result is similar to that observed at (negatively-charged) bare silica, indicating that the orientation of the rFVIII molecule is quite surface-specific.

rFVIII adsorption on (neutral) zinc sulfide

As shown in Figure A4, the adsorbed mass of rFVIII attained a value of about 0.55 μg/cm^2 after 1 h. The adsorbed mass decreased by about 0.05 mg/ml after rinsing, indicating in summary a somewhat lower affinity between rFVIII and the neutral surface than that observed between rFVIII and the charged surfaces studied to date. However, as we have seen a much more substantial reduction in rFVIII adsorption to PEO coatings which are also electronically neutral, we conclude that steric repulsion is a requirement for rFVIII repulsion. As shown in Figure A3 with nickel oxide coating, the adsorbed mass of rFVIII was about 0.75 μg/cm^2 in 30 mM NaCl, and reduced to 0.35 μg/cm^2 in 150 mM NaCl at 60 minutes of adsorption time. However, adsorbed mass on the Zinc sulfide-coated surface reduced from 0.5 μg/cm^2 to 0.35 μg/cm^2 when NaCl concentration was increased from 30 mM to 150 mM as shown in Figure 4. This indicates that at the near-neutral Zinc sulfide surfaces, salt concentration didn't have as much effect of reduction of

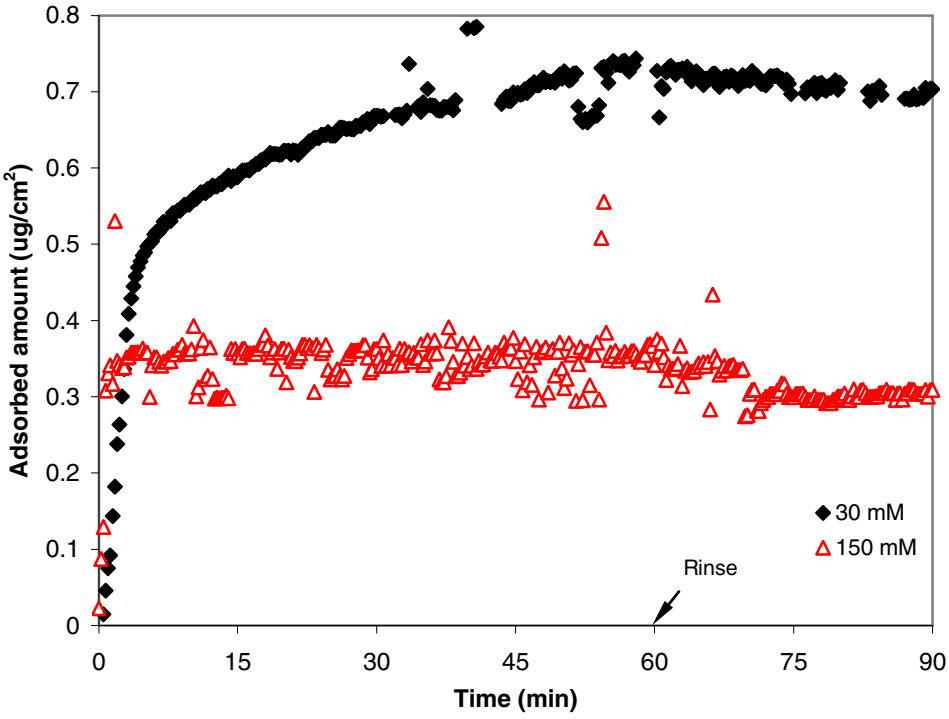

Figure A.3 rFVIII adsorption on positively-charged nickel oxide surfaces under various NaCl concentration.

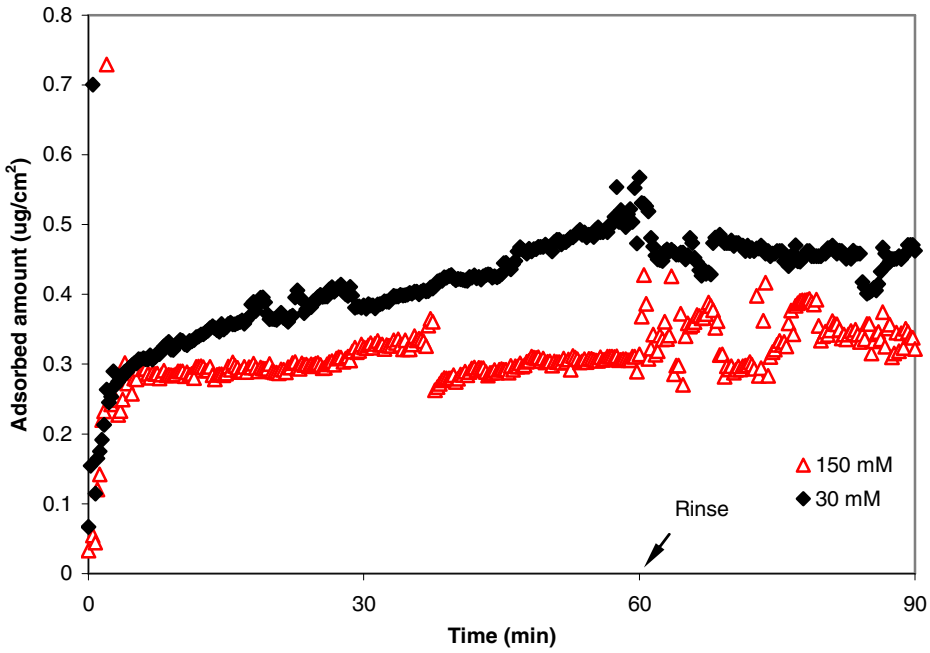

Figure A.4 rFVIII adsorption and elution on neutral hydrophilic zinc sulfide surfaces under various NaCl concentration.

rFVIII adsorption as at the positive charged nickel oxide surfaces to reduce rFVIII adsorption.

Conclusions

Ellipsometry results show that the F108 layer inhibits adsorption of rFVIII on hydrophobic surfaces. rFVIII has relative high affinity to positively charged nickel oxide surfaces than the neutral zinc sulfide coated surface. Adsorption to zinc sulfide showed a lower affinity than adsorption to the charged surfaces, but was appreciably higher than rFVIII adsorption to F108 layers. Higher ionic concentration greatly reduced adsorption of rFVIII on nickel oxide surface, and this can be attributed to shielding of charges involved in electrostatic interaction between the protein and surface, indicating attractive forces between opposite charges play an important role in rFVIII adsorption.

Acknowledgements

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APPENDIX B

ANIMAL EXPERIMENT

In this appendix we attached a protocol used for *in vivo* evaluation of implanted nisin-treated catheter and its results in summary tables. This experiment was performed on 16 sheep for five hours, three days and five days, in a randomized, double-blind manner. Samples were harvested and immediately sent for microbiology and activity analysis. This project was conducted between College of Veterinary Medicine and Department of Chemical Engineering in Oregon state University.

Sheep Study of Nisin-treated Catheters

Prepared by Jeff Tai

General Schedule

Date	Starting time	Task	P.I.C.
2/5, Sun	5 pm	§ Incubate catheters in F108	Jeff Tai
2/6, Mon	9 am	§ Prepare nisin solution and other reagents § Sterilize glassware and buffers § Label samples	Jeff Tai, Kari Gamble
2/7, Tue		§ 3-day catheter placement	Class, Kari Gamble, Jill Parker
2/9, Thu		§ 5-day catheter placement	Class, Kari Gamble, Jill Parker
2/10, Fri		§ 3-day catheter removal § Collect tissue and catheter samples § Activity assay on agar plates	Class, Kari Gamble, Jill Parker Jeff Tai
2/12, Sun	5 pm	§ Incubate catheters in F108	Jeff Tai
2/13, Mon	9 am	§ Prepare nisin solution and other reagents § Sterilize glassware and buffers § Label samples	Jeff Tai, Kari Gamble
2/14, Tue		§ 5-day catheter removal § 5-h catheter placement; 5-h catheter removal § Activity assay on agar plates	Class, Kari Gamble, Jill Parker Jeff Tai
2/16, Thu		§ 5-h catheter placement; 5-h catheter removal § Collect tissue and catheter samples	Class, Kari Gamble, Jill Parker

		§ Activity assay on agar plates	Jeff Tai

Inventory

Chemicals:

Nisin (Prime Pharma South Africa, Batch 20050116)
Sodium phosphate monobasic (Mallinckrodt 7892)
Sodium phosphate dibasic heptahydrate (Mallinckrodt 7914)
Pluronic F108 Prill (BASF 583062)
MRS broth (Merck, 1.10661.1)
DI Water

Glassware and other

Autoclave tray
Color labeling tapes
Corning “fleaker”, 500 ml (8)
Graduate Cylinder (2 liters, 1 liter)
Catheters, (64)
Aluminum Foil (1 roll)
Syringe filter (1 box)
Syringe pump (1)
200 mm test tubes (64)
Test tube racks
Elecrical. Pipettor
10 ml and 25 ml disposable pipettes
Screw-top test tubes for sample collecting (32)

Reagents

Reagent	Recipe	Sterilization	Total Qty Need
0.5 % (w/v) Pluronic F108 solution	Dissolve 5 g of F108 in 1 liter of DI water. Stir well to dissolve	Autoclave 15 min	4 liters
0.01 M monobasic sodium phosphate buffer	1.38 g in 1 liter of DI water	Autoclave 15 min	2 liters
0.01 M dibasic sodium phosphate buffer	2.68 g in 1 liter of DI water	Autoclave 15 min	2 liters
0.01 M sodium phosphate buffer pH 7	1.08 g dibasic and 0.53 g monobasic in every 1 liter of DI water	Autoclave 15 min	10 liters
MRS Broth	Suspend 52.2 g in 1 liter of DI water.	Autoclave 30 min and 15 min dry	1 liter
1 mg/ml nisin solution	For every liter of nisin solution, dissolve 1 g of nisin in 363.6 ml of 0.01 M monobasic phosphate buffer and stir-mix for 10 minutes. Then add 636.4 ml of 0.01 M dibasic sodium phosphate buffer and stir for another 10 minutes.	0.22 um syringe filter. Prepare one day before lab.	2 liters (500 ml x4)

F108 coating on catheters

1. Incubate sterilized F108 with catheters for at least 16 hour or overnight in 4 C coldroom. Cover the opening with aluminum foil or suitable cap.
2. Transfer all catheters to sterile 0.01 M phosphate buffer and stir for 10 minutes to elute un-adsorbed F108.
3. Transfer rinsed F108-coated catheter to another to 0.01 M sodium phosphate containing “fleakers” for nisin treatment or direct placement on sheep.

Nisin treatment

1. Incubate F108-coated or bare catheters at 1 mg/ml nisin solution for at least 30 minutes before placement.
2. Remove catheters from incubation container. Dry catheters by carefully touching the end of the tip to an sterile absorptive material.
3. Transfer catheters to another 500-ml "fleaker" of 0.01 M sodium phosphate buffer for placement on sheep.

Activity assay of catheter segments

1. Implanted catheters will be harvested from sheep and 3 cm segment from the tip of Teflon catheters will be cut and transferred to a sterile test tube. Test tubes will be mark with group identity and sent to microbiology lab in Vet Medicine for analysis.
2. The rest of the catheter segments will be inserted to a *Pediococcus* seeded agar plate for activity assay immediately after removal. Plate will be incubated for 48 hours and diameter of the kill-zone halo will be measure and recorded.

Table A.1 Activity of nisin treated catheter segment, 3-day and 5-day

Sample Number	Treatment	Catheter Dates		Study	Diameter (mm)			Area (mm^2)					Bacterial Culture	
		Insert	Removal		1	2	3	1	2	3	**Average**	Standard Deviation	anaerobic	aerobic
9154	Saline	2/7/2006	2/10/2006	3d								*Enterococcus spp*		
2623	Saline	2/7/2006	2/10/2006	3d								*S.sp (Coagulase-)*		
1483	Nisin	2/7/2006	2/10/2006	3d									n	*E.coli*
8137	Nisin	2/7/2006	2/10/2006	3d									n	n
3178	F108	2/7/2006	2/10/2006	3d									n	n
4807	F108	2/7/2006	2/10/2006	3d								*Corynebacterium spp*		
5714	F108 + Nisin	2/7/2006	2/10/2006	3d									n	n
7269	F108 + Nisin	2/7/2006	2/10/2006	3d									n	*E.coli*

Sample Number	Treatment	Catheter Dates		Study	Diameter (mm)			Area (mm^2)					Bacterial Culture	
		Insert	Remove		1	2	3	1	2	3	**Average**	Standard Deviation	anaerobic	aerobic
4715	Saline	2/9/2006	2/14/2006	5d									n	*E.coli*
9269	Saline	2/9/2006	2/14/2006	5d									n	*E.coli*
7138	Nisin	2/9/2006	2/14/2006	5d									n	*E.coli*
3481	Nisin	2/9/2006	2/14/2006	5d									n	*E.coli*
3622	F108	2/9/2006	2/14/2006	5d									n	*E.coli*
4159	F108	2/9/2006	2/14/2006	5d									n	*E.coli*
7884	F108 + Nisin	2/9/2006	2/14/2006	5d									n	n
8173	F108 + Nisin	2/9/2006	*1/12/2006*	5d	Catheter fell out prior to the 5d and disposed (NA)									

Table A.2 Activity of nisin treated catheter segment, 5-hours

Sample Number	Treatment	Catheter Dates		Study	Diameter (mm)			Area (mm^2)					Bacterial Culture	
		Insert	Remove		1	2	3	1	2	3	**Average**	Standard Deviation	anaerobic	aerobic
9682	Saline	2/14/2006	2/14/2006	5hr									n	n
4717	Saline	2/14/2006	2/14/2006	5hr	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.0	n	n
1532	Nisin	2/14/2006	2/14/2006	5hr	7.90	0.00	5.30	49.02	0.00	22.06	23.69	24.5	n	n
6264	Nisin	2/14/2006	2/14/2006	5hr	10.09	6.33	7.84	79.96	31.47	48.27	53.23	24.6	n	n
3623	F108	2/14/2006	2/14/2006	5hr	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.0	n	n
8175	F108	2/14/2006	2/14/2006	5hr	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.0	n	n
2440	F108 + Nisin	2/14/2006	2/14/2006	5hr	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.0	n	n
7358	F108 + Nisin	2/14/2006	2/14/2006	5hr	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.0	n	n

Sample Number	Treatment	Catheter Dates		Study	Diameter (mm)			Area (mm^2)					Bacterial Culture	
		Insert	Remove		1	2	3	1	2	3	**Average**	Standard Deviation	anaerobic	aerobic
4266	Saline	2/16/2006	2/16/2006	5hr	0.0	0.0	0.0	0.00	0.00	0.00	0.00	0.0	n	n
2531	Saline	2/16/2006	2/16/2006	5hr	0.0	0.0	0.0	0.00	0.00	0.00	0.00	0.0	n	n
3624	Nisin	2/16/2006	2/16/2006	5hr	0.0	5.1	0.0	0.00	20.11	0.00	6.70	11.6	n	n
5178	Nisin	2/16/2006	2/16/2006	5hr	6.6	11.0	5.6	34.00	95.21	24.19	51.13	38.5	n	n
5861	F108	2/16/2006	2/16/2006	5hr	0.0	0.0	0.0	0.00	0.00	0.00	0.00	0.0	n	n