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Title: Nisin Adsorption to PEO-PPO-PEO Tri-Block Copolymer Layers and its Resistance to Elution by Fibrinogen

Abstract approved:

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Joseph McGuire

While hydrophobic surfaces coated with the poly[ethylene oxide]-poly[propylene oxide]-poly[ethylene oxide] (PEO-PPO-PEO) surfactant Pluronic® F108 are highly resistant to plasma protein adsorption, the antimicrobial peptide nisin has been observed to adsorb in multilayer quantities at such surfaces, and the PEO chains themselves suggested to inhibit nisin exchange by blood proteins. But this had been investigated only with F108 bound by physical association between the hydrophobic surface and its apolar PPO block. In this work, nisin adsorption at hydrophobic, silanized silica surfaces coated with F108, and in separate experiments, coated with F108 that had been end-activated with nitrilotriacetic acid groups (EGAP-NTA), was detected *in situ*, by zeta potential measurements. The triblocks were covalently immobilized prior to the introduction of protein in each case. Zeta potential measurements were also used to evaluate fibrinogen adsorption, and the sequential adsorption behavior of nisin and fibrinogen, at bare hydrophobic and triblock-coated surfaces.

Silica microspheres (1 μm diameter) were silanized with either allyldimethylchlorosilane (ADCS) or trichlorovinylsilane (TCVS). Silanized microspheres were incubated overnight in a solution of F108 or EGAP-NTA to allow for self assembly of a PEO layer. Coated microspheres were then subjected to γ-irradiation under water or in the presence of the triblock coating solution. Layer stability was quantified by its resistance to elution by SDS. Zeta potential changes indicated that pretreatment with TCVS, and γ-irradiation performed in the presence of the triblock coating solution, produced more stable triblock layers than were produced with ADCS. For this reason silanization only with TCVS was used in protein adsorption experiments.

Introduction of fibrinogen to triblock-coated microspheres showed little change in zeta potential, indicating the presence of a steric repulsive barrier to fibrinogen adsorption. Introduction of nisin to triblock-coated microspheres showed a significant increase in zeta potential, a result of adsorption of the cationic nisin. In sequential adsorption experiments, the introduction of fibrinogen to "nisin-loaded" triblock layers caused a decrease in the zeta potential, consistent with the net negative charge of fibrinogen. This decrease was substantially more pronounced for TCVS-modified silica in the absence of triblock coatings, suggesting an enhanced resistance to nisin elution owing to its location in the PEO layer.

Key Words: nisin adsorption, zeta potential, Pluronic® F108, PEO-PPO-PEO triblock surfactant

Nisin Adsorption to PEO-PPO-PEO Tri-Block Copolymer Layers and its Resistance to Elution by Fibrinogen

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

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Matthew P. Ryder, Author

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Introduction

All materials used in blood and tissue contact are susceptible to bacterial adhesion. Once bacteria adhere to a surface, they replicate and become entrapped in a polysaccharide matrix, forming a biofilm. Once this occurs, both the host immune system and antimicrobial treatments become less effective against the bacteria due to mass transfer limitations associated with penetrating the biofilm matrix, partly because the physiology of the bacteria itself changes. In addition, bacteria that have formed a biofilm have increased capacity to develop resistance to antimicrobials. To reduce morbidity and mortality associated with medical device related infections, it is imperative to prevent bacteria from forming a biofilm.

Infections associated with medical devices can result in systemic infections that in the worst cases lead to multiple organ failure and even death, despite successful resolution of the original medical condition the patient was presented with. The cost of these infections per annum is in the billions of dollars. For example, a single instance of central venous catheter-related bacteremia has been estimated to cost upwards of $50,000 with an associated mortality rate up to 35% [1]. About 3 million of these catheters are used each year in the United States alone, and blood stream infections related to catheters occur in over 200,000 patients with over 80,000 of these taking place in an ICU. The cost of the ICU infections ranges between $296 million and $2.3 billion dollars per year, with an associated 2,400 to 20,000 deaths.

The process by which bacteria or proteins adsorb and remain on the surface of a medical device, such as a central venous catheter or a coronary stent is known as fouling. The former occurs via biofilm formation, while the latter occurs most often by proteins and platelets adhering to the surface, the platelets becoming activated and initiating the coagulation cascade.

Previous work done in the Biomaterials and Biointerfaces laboratory discovered that nisin (described below) adsorbs in multilayer quantities in PEO-PPO-PEO tri-block - hereafter referred to as tri-blocks - layers (described below). This observation was confirmed by ellipsometry tests, but results were tentative because the tri-blocks used were not covalently attached to the surfaces. Further experimentation suggested after adsorption of nisin fibrinogen preferentially located at the surface as well. Tentative results needed to be more carefully tested and confirmed.

In order to accomplish this we used silica microspheres and polished silica slides treated with trichlorovinyl silane (TCVS) to silanize the surface of the silica. We then covalently attached tri-blocks by self assembly and γ-irradiation as the antifouling layer of the coating. The tri-block layers consist of a hydrophobic base of poly[propylene oxide] and two longer hydrophilic tails of poly[ethylene oxide]; we also used a tri-block that had been end group activated with nitrilotriacetic acid (EGAP-NTA), previous work has shown EGAP-NTA to retain nisin better than unactivated tri-blocks, presumably because of the negative charge of the NTA end group. In solution, the hydrophobic base

of the tri-blocks will spontaneously and preferentially locate at a hydrophobic surface (see Figure I.1).

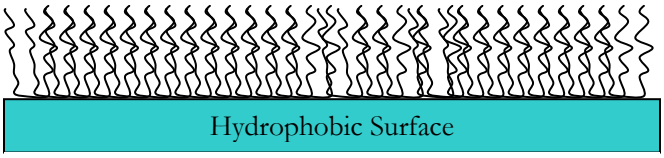

Figure I.1 *shows the tri-blocks preferentially located at a hydrophobic surface.*

The hydrophilic tails repel approaching proteins or bacteria by steric repulsive interactions. After the tri-blocks have preferentially located at the surface, it is entropically unfavorable for it to spontaneously desorb, and remains on the surface in aqueous solution. Once the surface had been incubated with the tri-blocks; and the tri-blocks had adsorbed to the surface, they were rinsed and the lantibiotic nisin was added to the solution. Nisin is a relatively small protein, made up of 34 amino acids, that acts to kill Gram positive bacteria. Nisin kills by forming open pores in the surface of a bacterium; it takes eight nisin proteins to form a single pore (see Figure I.2) [2].

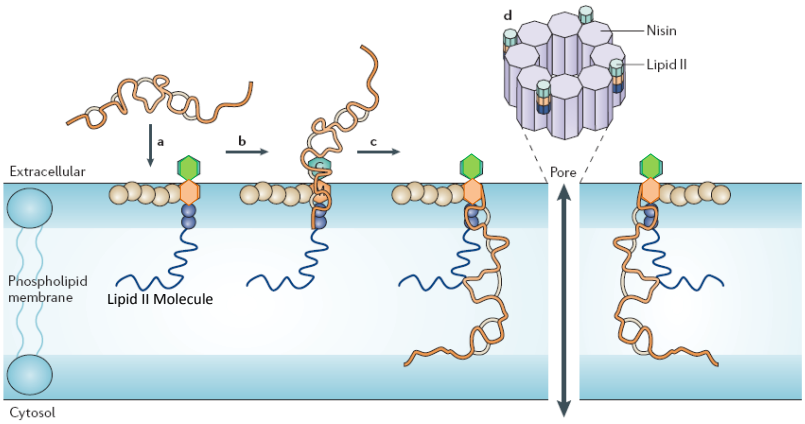

Figure I.2 *shows the mechanism by which Nisin forms pores in a target bacterium. (a) nisin approaches cell surface at Lipid II protrusion from the bacterial cell wall. (b) nisin attaches to Lipid II molecule. (c) nisin enters the cell through interaction with Lipid II. (d) 8 nisin molecules aggregate with 4 Lipid II molecules to form a pore in the bacterium surface, killing the cell.*

Contrary to the function of the tri-block coated surface, nisin is able to adsorb to the brush layer in aqueous solution [58]. Although theory suggests that small enough molecules should will be able to adsorb within the brush layer in monolayer quantities, it does not discuss multi-layer quantities. Multi-layer adsorption was measured by Tai et al using ellipsometry data [3].

Literature Review

Nonfouling vs antibacterial surfaces

Many device coating have been evaluated for their ability to reduce the instance of implant related infections. The approaches generally can be divided into two major categories. In the first category, surfaces are modified to prevent bacterial adhesion and/or protein adsorption. These approaches often involve minimization of adhesion or adsorption by steric repulsion. Many research groups have shown that surfaces grafted with PEO have significantly less tendency to be adsorbed or adhered to [4]. Additional work has focused on the use of PEO in block copolymers as surface modifiers, which for some such compounds create opportunity for "self assembling" surfaces, which rely on thermodynamic qualities of the solution to create the surfaces. The Pluronic® surfactants are representative of this type of synthetic block copolymer. These structures usually have a composition of PEO-PPO-PEO, the PEO being the hydrophilic "tails" and the apolar PPO being the hydrophobic "base" of the block copolymer. Through hydrophobic action, then, the PPO base essentially immobilizes the pendent PEO chains to a hydrophobic surface. In this way, almost any hydrophobic surface can be turned into hydrophilic, "protein-repelling" surfaces [5,6]. This process has been applied to decrease the surface adhesion of cells, including platelets and bacteria, and has been shown to reduce the adsorption of blood proteins as well [3,7]. Although hydrophilic coatings have been shown to reduce bacterial adhesion, problems with infection still occur.

In the second category, surfaces are impregnated or modified with agents that kill or prevent bacterial growth. Two commercially available short term catheters that fit into this category, for instance, have been shown to reduce infection rates. One is chlohexididine-silver sulfadiazine impregnated (CSI) and the other is minocycline-rifampin impregnated (MRI) [8]. These products, however, pose a serious risk for developing drug resistant strains of bacteria. For the CSI catheters, this risk is lower, but *in vitro* studies have found that exposure to the impregnating compound, chlorhexidine can result in increased bacteria to not only it, but other therapeutic antimicrobial agents. Other examples that fall into this category include antiseptic based coatings, but these have been associated with reports of anaphylactic shock [9-11]. The prophylactic use of antibiotic-coated implants seems to increase the risk of producting resistant strains of bacteria, while the use of other kinds of antibacterials, like antiseptics, provide inferior results when compared to clinical antibiotics [12].

Lantibiotics are antibiotic compounds that include one or more lanthionine rings. These structures are unique in that their physical structure significantly alters their mode-of-action from traditional antibiotics, suggesting they may offer means for preventing the rise of so-called "super bugs" or drug resistant microorganisms, and that cross-resistance is highly unlikely [13-16]. These compounds show vast variability in their inhibitory actions and have many characteristics that make them promising for biomedical applications [17]. Lantibiotics like nisin can adsorb to surfaces, maintain activity, and kill cells that have adhered *in vitro* [17-19]. Nisin is a 36 amino acid long

peptide, which has long been used as a food preservative and has been shown to be both potent and safe. Nisin is also the most extensively investigated lantibioted with reference to biomedical applications. The structure of nisin is shown in Figure L.1.

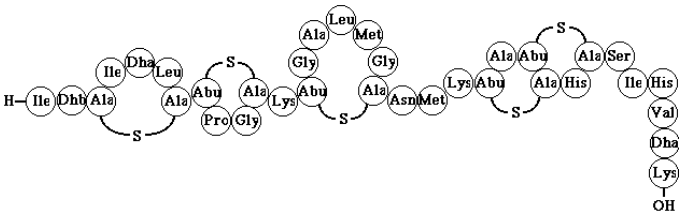

Figure L.1: *Structure of nisin. Abu: 2-aminobutyric acid; Dha: dehydroalanine; Dhb: dehydrobutyrine; Ala-S-Ala: lanthionine; Abu-S-Ala: β-methyllanthionine (adapted from Wiedemann et al., 2001).*

Nisin is able to kill Gram-positive bacteria at concentrations in the nanomolar range through a multistep process that destabilizes the phopholipid bilyaer of the cell, creating transient pores, thereby rupturing the cell [14, 15, 20, 21]. *Staphylococus aureus* and *Staphylococcus epidermidis* are the most frequently encountered biomaterial-associated pathogens [22-24], and both are Gram-positive bacteria. Additionally, nisin can be an effective inhibitor of many Gram-negative bacteria when combined with other compounds such as chelating agents [25].

Nisin has proven to be effective at preventing microbial adhesion on endotracheal suction catheters *in vitro* (using *Staphylococcus aureus, Staphylococcus epidermidis,* and *Enterococcus faecalis* (*Streptococcus faecalis*) as indicator organisms), prompting further studies *in vivo* evaluating nisin-treated intravenous catheters in sheep and tracheotomy tubes I ponies [19]. Nisin pretreated catheters for long term

placement (7 days) failed to retain antimicrobial activity, while short-term (3-5 h) catheters maintained activity. The nisin was bound by non-specific adsorption to otherwise untreated catheter materials in that work, and the activity loss was attributed to elution of nisin from the surface by blood protein exchange. Further studies were conducted on "tethering" nisin to solid surfaces for long-term antibacterial activity, in such a way as to allow the nisin to retain activity, i.e. access to bacterial membranes and molecular mobility necessary for binding, insertion, and pore formation [26]. In that study, thiol-modified nisin derivatives were synthesized, then chemically coupled to end-group activated PEO-PPO-PEO triblock polyers that had been attached to polystyrene microsphere surfaces. Nisin was secured to these groups through a disulfide linkage. Unreacted nisin was removed by dialysis and the coated microspheres were tested for activity against the Gram positive *Pediococcus pentosaceus*. Thiol-modified nisin was introduced to (unactivated) F108 in a similar manner for controlled comparison of antimicrobial activity. It was routinely found that the unactivated F108 - the control group - showed similar results to the end-group activated samples, despite thorough and repeated washings. These results suggested that a finite amount of nisin was entrapped in the PEO chains of the F108 triblocks [3].

It is well known that PEO resists protein interactions and F108 coated surfaces have been shown to prevent adsorption of proteins to coated surfaces [5, 27, 28]. This understanding leads to the expectation that nisin would not be able to interact with the PEO-PPO-PEO triblocks, other than at reactive sites on end-group activated samples.

The apparent entrapment of a substantial amount of nisin, therefore, is puzzling. Methods for producing these surfaces with entrapped nisin have been described [2,3].

Adsorption and repulsion of proteins by pendant PEO chains

There exists an abundance of literature describing the nonfouling mechanisms of material surfaces with pendant polymer chains. In particular, the function of pendant PEO has been studied extensively. The mechanisms commonly cited to describe the protein resistant nature of end-tethered PEO surfaces include hydration barriers and steric repulsion [29, 30]. Steric repulsion is based on PEO chain compression, which is representative of entropy loss, as the protein comes near the surface. This requires some minimum length of the pendant PEO chains in order to introduce entropy considerations. The hydration barrier mechanism is based on the idea that the binding of water to the PEO chains is sufficiently tight so as to keep approaching proteins from interacting with the surface. This is consistent with observations that very short PEO chains, as short as one or two monomer lengths, can provide protein resistance.

To date, debate continues on the specific chemical and physical origins of the protein resistant character of end-tethered PEO, yet consistent observations on the expected effects of several important factors on protein repulsion of pendant PEO have been reported. These factors include PEO chain density, which affects chain conformation and hydration, length, and the chemical character of the free end group [31-33]. In regard to chain density, two regimes can be distinguished [34]. At high chain density, where the distance between grafting sites D is relatively small, and $D < 2R_F$,

where R_F is the Flory radius of the polymer, the chains extend from the surface forming a "brush." In low chain density situations, the coils are sufficiently far away from one another to form random coils. In the brush configuration random coils are not formed, and protein repulsion is typically very good, and independent of chain length [27, 32, 33, 35]. Outside the brush regime, protein resistance increases with chain length, up to about 35 monomer units, beyond which repulsion is generally independent of chain length. It is understood that the basic requirement for protein repulsion is the formation of a brush layer.

In contrast to protein repulsion, there are few studies in the literature that describe the adsorption of small proteins to a PEO layer. It has been suggested that once a high enough chain density is achieved, the rejection capacity of the pendant polymer phase is determined by protein size, and is typically compared to the average distance between polymer chains [36, 37]. Further analysis of this tendency suggests that grafting densities consistent with the formation of a brush layer be achieved before discrimination of proteins based on size becomes evident [31]. A model for protein adsorption in a PEO brush based on kinetic and thermodynamic considerations predicts two possible modes of protein adsorption: primary, at the surface itself, and secondary adsorption, at the periphery of the grafted PEO chains [38]. Multilayer adsorption is not predicted, nor is protein association with the PEO chains in that simplified model. Based on surface force experiments involving compression of PEO brushes by protein-coated surfaces, however, it was suggested that a PEO brush may exhibit coexistence between

an inner, dense, hydrophobic phase and a dilute hydrophilic phases at the outer edge of the brush [39]. Such coexistence would give rise to an inner region that may be attractive for protein adsorption [38, 39]. Nisin adsorption within PEO layers may thusly be attributed to its high amphiphilicity.

A model based on a simplified difusion approach for protein interaction with PEO brushes was formulated by Fang et al. (2005) [41]. According to their model, adsorption and desorption kinetics depend on the relationship between protein size and brush layer thickness. Specifically, when the pendant chain layer thickness is greater than the size of the protein, both adsorption and desorption kinetics decrease with chain length. Their model indicated an adsorption time too large for any practical adsorption of proteins. Most interesting, their model suggests that increasing chain length provides a method for decreasing desorption rate, thereby entrapping the proteins between the surface and the free end of the pendant PEO chains. Based on that result, they suggested such a trapping mechanism may be used to design strategies for controlled release of proteins from surfaces.

As for the chemical character of the free end group, some studies have shown that protein adsorption is insensitive to end group chemistry, while others have suggested just the opposite. Mathematical models of PEO in the brush configuration indicate is highly unlikely that end-group chemistry affects the interaction with proteins. It was shown, for example, that chain ends are distributed through the brush, with a maximum occurring at about 70% of the total chain length [38]. Other experiments

showed that protein repulsion at PEO brushes is uniquely dependent on chain density, and independent of chain length and end-group chemistry [33]. Beyond a certain critical chain density, however, brushes with -OH end groups have been observed to retain nonfouling character while those with -OCH_3 end groups tended to promote protein adsorption. In the latter case, it was suggested that the high densities of terminal methoxy groups may have increased interchain association and/or adsorption-induced protein denaturation.

Most Relevant Literature

The studies most relevant to the research presented here were conducted by Tai et al. at Oregon State University. In those studies, *in situ* ellipsometry was used to verify the entrapment of nisin within pendant PEO layers by measuring the adsorption and elution of nisin of surfaces coated with Pluronic® F108 [3]. Ellipsometry is a sensitive technique for measuring the refractive index and thickness of very thin films. These properties are used to calculate adsorbed mass at the interface.

Figure L.2 provides a representative comparison of nisin adsorption and elution kinetics at bare hydrophobic and F108-coated surfaces. According to that study, nisin adsorption did not reach a plateau on either surface. According to that study, a monolayer would result in an adsorbed mass of 0.058 and 0.145 μg/cm^2, depending on whether the configuration was "side-on" or end-on," respectively. The patterns shown in Figure L.2 show that the nisin adsorption is more consistent with multi-layer adsorption. According to the article, nisin adsorption to the F108-coated surface was

generally slower than to the bare hydrophobic surfaces. The authors attribute this to steric inhibition by the pendant PEO chains. Once the surfaces had reached a steady state of nisin adsorption, the surfaces were exposed to protein-free buffer, in which nisin elution was similar on both surfaces initially. Elution was observed to continue only at the bare hydrophobic surface. This means nisin is resistant to elution from the brush layer in the case of the PEO coated surface, whereas on the bare hydrophobic surface, solvent accessibility to nisin readily elutes the protein from the surface.

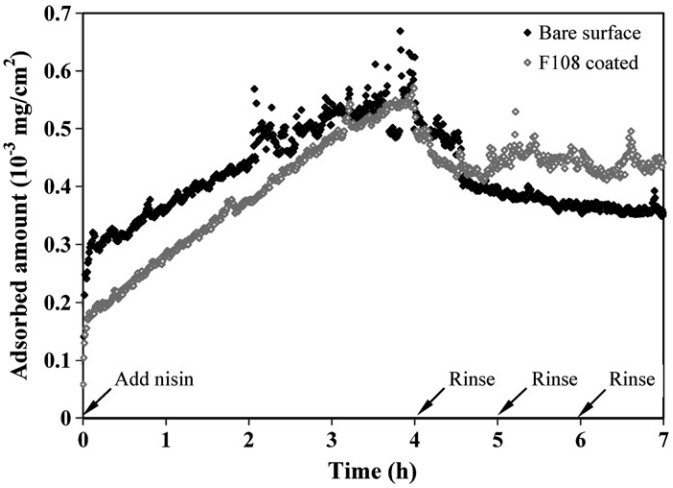

***Figure L.2:**. Comparison of nisin adsorption and elution kinetics at bare hydrophobic and F108-coated surfaces.*

The effect of pendant PEO chains in adsorption and elution can be further revealed by analysis of nisin interaction data with reference to a "history dependent" adsorption mechanism [40, 42]. A number of macromolecular species exhibit this type of adsorption behavior owing to the slow relaxation of non-equilibrium structures at the interface. Thus, for a given protein at a give surface, the rate of adsorption depends on

the formation history of the adsorbed layer. This is most relevant near monolayer surface coverage when protein-protein interactions begin to influence adsorption kinetics and available surface area. In one study, it was shown that, for a given mass density at a surface, if proteins were arranged in aggregates rather than individual species, more surface area would be available for further adsorption [3].

These results and results describing nisin activity when challenged by the model indicator strain *Pediococcus pentacaceus* were conducted using F108 triblocks bound to model and catheter materials by hydrophobic association alone. In order for these types of materials to be viable for use in medical devices such as central venous catheters or coronary stints, these triblocks must be covalently attached to the surface to prevent eventual elution and loss of functionality. This work describes a method derived from work done by McPherson et al. (1997) and Park et al. (2000), using pretreatment with a vinyl-containing silane and subsequent γ-irradiation [29, 43].

Materials and Methods

Protein and surfactant solutions

Nisin (MW 3510 Da) was obtained from Prime Pharma (Batch number 20050810, Gordons Bay, South Africa) and was dissolved in filtered 0.2 µm, 10 mM sodium phosphate buffer with 0.15 M NaCl (10 mM PBS with 150 mM NaCl), adjusted to a pH of 7.4, physiologic conditions. Nisin has an isoelectric point in the alkaline range (above 8.5) and is therefore positively charged at neutral pH. Fibrinogen (Sigma-Aldrich, Lot # 092K7602) was dissolved in filtered 0.2 µm 10 mM PBS with 0.15 M NaCl. Solutions of nisin and fibrinogen (at 5.0 mg/mL and 2.5 mg/mL, respectively) were prepared immediately before use. The Pluronic® surfactant F108 (MW 14,600, with two end blocks of PEO that are 141 monomer units in length and a center block of PPO that is 44 monomer units in length, i.e., HO-$(CH_2\text{-}CH_2\text{-}O)_{141}$-$(CHCH_3\text{-}CH_2\text{-}O)_{44}$-$(CH_2\text{-}CH_2\text{-}O)_{141}$-H) was obtained from Allvivo Vascular (Anti-Link™, Lot WPHA512E) and was dissolved in 10mM PBS with 150mM NaCl (pH 7.4) as needed. End Group Activated Pluronic with nitrilotriacetic acid was also obtained from Allvivo Vascular (EGAP-NTA, Lot A0907N10) and was dissolved in 10 mM PBS with 150 mM NaCl (pH 7.4) as needed.

Surface modification

Silica microspheres (1 µm AngstromSphere Monodispersed Silica Powder, Fiber Optic Center Inc.) were used as base surface for modification. The microspheres were washed with solution of HPLC-H_2O:NH_4OH 30%:H_2O_2 30% (5:1:1 volume ratio) at 80 °C for 10 min followed by HPLC-H_2O: HCl 37%:H_2O_2 30% (5:1:1 volume ratio) at 80 °C for 10

min to remove particulates and organic residues [29]. They were then rinsed with HPLC-H_2O three times using a volume equal to the original wash solutions, and dried in a glassware oven at 110 °C for minimum of one hour. Washed (bare) Si microspheres were stored dessicated until use. From this point, two different silanes were used to render the silica surfaces sufficiently hydrophobic for tri-block coating. One silane used was Triochlorovinylsilane (TCVS, Product number 104876, Aldrich) and the other was Allylchlorodimethylsilane (ADCS, Alfa Aesar). A 5%(v/v) solution of silane in dry chloroform was prepared immediately before use. Silane solution was added to the bare Si microspheres and rotated at room temperature for three hours. Once silane incubation was complete, the samples were washed three times each with dry chloroform, dry ethanol and HPLC-H_2O, respectively. A washing consists of solution being added to the microspheres, vortexing for a minimum of three minutes, sonication for a minimum of one minute, followed by shaking vials by hand until the solution appeared well mixed. After this point, the samples were centrifuged and the wash solution was removed. Observationally, once the spheres reached the water washes, they resisted pellet formation, and remained at the air-water interface, presumably due to the hydrophobic nature of the coated spheres, and the hydrophobicity of these samples was noted by their inability to form a suspension within an H_2O-only environment after centrifugation. The samples were dried in a glassware oven overnight (at least 12 hrs). Silanized microspheres were stored dessicated until needed.

Surface coating with tri-blocks

TCVS- or ADCS-treated samples were coated with tri-blocks by incubation with 0.50% F108 in 10 mM PBS with 150 mM NaCl. Incubation took place overnight (at least 16 hrs), in 2 mL microcentrifuge tubes. Microspheres remained in their original centrifuge tube through experimentation, only solutions were changed. The coated samples were then rinsed, *in situ*, at least twice with 2 mL of 10 mM PBS with 150 mM NaCl. Two different silanes were used to discover which would provide a better surface for further modification.

PEO coatings were also prepared by covalent attachment of the tri-blocks. McPherson et al. [29] and Park et al. [43] described pretreatment with a TCVS and subsequent γ-irradiation to covalently bind PEO-PPO-PEO triblocks (via the PPO block) to glass, metal and pyrolytic carbon surfaces. 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 [29]. In this work, the tri-blocks were covalently attached according to methods similar to those cited above. The silanized samples were incubated with tri-blocks overnight. Half of the samples were rinsed with 10 mM PBS with 150 mM NaCl prior to γ-irradiation (to achieve 0.3 MRad at the OSU Radiation center, using $Cobalt_{60}$; samples were typically irradiated for around 6.5 hours) while the other half remained in a solution of 10 mM PBS with 150 mM NaCl and 5% tri-blocks prior to γ-irradiation. After γ-irradiation, all samples were rinsed twice with 2 mL of 10 mM PBS with 150 mM

NaCl. Only samples of TCVS treated Si microspheres were coated with EGAP-NTA. Both ADCS and TCVS treated samples were coated with F108.

Preparation of protein loaded samples

TCVS- or ADCS-treated samples (tri-block coated or uncoated) were incubated for 4 hr in solutions of 10mM PBS w/150mM NaCl containing 5mg/mL nisin or 2.5 mg/mL fibrinogen then rinsed with 2 volumes 10mM PBS w/150mM NaCl. Half of the nisin treated samples were then also contacted with fibrinogen solution following the same incubation method. Each of these samples were also contacted in a solution of 5% sodium lauryl sulfate (SDS) in 10 mM PBS with 150 mM NaCl. See schematic in Appendix XX for further detail.

In situ *Zeta Potential Analysis*

For the purpose of zeta potential measurement, 10 µL of ADCS or TCVS treated sample solutions containing around 10% spheres in 10 mM PBS with 150 mM NaCl were placed into disposable cuvettes with 2 mL 1mM KCl (pH ≈7.55). The samples were tested individually in the zeta potential analyzer (Model: ZetaPALS, Brookhaven Instruments Corp.) and analyzed for 5 cycles of 30 readings. Briefly, zeta potential measurement occurs by placing solutions with known particle sizes between an anode and a cathode. A voltage is applied between the electrode plates and the particles will move at a certain velocity depending on their surface charge. This movement is detected by use of a laser light passing through the suspension, and zeta potential is calculated based on these values.

Results and Discussion

Covalent attachment of tri-blocks to silica microspheres: TCVS v ADCS

Both TCVS and ADCS were tested because previous studies had not discerned which silane was better for further surface modification. McPherson et al. [29] and Park et al. [43] give precedent for silanization of TCVS, however, because TCVS is much more toxic than ADCS, and ADCS does not polymerize during silanization, it was thought that ADCS would prove more effective and reproducible for further modification.

Figure R.1 provides zeta potential measurements recorded for covalent attachment of F108 on both TCVS and ADCS silanized silica microspheres. TCVS coated spheres in all samples showed an overall less negative surface charge. This is likely a result of the poly-functional quality of TCVS compared to the mono-functional nature of ADCS. Observationally, this resulted in a higher hydrophobic nature from the TCVS coated samples.

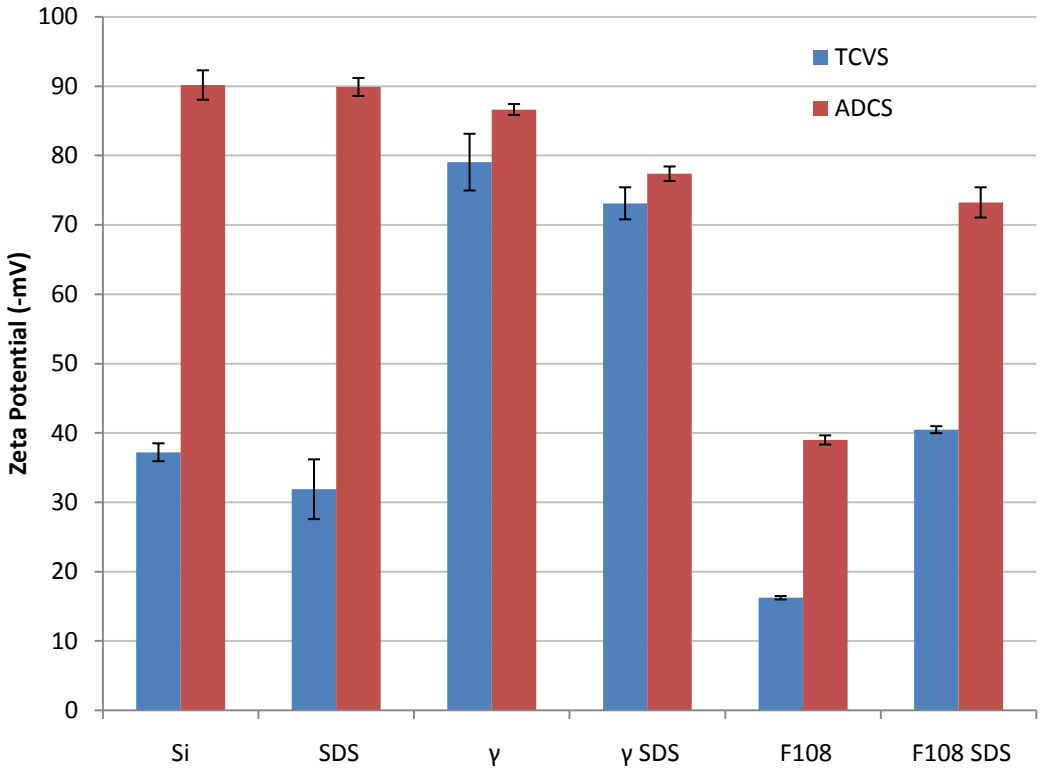

Figure R.1: Comparative zeta potential values for surfaces coated with TCVS or ADCS. Si represents surfaces that had been silanized only. γ labeled surfaces were silanized, then irradiated to 0.3 Mrad. F108 labeled samples had been silanized, then incubated with F108 overnight. Samples labeled with SDS were challenged with a 5% solution in 10 mM PBS with 150 mM NaCl for a minimum of 1 hr.

As shown in Figure R.1, the zeta potential of the surfaces remained unchanged for surfaces that had been silanized only and then treated with SDS. The same is true for surfaces that had been silanized followed by γ-irradiation. In the case of silanized surfaces coated with F108 saw a significant zeta potential increase in negativity upon challenge by SDS. This is an expected result as SDS can associate with both the surface and the hydrophobic base of the tri-block. This removes the thermodynamic drive for the tri-block to interact with the surface and is thus eluted.

It was originally hypothesized that ADCS would provide a better surface because of the mono-functional units, however data in Figure R.2 suggest that TCVS coated surfaces that have F108 covalently attached are more resistant to elution than the ADCS counterpart. Two treatment types were tested; samples labeled "w" were incubated in a solution of F108 and then washed three times with PBS (see Materials and Methods) prior to γ-irradiation. Samples labeled "u" were γ-irradiated in an F108 solution.

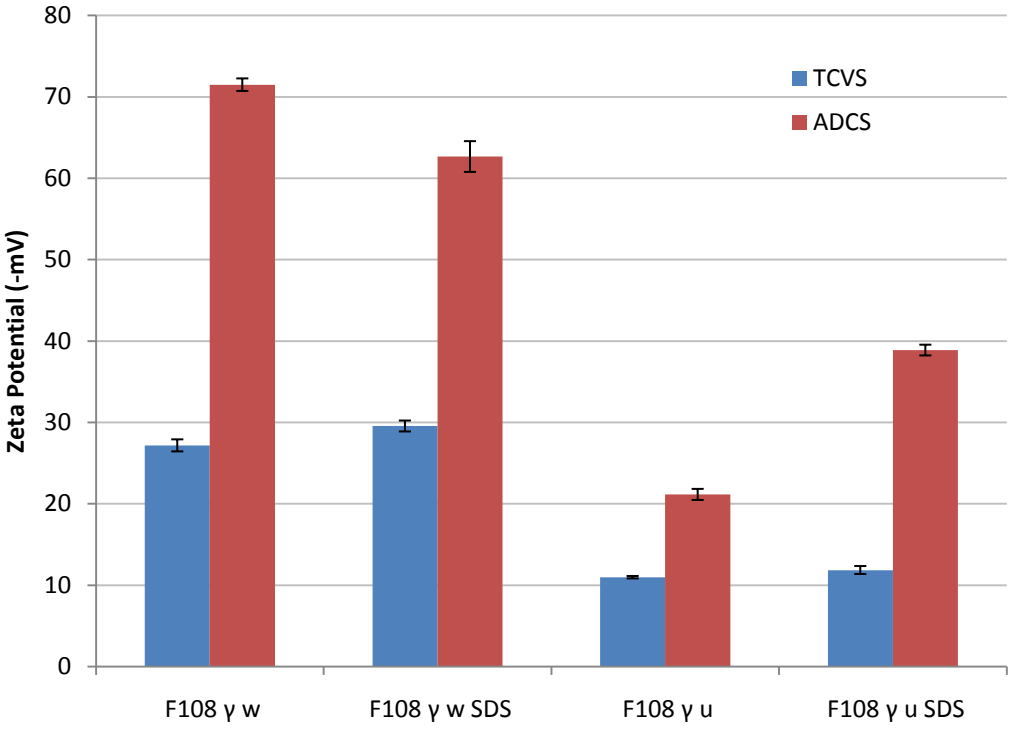

Figure R.2: Zeta potential results for silanized samples that had been coated with F108 and then γ-irradiated. Two treatment types were tested; samples labeled "w" were incubated in a solution of F108 and then washed three times with PBS (see Materials and Methods) prior to γ-irradiation. Samples labeled "u" were γ-irradiated in an F108 solution.

The washed samples showed more negative zeta potential for both TCVS and ADCS coated microspheres than the unwashed samples, indicating the unwashed samples provided a fuller coating with the F108, and therefore a better surface for

antifouling and protein repelling. Furthermore, in the case of the unwashed samples, when they were challenged by SDS, the TCVS coated samples showed no significant increase in negativity when compared to the zeta potential of the ADCS coated samples.

Because the only chemical difference between the F108 and EGAP-NTA tri-blocks is that one end, furthest from the PPO base, has been modified, TCVS coated surfaces would yield similar results if treated with EGAP-NTA rather than F108, and were therefore not tested.

Covalent attachment of tri-blocks to silica microspheres: challenge by SDS

Figure R.3 shows comparative results between samples treated with a 5% (in PBS) SDS solution and those treated with PBS only, after which the samples were washed with PBS. In all samples, the surfaces were first silanized with TCVS. In the case of TCVS silanized samples, challenge by SDS does not yield a significant change in the zeta potential of the surface. A significant increase in negativity is seen when the surfaces are γ-irradiated. This increase can be attributed to vinyl groups being broken by the radiation, which results in two free hydroxyl groups on the surface, the negatively charged oxygen gives rise to the increase in negative zeta potential. The samples silanized with TCVS then incubated with F108 show that the SDS removes the F108 from surface, as discussed for Figure R.1. The samples silanized with TCVS then incubated with F108 followed by γ-irradiated however, show no significant change in zeta potential, indicating the F108 is non-elutable and therefore covalently bound to the surface. The next two sample types complicate these results because if the same

analysis is applied to the EGAP-NTA samples, it would appear SDS removes the tri-block whether the samples are γ-irradiated or not. Observationally, the samples that were silanized with TCVS, then incubated with EGAP-NTA tended to resist pellet formation upon centrifugation. Bead loss considerations hindered the ability to wash away the SDS solution using PBS, and these results may simply show SDS hovering near the boundary layer of the tri-blocks. As will be shown, later results did not indicate that EGAP-NTA proved less effective than F108 for nisin retention or the repelling of proteins.

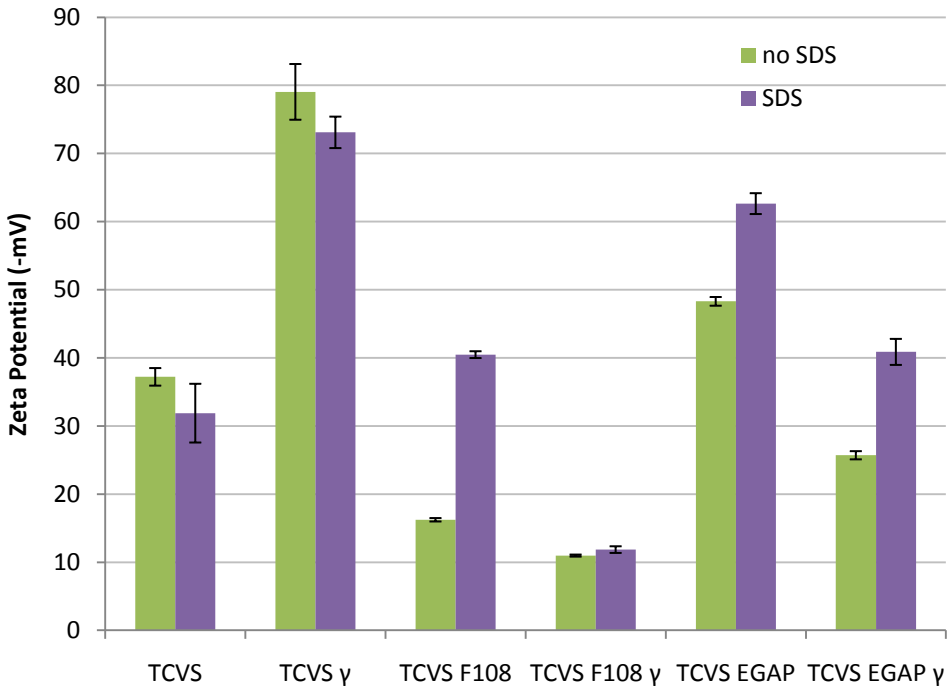

Figure R.3: Zeta potential results comparing samples treated with SDS and those treated with PBS. In all cases of γ-irradiated tri-block samples, the solutions were irradiated in solutions of tri-blocks.

Nisin adsorption: challenge by fibrinogen

Figure R.4 provides zeta potential results for surfaces silanized with TCVS and left otherwise uncoated. Data from Tai et. al. provided evidence that nisin adsorbs to bare surfaces and tri-block coated surfaces in multi-layer quantities [3]. This is seen in the comparison between the TCVS sample and the TCVS nisin sample in Figure R.4. The positively charged nisin significantly reduces the negativity of the zeta potential of the surface. The TCVS fibrinogen sample also appears to decrease the negativity of the surface, despite the net negative charge of fibrinogen. Zeta potential analysis relies on density of surface charge, not just overall surface charge. A fibrinogen coated surface would have areas of high negative charge, and other areas of low negativity, depending on how fibrinogen adsorbed to that surface. This would reduce the overall negative charge density, and make the surface appear more neutral from the standpoint of zeta potential analysis.

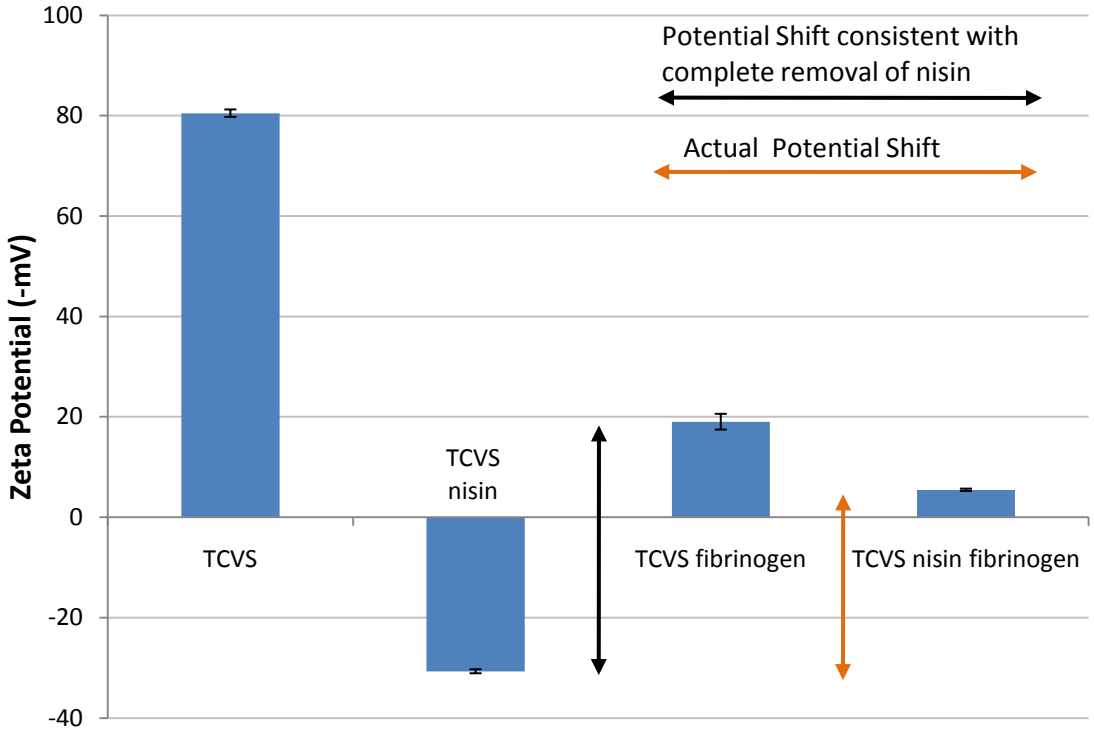

Figure R.4: Zeta potential measurements for TCVS coated surfaces incubated with nisin, fibrinogen or nisin followed by fibrinogen challenge.

The nisin coated surface has a net zeta potential of 30.7 mV which represents the highest value the surface charge could reach because the incubation time for the surfaces in solution with nisin was sufficiently long enough such that all the nisin that would adsorb, had adsorbed. This zeta potential is considered consistent with full coverage of the surface by nisin. The fibrinogen coated surface has a net zeta potential of -19.0 mV. Again, the incubation time was sufficiently long so that all the fibrinogen that was going to adsorb to the surface had adsorbed, and this value is considered consistent with complete coverage by fibrinogen. The TCVS nisin fibrinogen sample represents a solution of TCVS coated spheres that were incubated in a solution with nisin, rinsed, and then incubated in a solution of fibrinogen, and then rinsed. The zeta

potential of this surface was -5.46 mV. This means the surface started at 30.7 mV, and upon challenge by fibrinogen, decreased to -5.46, a change of 36.2 mV. The potential change for complete removal of nisin and replacement by fibrinogen would have been 49.7 mV. This is a represents 73 % nisin removal on surfaces coated with TCVS only.

Figure R.5 provides zeta potential results for surfaces silanized with TCVS and incubated with F108 tri-blocks. Similar to results for uncoated samples, the adsorption of nisin significantly reduces the negativity of the zeta potential. This seems to indicate that nisin had adsorbed into the tri-block layer because the samples were washed thoroughly prior to analysis. The adsorption of fibrinogen increased the negativity of the F108 coated surface, however the difference between the fibrinogen challenged sample and the F108-only sample is 1.96 mV, insignificantly different. This may indicate that there is not any fibrinogen near the solution at all.

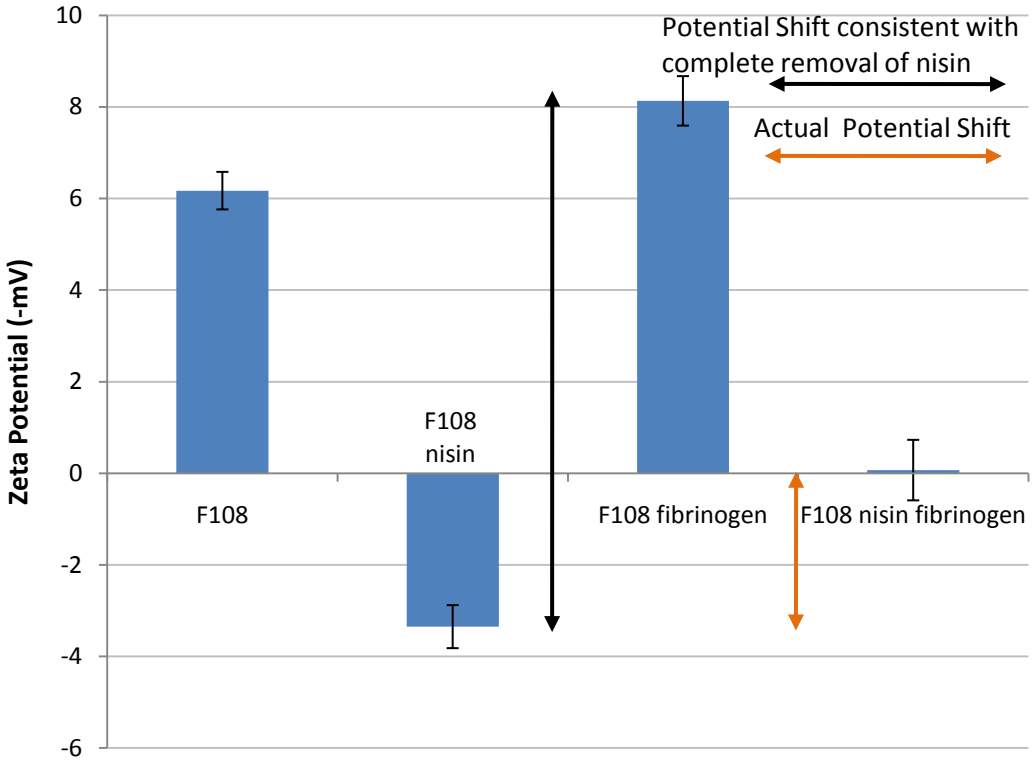

Figure R.5: Zeta potential results for TCVS coated samples with covalently attached F108 and contacted with nisin, fibrinogen, or nisin followed by challenge with fibrinogen

Following the same logic as used for Figure R.4, the sample consistent with maximum adsorption of nisin (F108-nisin) has a zeta potential value of 3.35 mV, while the F108-fibrinogen sample, consistent with maximum adsorption of fibrinogen has a zeta potential of -8.13 mV; a maximum potential shift of 11.48 mV. The F108-nisin-fibrinogen sample has a zeta potential of 0.068 mV, a change from maximum nisin adsorption of 3.41 mV. This is consistent with 30 % removal of nisin. When compared to the 73 % removal of nisin in the uncoated samples, we see that the F108 coated samples appear to retain most of the nisin initially adsorbed. Another possibility, however, is that F108 coated surfaces contacted with nisin increase the probability that

fibrinogen will associate with that surface, and these results are consistent with no removal of nisin, and association of fibrinogen with the boundary layer.

Figure R.6 provides zeta potential analysis results for TCVS silanized surfaces with covalently attached EGAP-NTA. Again, the adsorption of nisin significantly reduces the negativity of the zeta potential. This seems to indicate that nisin had adsorbed into the tri-block layer because the samples were washed thoroughly prior to analysis. The adsorption of fibrinogen decreased the negativity of the EGAP-NTA coated surface, however the difference between the fibrinogen challenged sample and the EGAP-NTA-only sample is 0.63 mV, insignificantly different, as indicated by the standard deviation of 0.6 mV. This may indicate that there is not any fibrinogen near the solution at all.

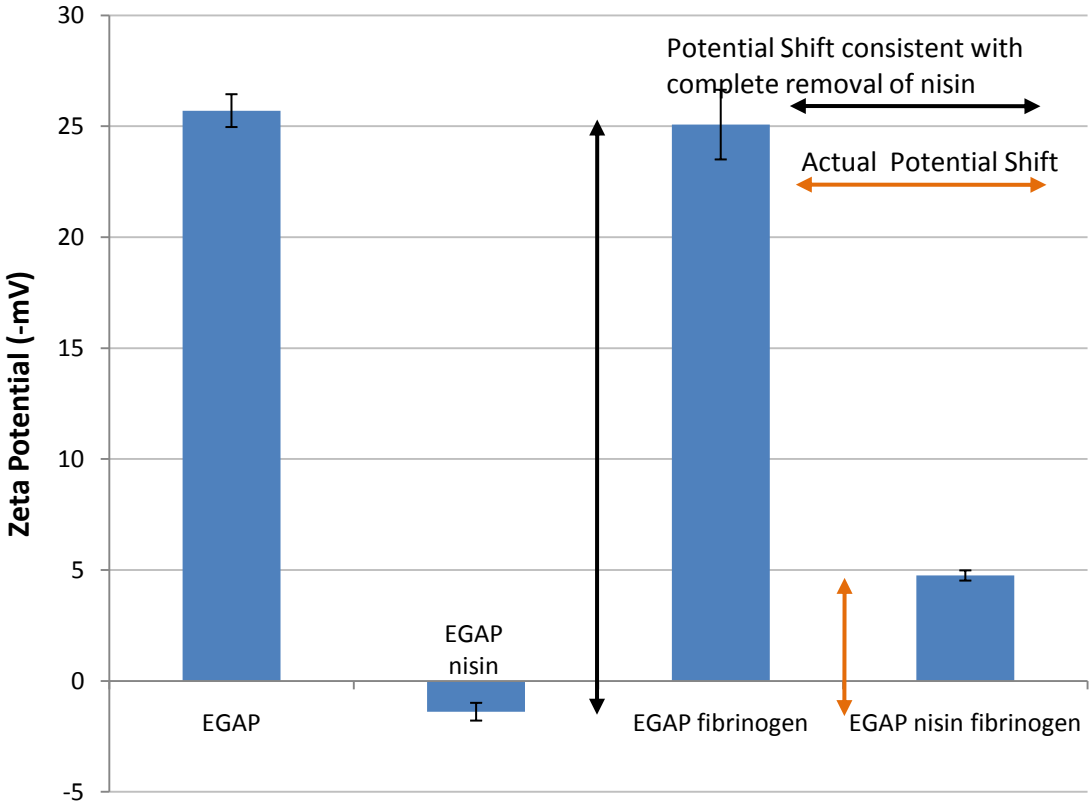

Figure R.6: Zeta potential results for TCVS coated samples with covalently attached EGAP-NTA and contacted with nisin, fibrinogen, or nisin followed by challenge with fibrinogen

Following the same logic as used for Figure R.4 and R.5, the sample consistent with maximum adsorption of nisin (EGAP-nisin) has a zeta potential value of 1.39 mV, while the EGAP-fibrinogen sample, consistent with maximum adsorption of fibrinogen has a zeta potential of -25.1 mV; a maximum potential shift of 26.5 mV. The EGAP-nisin-fibrinogen sample has a zeta potential of 4.75 mV, a change from maximum nisin adsorption of 6.14 mV. This is consistent with 23 % removal of nisin. When compared to the 73 % removal of nisin in the uncoated samples, we see that the EGAP coated samples appear to retain most of the nisin initially adsorbed. Again, these results are

also consistent with no removal of nisin, and association of fibrinogen with the boundary layer.

From these results we can see it is likely that triblock coatings stabilized by γ-irradiation not only show substantially lower levels of protein adsorption compared to untreated and silane-modified substrates, but also retain their protein repelling properties even after vigorous washing with SDS [43]. These surfaces, which were shown to adsorb nisin in multi-layer quantities by Tai et. al. [3] for surfaces that had non-specifically adsorbed tri-blocks maintained this ability upon covalent attachment of those tri-blocks.

Conclusion

This study effectively shows that surfaces containing pendant tri-block layers covalently attached to hydrophobic surfaces effectively protect nisin from elution by challenge with fibrinogen. The retention of nisin in covalently attached PEO-PPO-PEO triblock surfactants was quantified by *in situ* zeta potential analysis. From earlier studies, we understand that although the function of these layers is to prevent protein adsorption, nisin is able to adsorb in multilayer quantities, and this study suggests this adsorbed quantity may be resistant to challenge by fibrinogen. Comparison of zeta potential effects of nisin pre and post challenge suggest that fibrinogen reduces the absorbed mass by no more than 30% on tri-block coated surfaces. Analysis of the layer itself suggests γ-irradiation is an effective method for covalently attaching tri-blocks to silanized surfaces, and that TCVS makes a more viable substrate than does ADCS for covalent attachment. One question remains, however, related to whether nisin is truly eluted from the brush layer upon challenge by fibrinogen, or if the fibrinogen is more likely to adsorb to the surface due in part to the entrapment of nisin in the tri-block layer. Early experimentation has been inconclusive. We suggest a model, however of what is occurring at the surface, as shown in Figures C.1 through C.2.

In Figure C.1, we show a tri-block layer without any proteins adsorbed or entrapped. We believe in this scenario, that the pendant PEO chains do not extend to their full length, but only a percentage of that potential length, as suggested in the literature [38].

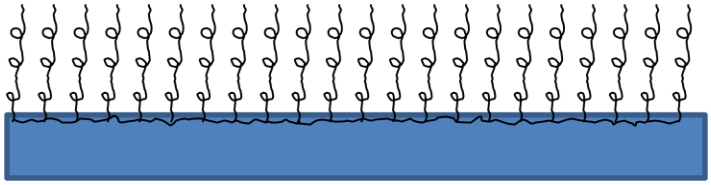

Figure C.1: This figure shows the pendant PEO chains in a tri-block layer, without any protein interaction.

Upon introduction of nisin, which is able to adsorb to the tri-block layer, we hypothesize the layer is thermodynamically induced to extend further into solution as nisin fills the free space within, as shown in Figure C.2.

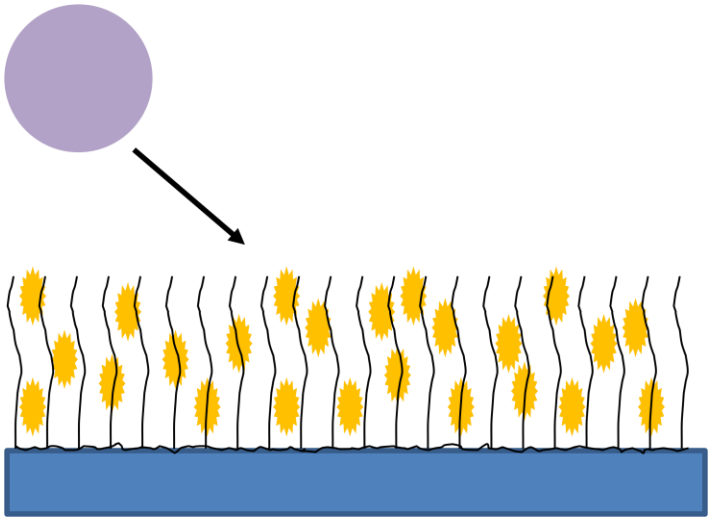

Figure C.2: This figure shows the pendant PEO chains with adsorbed nisin, prior to challenge by fibrinogen.

Finally, as shown in Figure C.3, upon challenge by fibrinogen, the uppermost layer of nisin elutes from the tri-block layer as it is compressed by the approaching blood protein. Fibrinogen would be unable to remain at the surface, and would diffuse through the bulk solution along with that upper layer of nisin, which is consistent with a 30% change in zeta potential. At this point, the uppermost units of the PEO are able to regain some mobility, and the overall surface covering shortens.

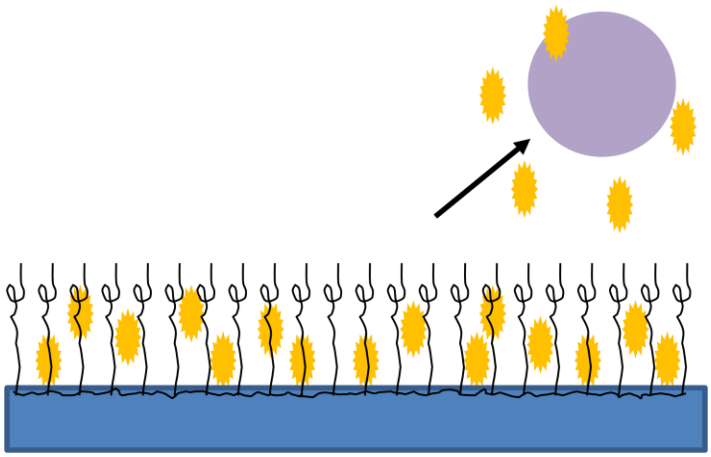

Figure C.3: Challenge by fibrinogen has compressed the pendant PEO chains, eluting the uppermost layer of adsorbed nisin.

This remains merely conjecture, as we have little data to support this hypothesis, but experimentation is in progress that we expect will reveal the mechanisms by which fibrinogen interacts with a nisin-loaded PEO layer.

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Appendix A

Sample Scheme

The sample scheme in Figure AA.1. outlines the method of surface treatment for the test samples. Samples silanized with ADCS were further treated with F108 only. Because of the structural similarity between the tri-blocks, EGAP-NTA was not used for ADCS coated microspheres.

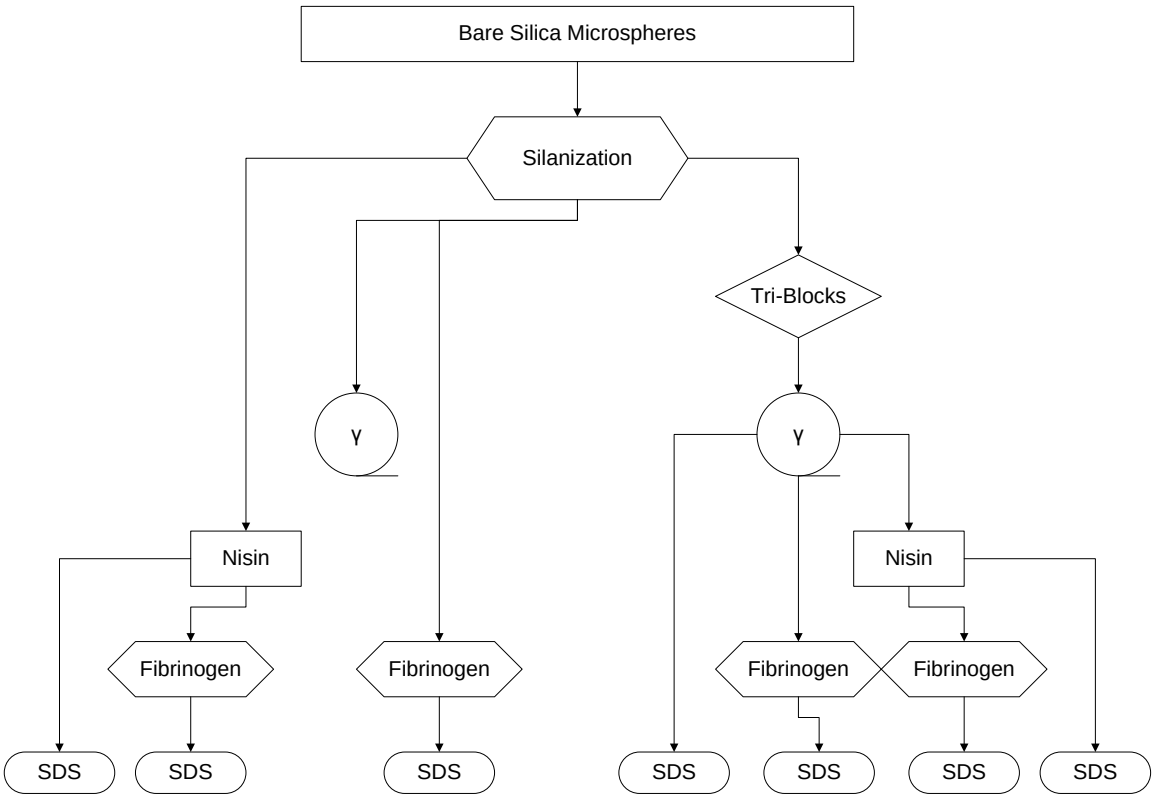

Figure AA.1: This figure shows the sample scheme for the testing done on silica microspheres. Each point an arrow touches represents a sample scheme that zeta potential measurements were taken.

Appendix B

Raw Data: TCVS v ADCS

Table AB.1. shows recorded data for samples silanized with TCVS or ADCS and subsequent surface coatings. Data was recorded on July 17, 2008.

Table AB.1: Data for TCVS and ADCS treated samples. Data recorded July 17, 2008.

	Zeta (-mV)	Std Err
Bare Si	69.9	1.22
TCVS	37.21	1.29
TCVS SDS	31.88	4.31
TCVS γ	79.04	4.09
TCVS γ SDS	73.1	2.31
TCVS F108	16.23	0.25
TCVS F108 SDS	40.47	0.5
TCVS F108 γ w	27.17	0.74
TCVS F108 γ w SDS	29.55	0.67
TCVS F108 γ u	10.97	0.15
TCVS F108 γ u SDS	11.85	0.49
ADCS	90.16	2.12
ADCS SDS	89.88	1.29
ADCS γ	86.63	0.78
ADCS γ SDS	77.37	1.05
ADCS F108	38.98	0.66
ADCS F108 SDS	73.23	2.18
ADCS F108 γ w	71.49	0.77
ADCS F108 γ w SDS	62.66	1.89
ADCS F108 γ u	21.14	0.68
ADCS F108 γ u SDS	38.88	0.66

Raw Data: Fibrinogen and SDS challenge of tri-block and nisin coated surfaces

Table AB.2. shows the raw data for samples silanized with TCVS then treated with either tri-block. Subsequent surface coatings are also shown. Data was recorded September 1, 2008.

Table AB.2: Data for samples upon addition of indicated substrate. Data recorded September 1, 2008.

	Zeta (-mV)	Std Err
TCVS	80.49	0.74
TCVS γ	27.61	1.25
TCVS γ nisin	-30.69	0.4
TCVS γ nisin SDS	38.27	9.56
TCVS γ fibrinogen	19.03	1.57
TCVS γ fibrinogen SDS	58.83	1.44
TCVS γ nisin fibrinogen	5.46	0.23
TCVS γ nisin fibrinogen SDS	63.04	4.22
F108 γ	6.17	0.41
F108 γ SDS	13.93	0.69
F108 γ nisin	-3.35	0.47
F108 γ nisin SDS	9.37	2.29
F108 γ fibrinogen	8.13	0.54
F108 γ fibrinogen SDS	18.13	0.4
F108 γ nisin fibrinogen	0.0677	0.661
F108 γ nisin fibrinogen SDS	7.18	1.36
EGAP	48.29	0.64
EGAP SDS	62.64	1.53
EGAP w γ	54.39	0.72
EGAP w γ SDS	65.14	0.66
EGAP γ	25.7	0.6
EGAP γ SDS	40.87	1.91
EGAP γ nisin	-1.39	0.972
EGAP γ nisin SDS	17.34	2.53
EGAP γ fibrinogen	25.07	0.43
EGAP γ fibrinogen SDS	34.76	0.59
EGAP γ nisin fibrinogen	4.75	0.5
EGAP γ nisin fibrinogen SDS	22.53	2.01

Appendix C

Challenge by SDS

The following three figures are comparable, respectively to figures R.4, R.5, and R.6. They show those same sets of data after subsequent challenge by SDS. Methods for challenge by SDS were similar to challenge by fibrinogen.

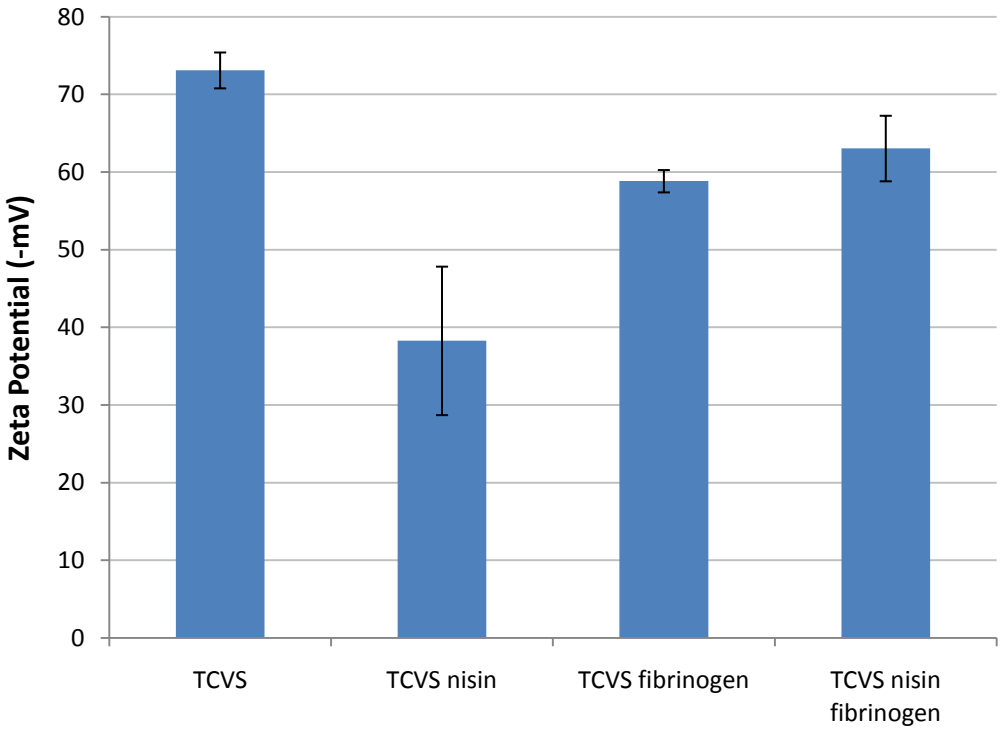

Figure AC.1: Zeta potential measurements for TCVS coated surfaces incubated with nisin, fibrinogen or nisin followed by fibrinogen challenge. All samples were γ-irradiated after TCVS silanization and each was challenged by SDS.

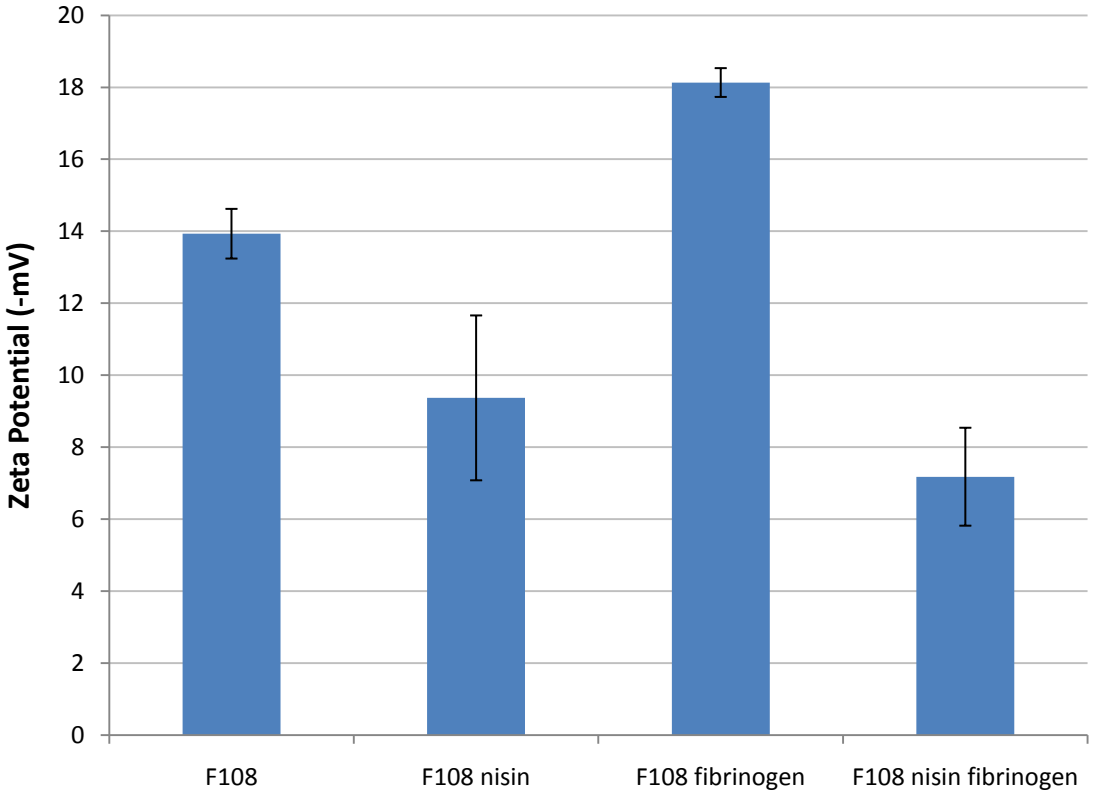

Figure AC.2: Zeta potential results for TCVS coated samples with covalently attached F108 and contacted with nisin, fibrinogen, or nisin followed by challenge with fibrinogen. All samples were γ-irradiated after TCVS silanization and each was challenged by SDS.

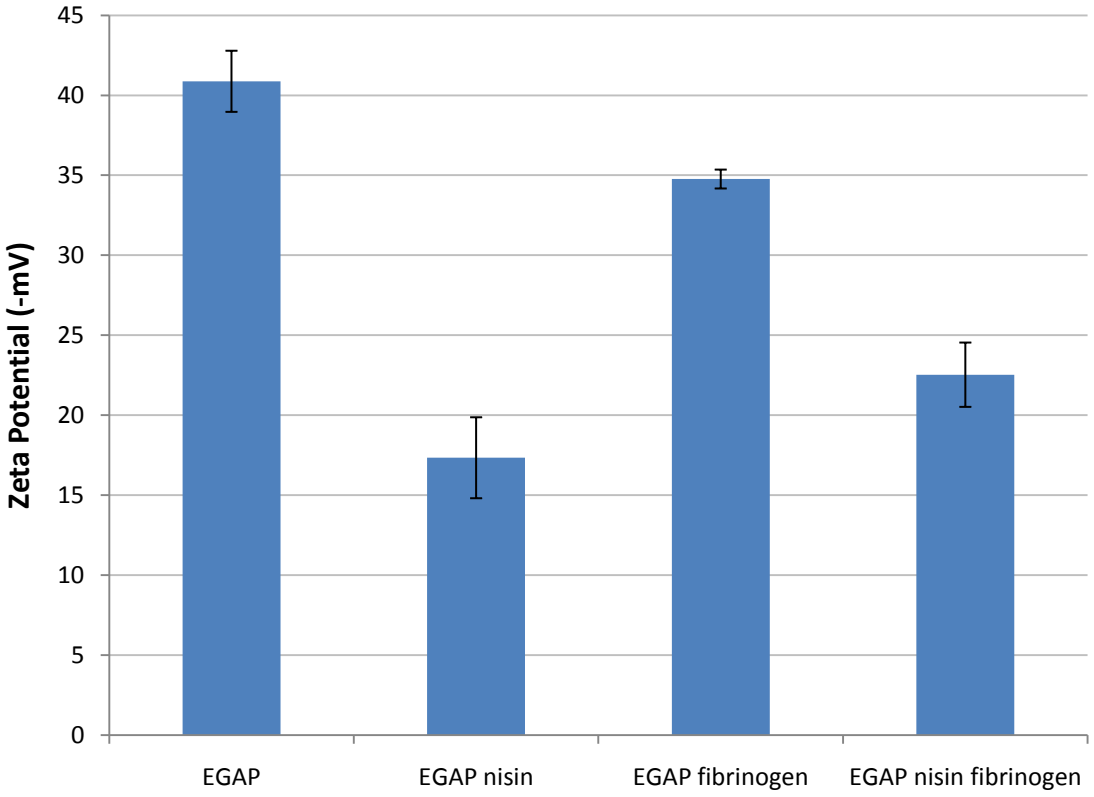

Figure AC.3: Zeta potential results for TCVS coated samples with covalently attached EGAP-NTA and contacted with nisin, fibrinogen, or nisin followed by challenge with fibrinogen. All samples were γ-irradiated after TCVS silanization and each was challenged by SDS.