



AN ABSTRACT OF THE THESIS OF

Julie A. Auxier for the degree of Honors Baccalaureate of Science in Bioengineering presented on June 9, 2011. Title: Quantification of fibrinogen adsorption to nisin-loaded polyethylene oxide layers.

Abstract approved: \_\_\_\_\_  
Joseph McGuire, Ph.D.

Antimicrobial coatings used to reduce the risk of infection caused by tissue-contacting medical devices must address specific biocompatibility requirements including prevention of thrombosis and rise of bacterial resistance. An antimicrobial-loaded poly(ethylene-oxide) (PEO) coating has been tentatively shown to be non-fouling with sustained antimicrobial activity. However, the presence of the antimicrobial, nisin, may encourage protein adsorption at the interface. Past research has suggested that fibrinogen, a protein involved in thrombosis, may mediate elution of nisin integrated within the brush layer, or adsorb to the outer surface of the nisin-loaded brush layer. The work described here was motivated by the need to better understand interactions between nisin-loaded PEO and the procoagulant fibrinogen. Fibrinogen adsorption was quantified using enzyme-linked immunosorbant assay (ELISA), detection of fluorescein isothiocyanate (FITC) labeled fibrinogen, and zeta potential analysis. ELISA and FITC-labeled fibrinogen tests measured decreased fibrinogen levels at PEO coatings with no significant difference between nisin-free and nisin-loaded PEO coatings. These results indicate that the presence of nisin does not significantly promote fibrinogen adsorption to an extent beyond that expected for a PEO layer without entrapped nisin. Zeta potential detection corroborated these results, showing no significant differences between zeta potential of nisin loaded PEO layers detected after repeated elution challenges, independent of the presence of fibrinogen.

Key Words: PEO brush layer, nisin, fibrinogen, ELISA, FITC, zeta potential  
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Quantification of fibrinogen adsorption to nisin-loaded polyethylene oxide layers

by

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

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Julie A. Auxier, Author

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## TABLE OF CONTENTS

Chapter	Page
<b>Introduction .....</b>	<b>1</b>
Cardiovascular medical devices difficulties .....	1
Hemostasis and protein adsorption .....	1
Non-fouling coating use and structure .....	2
Nisin antimicrobial properties .....	4
Consequences of nisin integration into brush layer .....	6
Statement of purpose .....	8
<b>Materials and Methods .....</b>	<b>9</b>
Introduction to methods .....	9
Silica surface modification and coating construction .....	10
Experimental Controls and Variables .....	13
Enzyme-linked immunosorbant assay (ELISA) modification and application .....	14
Fluorescein isothiocyanate (FITC) labeling and detection of fibrinogen .....	17
Zeta potential surface measurements .....	18
<b>Analysis and Discussion.....</b>	<b>20</b>
ELISA analysis .....	20
FITC-labeled fibrinogen analysis .....	22
Zeta potential analysis .....	25
Limitations of tests .....	28
<b>Conclusions and Recommendations .....</b>	<b>28</b>
<b>Bibliography .....</b>	<b>31</b>

## LIST OF FIGURES

<b>Figure</b>		<b>Page</b>
[1]	Tri-block brush layer assembly.....	4
[2]	Nisin molecular structure.....	5
[3]	Nisin entrapment in PEO brush layer.....	6
[4]	Theoretical consequences of nisin entrapment.....	7
[5]	Vapor Deposition OSCAR apparatus design.....	11
[6]	PEO brush layer covalent bonding to model surface.....	12
[7]	ELISA process comparison.....	15
[8]	ELISA fibrinogen adsorption.....	21
[9]	FITC-labeled fibrinogen on coated and non-coated microspheres.....	23
[10]	FITC-labeled fibrinogen absorbances.....	24
[11]	Zeta potential of a hydrophobic surface and tri-block coated surfaces.....	26
[12]	Zeta potential of antimicrobial coatings challenged with fibrinogen.....	27

## **Introduction**

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### **Cardiovascular medical device difficulties**

Thrombus formation, bacterial adhesion, and infection at the site of a medical device are continual issues in healthcare. Cost benefits and improved patient care drive the development and use of anti-coagulant medications and antimicrobial coated devices. However, long term anti-coagulant use poses additional health problems for patients. Also, such coatings pose a very serious risk of bacterial resistance and are currently recommended only for high risk patients. To overcome the limitations of existing coating approaches, material surface modifications must (i) kill bacteria upon contact with the device, (ii) be biocompatible and not adversely affect the patient, (iii) be readily applied to a variety of materials and irregularly shaped objects, (iv) prevent thrombus formation and occlusion, and (v) not stimulate bacterial resistance.

### **Hemostasis and Protein Adsorption**

The body responds to disruptions in blood vessel endothelial cells with hemostasis. Tissue injury and the presence of a foreign body may induce hemostasis. Hemostasis occurs through a series of protein and chemical events broken into primary hemostasis (vasoconstriction and platelet plug formation) and secondary hemostasis (blood coagulation). Vasoconstriction occurs immediately to limit blood loss. The platelet plug is formed when platelets stick to the injured endothelial extracellular matrix by von Willebrand factor and the protein thromboxane causes platelet activation and

initiates the clotting cascade. Fibrinogen locates at the platelets and provides stability between platelets. Secondary hemostasis primarily consists of fibrinogen conversion to fibrin by thrombin. Thrombomodulin stops the clotting process by binding thrombin and converting it to a strong anticoagulant.<sup>1</sup> Hemostasis continues until the injury heals, but medical device-blood interfaces cannot be healed by this method. Only the primary proteins have been highlighted; however, it should be noted that many other proteins are involved in the clotting process and these proteins participate by adsorption and conformational changes.

### **Non-fouling coating use and structure**

Thrombosis may be averted by making the medical device surface “non-fouling” resistant to protein adsorption. At high concentrations the polymer surfactant will quickly adsorb to the interface and at a high enough surface density specific to the polymer construction, the coating will assume a “brush” configuration.<sup>1</sup> In the brush configuration, the coating hinders protein adsorption through steric repulsion and thus assumes a “non-fouling” character.

The efficiency of a non-fouling coating depends on the brush characteristics, namely thickness and polymer grafting density.<sup>2</sup> These properties in turn rely on other physico-chemical properties such as adsorbing protein size and concentration, grafting and free energies, and degree of polymerization.<sup>2</sup>

Protein adsorption to a polymer layer requires the protein surpasses an adsorption energy threshold. The repulsive energy may be described as a combination of the bare

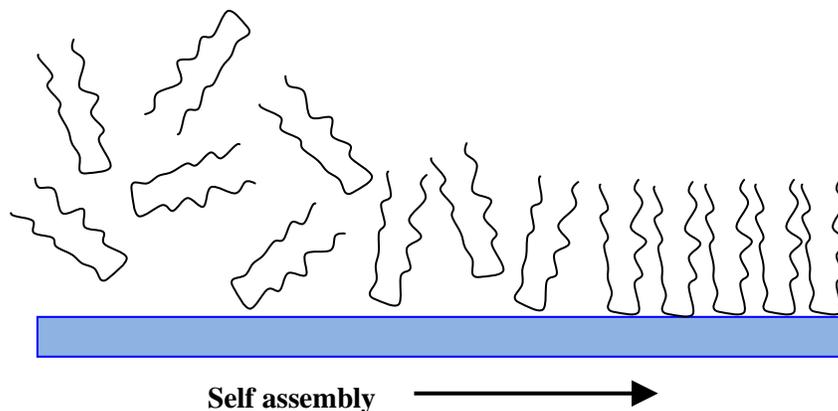
surface and brush electrostatic and Van der Waals energies as a function of distance from the surface,  $z$ , (1)

$$U_{effective}(z) = U_{bare}(z) + U_{brush}(z) \quad (1)$$

where  $U_{effective}$  is the apparent total surface energy,  $U_{bare}$  is the energy of the surface, and  $U_{brush}$  is the energy of the applied polymer coating.<sup>2</sup>

A polymer consisting of a hydrophobic and hydrophilic portion will self-assemble at a hydrophobic surface in aqueous solution. Co-polymers with a tri-block configuration are widely known for easy, self-assembly of brush layers. The co-polymer may be tethered to the surface of interest by either covalent or non-covalent association with pendent chains solubilized in the surrounding fluid (**Figure 1**).

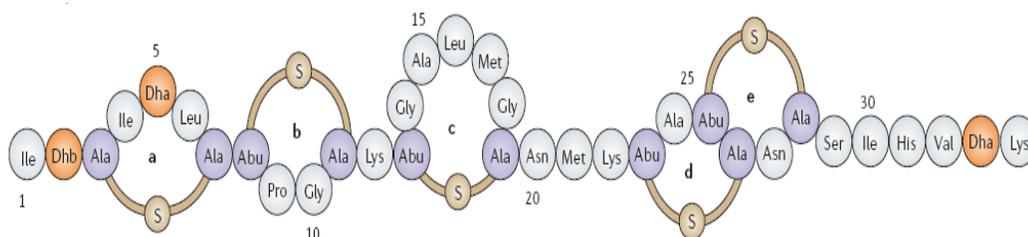
It is known that Pluronic<sup>®</sup> F108 creates the protective brush layer on a hydrophobic surface through its tri-block PEO-PPO-PEO (*poly(ethylene-oxide)–poly(propylene-oxide)–poly(ethylene-oxide)*) structure.<sup>3,4</sup> F108 and other Pluronics are FDA approved and used in biopharmaceutical drug formulation.<sup>1</sup> The PEO of the tri-block is hydrophilic whereas the PPO is hydrophobic; the amphiphilicity allows the tri-block to self-assemble to the hydrophobic surface in an aqueous medium (**Figure 1**). It has been demonstrated that F108 can be tethered to a surface permanently through gamma irradiation, providing surface protection for an extended time.<sup>5</sup>



**Figure 1:** The amphiphilic tri-block Pluronic® F108 consists of a hydrophobic poly(propylene-oxide), PPO, middle segment. Two hydrophilic pendent chains made of poly(ethylene-oxide), PEO, extend from the PPO segment. The tri-block will hydrophobically associate with a hydrophobic surface in an aqueous solution, shown progressively from left to right. The resulting ‘brush’ configuration, shown on the right, prevents proteins and other macromolecules from adsorbing to the surface.

### **Nisin antimicrobial properties**

The lantibiotic nisin is a small amphiphilic, cationic peptide that is an effective inhibitor of Gram-positive bacteria. It is produced by strains of *Lactococcus lactis*.<sup>6-8</sup> The peptide (1.8-4.6 kDa) is synthesized by ribosomes and post-translationally modified to create the characteristic lanthionine rings (**Figure 2**).<sup>9-10</sup>



**Figure 2:** Nisin is a lantibiotic synthesized by *Lactococcus lactis* ribosomes that undergoes post-translational modification to form the characteristic lanthionine rings. Nisin permeabilizes Gram-positive bacterial membranes to inactivate bacteria without inducing bacterial resistance.

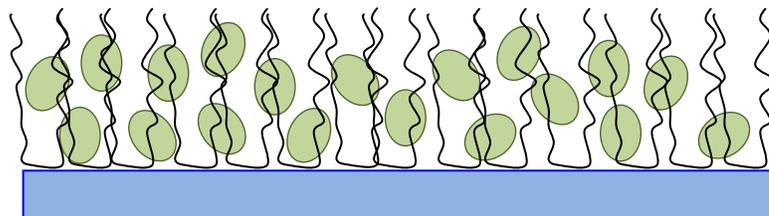
The nisin mode of action is not conducive to bacterial resistance. Nisin binds to a bacterial membrane and causes sulfhydryl group modifications thereby creating a pore through which low molecular weight particles rush out of the cell.<sup>11,12</sup> It has been suggested that nisin binds to the lipid II receptor to initiate membrane permeabilisation.<sup>6,13-14</sup> Interestingly, vancomycin, a widely used broad spectrum antibiotic, also binds to lipid II and has been shown to inhibit nisin function on the membrane.<sup>6</sup> Nisin inactivates Gram-positive bacteria (eg. *S. aureus*) much more efficiently than Gram-negative bacteria (eg. *E. coli*).<sup>15</sup> Nisin has low toxicity for humans and is widely used as a food preservative.<sup>16</sup>

The peptide is stable for wide temperature and pH ranges. Stability has been shown to increase with decreasing pH; nisin has been shown to retain function after being autoclaved at pH 2.<sup>17</sup> However, activity decreases with age, most likely due to degradation. Nisin degrades into two products. Notably, one of the degradation products has also exhibited antimicrobial activity and so observed activity retention under

strenuous conditions (extreme temperature and pH) may be in part due to activity from the active degradation product and not the intact nisin molecule.<sup>18</sup>

### Consequences of nisin integration into brush layer

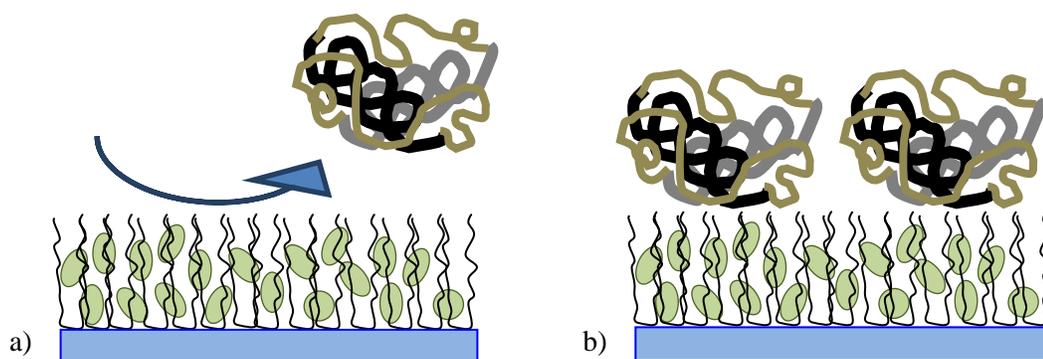
Previous research in the McGuire laboratory has shown that nisin integrates into a Pluronic® F108 brush layer and retains antimicrobial function (**Figure 3**).<sup>3,19</sup>



**Figure 3:** Nisin overcomes the repulsive pendent chains and integrates into the brush layer. Previous work has shown that nisin easily integrates into the brush and resists elution by buffer washing.<sup>3,19</sup>

By definition, the brush layer should fully repulse proteins yet nisin integrates into the surface. The mechanism by which this integration occurs is still ambiguous but related laboratory work is pursuing to characterize the mechanism. Halperin suggests that small proteins weakly disturb the energy of the brush coating: specifically proteins with a protein radius,  $R$ , to length,  $L_o$ , ratio much less than 1 ( $R/L_o \ll 1$ ). These small proteins may preferentially penetrate the brush for strong short range attractive forces as a function of polymer density,  $\sigma$ .<sup>2</sup> However, the presence of the peptide may reduce the polymer lateral mobility and normal compressibility. Therefore, loading nisin into the

brush layer can be expected to reduce the mobility of the chains and interfere with its repulsive capability, potentially allowing protein adsorption (**Figure 4**).



**Figure 4:** a) Ideally nisin entrapment in the PEO-brush will leave a few monomers exposed to maintain non-fouling properties. b) Nisin entrapment in the PEO-brush may hinder the steric repulsive character of the pendant chains and so cause a decrease in protein repulsion. Also, because nisin is cationic, the change in electrostatic forces may potentially encourage protein adsorption.

As previously mentioned, fibrinogen is a key component of the clotting cascade that leads to thrombosis. Fibrinogen is used as a model protein when evaluating the biocompatibility of a device or process. If fibrinogen adsorption is detected at the surface or during the process of interest, the biocompatibility may be evaluated according to the degree of fibrinogen adsorption. It is essential for cardiovascular interventional techniques and devices will not encourage fibrinogen adsorption.

## Statement of Purpose

The preferential location of procoagulant proteins, such as fibrinogen, at a peptide-loaded PEO layer would significantly reduce the viability of use as a medical device coating.<sup>3</sup> Since nisin is a cationic peptide, fibrinogen may be more attracted to non-fouling surfaces with entrapped nisin. This study focused on the possibility of increased fibrinogen adsorption at the coating due to the presence of nisin.

Results from Ryder et al. indicate that nisin is more resistant to elution by fibrinogen when entrapped in PEO layers than when simply adsorbed at an uncoated surface.<sup>20</sup> However, results did not exclude the possibility of fibrinogen adsorption at regions of the nisin-loaded PEO layer, seemingly where PEO functionality could be compromised and where attractive electrostatic interactions due to nisin are significant. The results could indicate either fibrinogen adsorption to the surface of the coating or fibrinogen displacement nisin from the brush coating.

This study sought to quantify fibrinogen adsorption more directly on nisin-loaded PEO coatings. It was hypothesized that nisin entrapment would not significantly reduce the PEO layer steric repulsive character and thus no significant increase in fibrinogen adsorption would be observed.

## Materials and Methods

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### Introduction to methods

Silica surfaces were prepared using methods previously established.<sup>20</sup> Trichlorovinylsilane (TVCS) adds silane groups to the surface of the silica and so increases surface hydrophobicity. The F108 tri-blocks may hydrophobically associate to the surface and through radiation may be covalently bound to the silane groups on the silica. The covalent bond should prevent F108 desorption from the surface, thereby preserving the brush.

The modified silica surfaces were challenged with a pure fibrinogen solution and tested for fibrinogen adsorption using enzyme-linked immunosorbent assays (ELISA), fluorescein isothiocyanate (FITC) labeled fibrinogen, and zeta potential tests. ELISA tests employed a protocol modified from traditional sandwich ELISA; system constraints presented difficulties not normally encountered with traditional ELISA. FITC-labeled fibrinogen was synthesized successfully in laboratory and used for preliminary tests. However, uniform number of FITC residues per fibrinogen molecule could not be controlled with laboratory synthesized tagged fibrinogen. Therefore, manufactured FITC-labeled fibrinogen was used for more quantitative measurements. Zeta potential measurements were conducted as an addendum for further clarification and insight on fibrinogen adsorption.

## **Silica surface modification and coating construction**

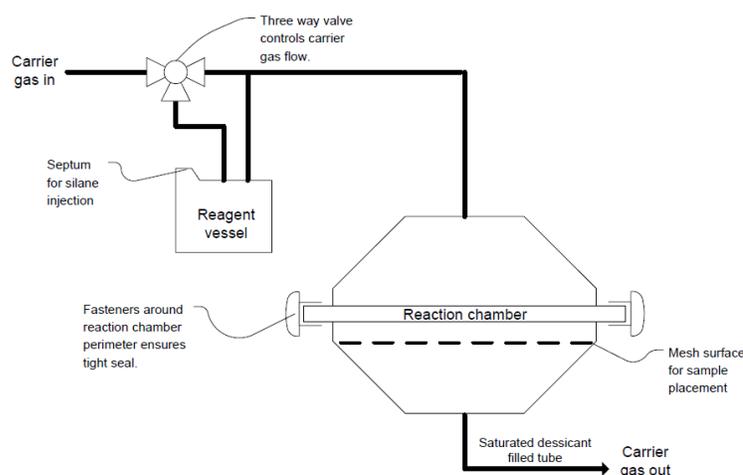
Silanization is a process during which organosilanes react with a silica or glass surface. A siloxane (specifically the Si-O moiety) on the organosilane reacts with the hydroxyl on the silica surface. The reaction yields a hydrophobic surface. The highly hydrophobic surface is crucial in the formation of the tri-block brush layer. There are many forms of organosilanes, also referred to as silanes, each suitable for certain conditions. Previous laboratory work has shown that trichlorovinylsilane (TCVS) works well with the silica surfaces used.<sup>20</sup>

Silica surfaces were used in both 1  $\mu\text{m}$  microsphere (FiberOpticCenter, New Bedford, MA) and wafer (1  $\text{cm}^2$ , WaferNet, San Jose, CA) forms. Silica microspheres were used for FITC-labeled fibrinogen and zeta potential tests. Both forms were cleansed with a piranha wash using a 1:1:5  $\text{H}_2\text{O}$ :  $\text{HCl}$ :  $\text{H}_2\text{O}_2$  acid wash for 15 minutes at  $80^\circ\text{C}$  followed by a 1:1:5  $\text{H}_2\text{O}$ :  $\text{NaOH}$ :  $\text{H}_2\text{O}_2$  base wash for 15 minutes at  $80^\circ\text{C}$ . This wash strips the surface of all organic material. The wafers and microspheres were then cured in a  $60^\circ\text{C}$  oven.

Silica microspheres were prepared according to the protocol described in Ryder et al.<sup>20</sup> Trichlorovinylsilane (97% Aldrich) was added to the microspheres under chloroform and incubated for 3-4 h. Microspheres were rinsed with three volumes of sodium phosphate buffer with 0.15 M NaCl (PBS) and cured in a  $60^\circ\text{C}$  oven. Microspheres were kept dry and away from light until needed.

Silica wafers were silanized by vapor deposition with an organosilane carrier gas assisted reactor (OSCAR). Wafers were placed in the reaction chamber and TCVS added

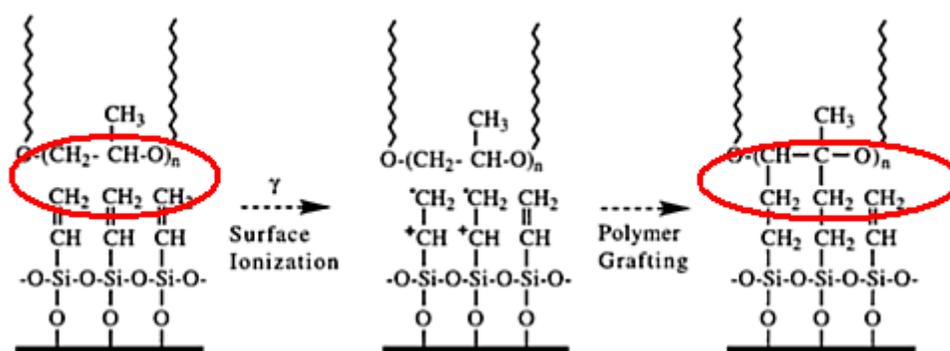
to the reagent vessel as nitrogen gas flowed through the system (**Figure 5**). The reaction continued for at least 4 h up to overnight. The wafers were rinsed with three volumes of PBS. The wafers were also cured in a 60 °C oven and stored away from light until needed. Silanized silica surfaces were kept away from UV light as a precautionary measure for silanes degrade in the presence of ultra-violet light.



**Figure 5:** The organosilane carrier gas reactor (OSCAR) fed inert gas through a reagent vessel containing the TCVS to the reaction chamber. The gas filled the chamber and evenly deposited TCVS on the wafers placed on the mesh surface. Carrier gas flowed out through a tube filled with saturated desiccant.

Tri-block polymers associate to the silanized, hydrophobic surface when in an aqueous solution. A 0.01 g/mL solution of Pluronic® F108 in 10 mM sodium phosphate buffer with 0.15 M NaCl was added to the silica for 4-12 h at room temperature. Brush formation occurs quickly; ample time was allowed for the surface-solution system to reach an equilibrium state. Microsphere solutions were rotated end-over-end and wafers were agitated on a rocker for the duration of the F108 incubation.

The association was made permanent through via 0.3 Mrad gamma,  $\gamma$ , radiation [McPherson]. during which the ethyl carbons of silanized surface assume a positive charge and form carbon-carbon bonds with the carbons on the PPO tri-block backbone (Figure 6). The silica surfaces were kept in the F108 solution during the radiation treatment. Surfaces were subsequently rinsed in a series of three distilled water washes to remove loose tri-blocks. The surfaces were kept in distilled water until use.



**Figure 6:**<sup>5</sup> Tri-block polymers hydrophobically associated to a silanized surface (shown left) undergo covalent bonding when exposed to sufficient gamma radiation. The radiation first causes the silane carbons to ionize. The ethyl carbons then covalently bond with the carbons of the hydrophobic tri-block segment (PPO in this study). The resulting carbon-carbon bond keeps the polymer brush layer sufficiently bound to the surface to prevent elution.

Tai presents compelling data that indicates nisin becomes entrapped in the brush layer; a 4 h nisin incubation time is ample time for maximum nisin entrapment according to ellipsometry data.<sup>19</sup> Surfaces with and without F108 coatings were incubated in a 0.05 mg/mL solution of commercial nisin (Sigma-Aldrich N5764) in PBS for 4 h at room temperature, pH 7.4. Incubations were set up in the same manner as F108 incubations.

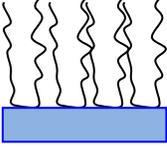
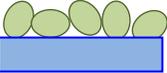
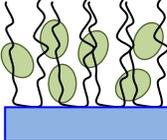
All samples were subsequently rinsed with three volumes of PBS to remove excess nisin. Nisin was added to the brush layer only when surfaces would be tested shortly thereafter.

Throughout the surface modification process the hydrophobicities of the wafers and microspheres noticeably changed. Solution beading or spreading on the wafers and microsphere solubility was noted at each step. Surface hydrophobicity observations were used as an additional step-by-step check to assure the coatings were constructed properly.

### **Experimental Controls and Variables**

Proper controls were used for each experimental technique (**Table 1**). The surfaces with nisin-loaded F108 were the variable surface in all testing. The control “bare” coatings with only silanized silica served as the negative control. The positive controls included the coatings with only F108 or only nisin added to the surface. Each positive control indicated normal protein interaction with either a brush or a peptide coated surface, respectively. Note that zeta potential experiments only investigated the variable layer and negative control. All experiments were run in triplicate.

**Table 1:** Experimental surface treatments for ELISA, FITC-labeled fibrinogen, and zeta potential experiments.

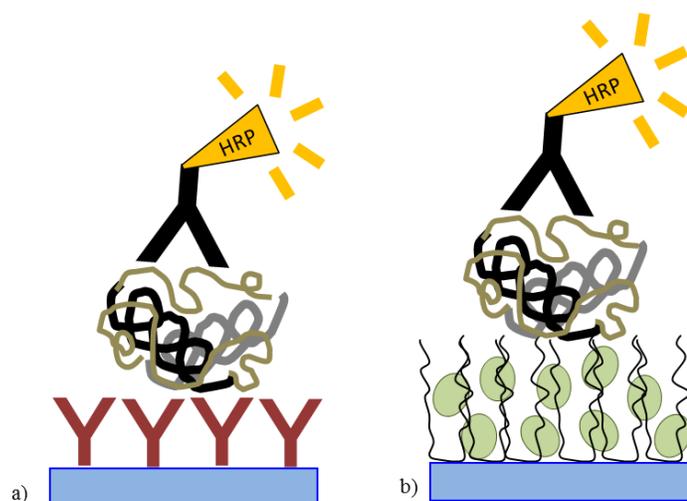
<i>Surface Treatment</i>	<i>Purpose</i>	<i>Surface</i>
Silanized silica, “bare”	Negative control	
F108 covalently attached to silanized silica	Positive control	
Nisin adsorbed to silanized silica	Positive control	
Nisin-loaded F108 covalently attached to silanized silica	Variable	

Fibrinogen was used as the model protein for blood protein adsorption. Fibrinogen adsorption was the measure of success evaluated in ELISA and FITC-labeled fibrinogen tests. In zeta potential experiments fibrinogen was used as the challenge to elute nisin from the PEO layer.

### **Enzyme-linked immunosorbent assay (ELISA) modification and application**

Enzyme-linked immunosorbent assays utilize specific antibodies to identify target proteins in a sample. The ELISA test was adapted from a basic sandwich ELISA protocol. The target proteins are usually free-floating in solution and so must be tethered to a surface for detection. The tethering and detecting process is referred to as a sandwich ELISA (**Figure 7**). A primary antibody is first adhered to the bottom of a well.

The protein then specifically binds to the antibody and other proteins are washed away in rinsing steps. A secondary antibody with the colorimetric tag horseradish peroxidase (HRP) specifically binds to the protein. Finally, a colorimetric substrate is used to visualize the secondary antibody. The reaction may be quenched with sulphuric acid and protein quantified in the sample spectrophotometrically.



**Figure 7:** a) The traditional sandwich ELISA binds a protein-specific primary antibody to a surface. The protein binds to the primary antibody (maroon) and then a protein-specific, fluor-tagged secondary antibody binds to the protein. The fluor-tag, HRP, produces a color of measurable intensity that correlates to the amount of protein present. b) The adapted ELISA requires only the secondary antibody for the target protein is already present on the surface. Note, the loosely associated fibrinogen presented difficulties attaining conclusive data.

Because the presence of fibrinogen was the marker of coating repulsive character, the fibrinogen would already be present on a surface and not require the primary antibody. However, any adsorbed fibrinogen would only loosely be bound to the surface, unlike the tightly bound protein to the primary antibody in traditional sandwich ELISA.

The adapted protocol therefore used only the second, fluorescence-tagged antibody and conducted the entire fibrinogen challenge and detection protocols in one well with careful washings to avoid mechanistic removal of fibrinogen.

F108-coated and uncoated wafers, with and without nisin added, were adhered to the bottom of BSA-blocked wells in a polystyrene micro-test plate (**Table 1**). The wells were blocked for 1 h with a 25 mg/mL BSA in PBS solution and then rinsed with three volumes of PBS. The rigorous washings between incubations involved forcefully inverting the well plate to remove excess solution before the next incubation. Therefore, the wafers were adhered to the bottom of the blocked wells with vacuum grease. Samples were covered with a 1% fibrinogen in PBS solution for 1 h then rinsed with three volumes of PBS.

Antibodies were purchased from US Biological and all solutions were made according to US Biological specifications. An HRP-linked antibody (US Biological) was added to HBS-BSA diluent. The antibody solution was added for 1 h and rinsed with three volumes of PBS. HEPES buffer was prepared without Tween 20. Tween 20 is a surfactant often added to protein solutions to prevent loose, non-specific binding. Tween 20 was omitted in the solution because the loose protein binding was the measured outcome. Bound HRP was quantified by adding o-phenylenediamine and  $H_2O_2$  until color developed (approximately 20 min). The reaction was quenched with sulfuric acid and equal aliquots of the supernatant colorimetric solution were transferred to a 96-well plate before absorbance was measured at 490 nm to determine amount adsorbed.

## **Fluorescein isothiocyanate (FITC) labeling and detection of fibrinogen**

Fluorescein isothiocyanate (FITC) is a fluorescent molecule often used for biomolecule labeling. Green in solution, FITC will absorb light and fluoresce to a vivid yellow/orange color. Notably, the molecule is destroyed by ultraviolet light and therefore requires handling in darkened rooms. FITC concentration is measured by the fluorescent intensity. The absorbance intensity is proportional to FITC concentration which in turn is proportional to target molecule concentration (when accurately synthesized). FITC is best applied for semi-qualitative analysis: if the target molecule is present and how it is relative to similarly treated samples.

Fibrinogen was labeled with fluorescein isothiocyanate according to Cierniewski et al.<sup>21</sup> Briefly, a 1% fibrinogen (Sigma Aldrich) solution in PBS was adjusted to pH 9.0 with 0.5 M Na<sup>+</sup> carbonate-bicarbonate buffer. A 0.2 mg/mL FITC in acetone solution was added to the fibrinogen solution. The coupling reaction was kept on ice for 1 h.<sup>22</sup> The solution was then transferred to a dialysis cassette and exhaustively dialyzed in 1 L PBS. PBS dialysate was changed approximately every 12 h. Dialysis continued until the dialysate was completely clear. FITC-labeled fibrinogen also was obtained from Molecular Innovations (FIB-FITC-909).

Silica microspheres of the same layer construction as the ELISA tests (**Table 1**) were tested against FITC-labeled fibrinogen. Aliquots of each sample (0.05 g microspheres/aliquot) were incubated with a 1.0 mg/mL FITC-labeled fibrinogen solution in HPLC water overnight at room temperature. The samples were then thoroughly washed via vortex and centrifugation with three volumes of PBS to remove excess fibrinogen. This procedure was conducted first with the laboratory synthesized FITC-

labeled fibrinogen to verify the labeling would yield measurable results (determined visually by the presence of a yellow tint on bare microspheres). The procedure was then repeated with the Molecular Innovation FITC-labeled fibrinogen.

Absorbance readings are necessary to measure the amount of FITC present on the microspheres and the microspheres' opacity prevents a spectrophotometer from accurately measuring the samples. Silica can be dissolved with a concentrated sodium hydroxide solution. By dissolving the microspheres and measuring proper controls the microspheres would no longer inhibit the reading and the amount of FITC present could be measured.

Microspheres were dissolved with a strong, 10 N, sodium hydroxide solution before absorbance was measured at 490 nm. An image of the microspheres prior to dissolution was also analyzed with Image J software.

### **Zeta potential surface measurements**

Zeta potential tests compared nisin elution from the PEO layer in samples challenged with either buffer or fibrinogen solution. Pluronic F108 and F68 coated surfaces were studied in this test. Microspheres with nisin-loaded PEO layers were challenged with either PBS or 1% fibrinogen for 1 h prior to zeta analysis. The buffer and fibrinogen challenges were repeated thrice for each sample to determine the nisin elution over repeated challenges.

The zeta potential of coated microspheres was measured with a Brookhaven ZetaPALS analyzer in the same method as described in Ryder et al. The instrument

measures surface charge of a particle by measuring the velocity of the particles as the progress towards the electrode.

The electrode was cleaned by sonication and rinsing with purified water prior to each run. The cuvette was also rinsed with purified water before each run. The microsphere solution was diluted to 10 mg/L in 1 mM KCl in the cuvette. The electrode was attached to the cuvette, and assembly connected to the ZetaPals instrument. Each trial included 10 runs, 20 cycles. The electrode was properly cleaned and stored after each run.

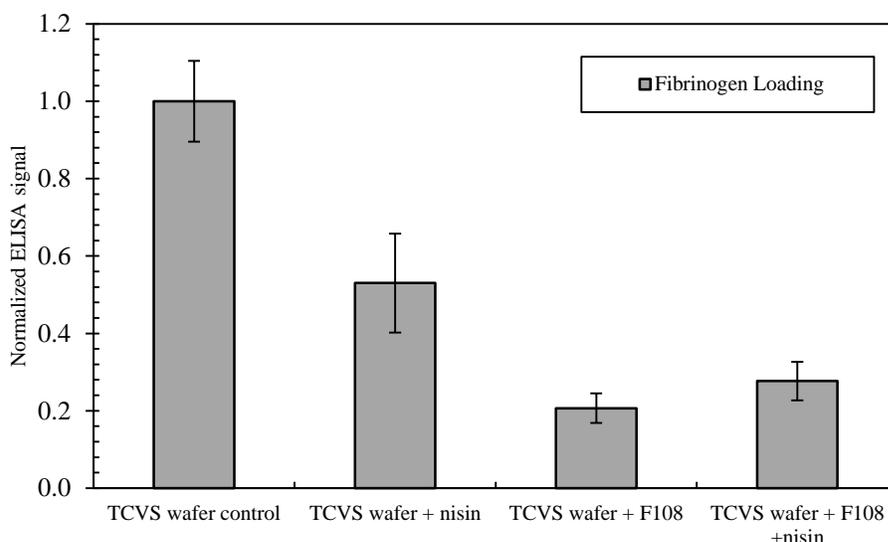
## Results Analysis and Discussion

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The test samples were those treated with F108 and nisin; all other treatments were controls for the purpose of discerning coating functionality (**Table 1**).

### ELISA analysis

The fluorescence signal emitted by the sample is directly proportional to the amount of fibrinogen present in the sample and the results shown may be analyzed as a semi-quantitative comparison. The fluorescence signal was normalized to the negative, “bare” control. The results shown are an average of all trials ( $\pm 1$  standard error). The ELISA results shown in **Figure 8** suggest that the presence of nisin in the PEO layer instigated fibrinogen adsorption to a level not substantially greater than with PEO alone.



**Figure 8:** Relative fibrinogen adsorption on uncoated and F108-coated silanized surfaces in the presence and absence of adsorbed nisin. Both coated and uncoated surfaces were gamma irradiated (uncoated in PBS, coated in F108 solution) prior to fibrinogen challenge. The absorbance values have been normalized to the uncoated, “bare” silica. The presence of F108 clearly hinders fibrinogen adsorption and the presence of nisin does not appear to instigate increased fibrinogen adsorption.

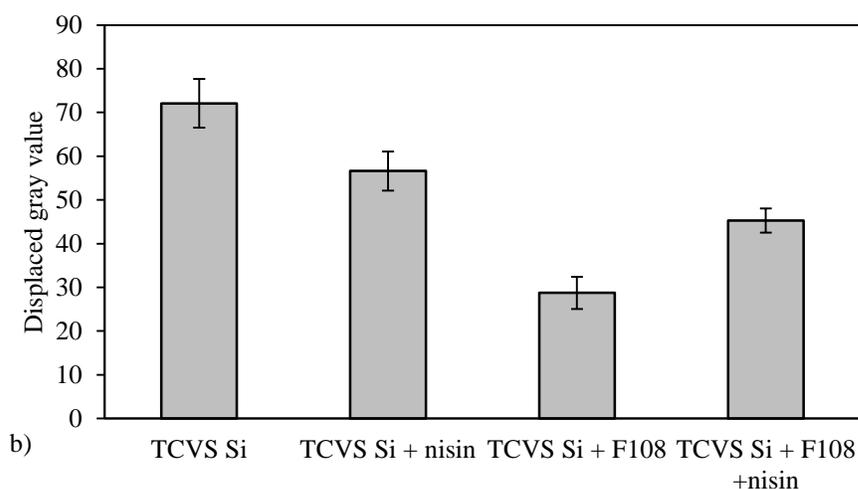
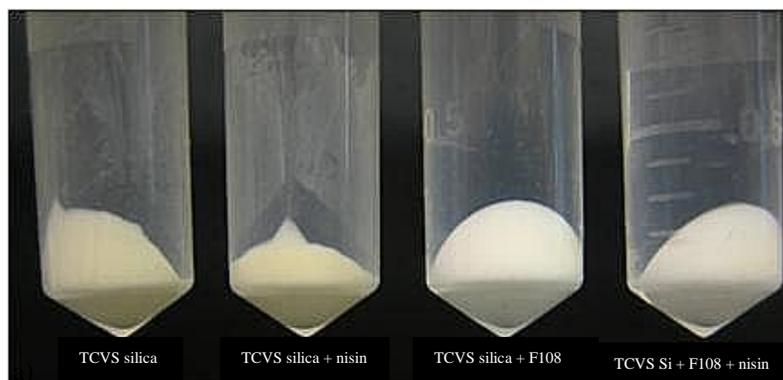
The decrease observed signal from the bare TCVS wafer (control) to the TCVS wafer with nisin may be attributed to the surface area nisin occupies on the surface onto which fibrinogen may no longer adsorb. The fibrinogen (340 kDa) may actually displace the nisin (approximately 7 kDa) over time. Competitive adsorption of fibrinogen or nisin at the surface could be modeled with further testing, but that pursuit is outside the scope of this proposal.

As expected, the F108 coated surface showed the lowest mean absorbance signal and thus lowest fibrinogen adsorption. However, there was apparent fibrinogen adsorption on each tested F108-coated surface. Said adsorption may be explained by irregularities in the PEO layer that compromise fibrinogen repulsion but may also be

explained as a product of the ELISA technique itself. The ELISA technique faced difficulties ensuring only specific adsorption by HRP-labeled anti-fibrinogen.

### **FITC-labeled fibrinogen analysis**

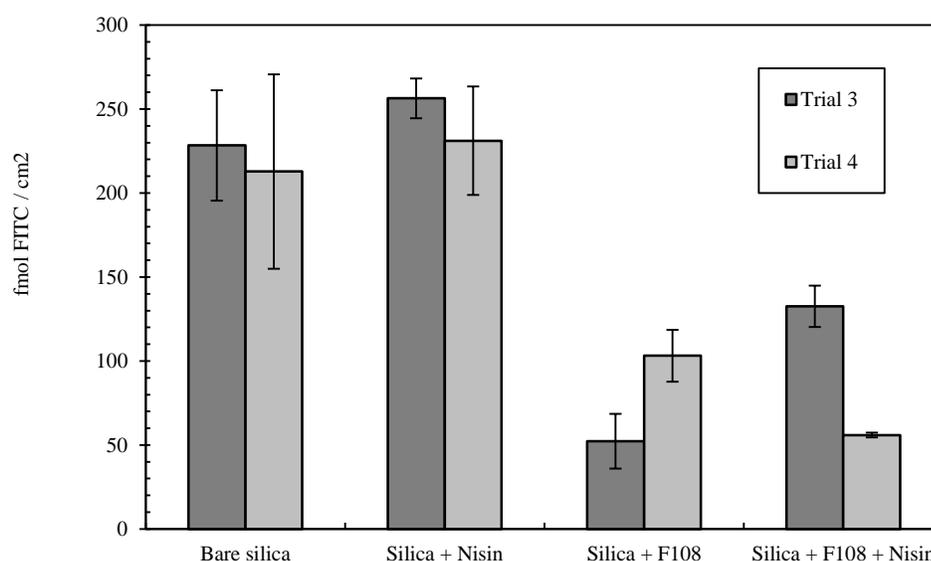
**Figure 9a** distinctly shows FITC-labeled fibrinogen, indicated by the yellowed tint, more prominently adsorbed to surfaces without the F108 coating than the F108-coated surfaces. When quantified with ImageJ, the mean gray scales in **Figure 9b** confirmed a significantly lower brightness for surfaces without F108 that equates to higher fibrinogen adsorption. The highest value on the gray scale is black, values decrease with color saturation with the lowest gray scale value being white. Thus, brightness is inversely proportional to amount of adsorbed fibrinogen. Displaced gray value is equivalent to how far the sample region is from pure white color; plotting this value more clearly indicates how colored the sample was with FITC. Unfortunately, the ImageJ analysis lacked accuracy, possibly due to curvature of tubes and overhead lighting, and thereby quality representation of true sample fluorescence.



**Figure 9:** Silica microspheres with bare, F108, nisin, and combination F108 and nisin coatings were challenged with FITC-tagged fibrinogen. a) The distinct yellow colors of the two samples without F108 indicate the tagged fibrinogen adsorbed to the microsphere surfaces. Conversely, the two F108 coated samples appear predominantly white, thereby indicating fibrinogen did not adsorb to the surface and the presence of nisin did not cause increased fibrinogen adsorption at the tri-block coating. b) The color intensity was measure with ImageJ in terms of gray value. Displaced gray value is equivalent to how far the sample region is from pure white. The method lacked accuracy and quality representation of true sample fluorescence.

The manufactured FITC-labeled fibrinogen showed more function than the lab synthesized FITC-labeled fibrinogen; however, both demonstrated enough function to differentiate between surface treatments. The amount of fibrinogen present on the

microspheres was calculated per surface area after microsphere dissolution (**Figure 10**). Trials 1 and 2 tested the laboratory synthesized tagged fibrinogen while trials 3 and 4 tested the manufactured fibrinogen. The manufactured FITC-labeled fibrinogen contained consistent number of FITC molecules per fibrinogen while the laboratory-synthesized fibrinogen FITC to fibrinogen ratio was unknown and not quantifiable. Hence, trials 1 and 2 confirmed the test protocol would yield discernable results, but only trials 3 and 4 were used for analysis.



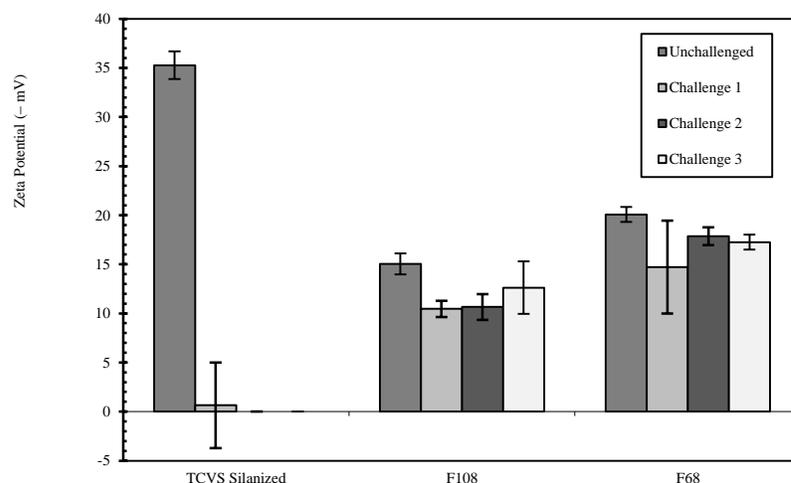
**Figure 10:** The molar quantity of FITC present on the surface of the microsphere samples was calculated from the absorbance measurements of each respective solution of dissolved microspheres, surface coatings, and tagged fibrinogen. The amount of FITC present on the non-F108 coated microspheres is on average more than microspheres with the F108 coating. Most importantly, there seems to be no discernable change in fibrinogen adsorption when nisin is integrated into the brush layer. Trials 1-2 used the laboratory synthesized FITC-labeled fibrinogen which may have contained inconsistent FITC residues per fibrinogen. As such, only trials 3-4 are shown above.

The results from these tests indicate the Pluronic® F108 modified surfaces significantly repel fibrinogen. Conversely, nisin coated surfaces appear to encourage fibrinogen adsorption. Electrostatic interactions may account for this increase since cationic nisin would decrease the hydrophobicity of the silanized silica surface and thus more readily attract fibrinogen. Furthermore, the nisin-loaded, Pluronic® F108 modified surfaces lost a fraction of the lone Pluronic® F108 non-fouling character. However the increases due to nisin presence are not significant and the nisin-loaded, Pluronic® F108 modified surfaces retained non-fouling properties to a degree equal or less than only F108 coated silica.

Similar to ELISA results, the presence of nisin in either sample with or without F108 did not significantly change the apparent amount of adsorbed fibrinogen as compared to equivalent samples without nisin.

### **Zeta potential analysis**

Zeta potential tests were conducted in an effort to determine the effect of fibrinogen on nisin elution from the PEO layer. A brush layer surface charge can be expected to increase when nisin integrates into the layer. Likewise, the surface charge of the microsphere surface can be expected to decrease as nisin leaves the layer. The hydrophobic surface has been measured to at approximately -35 mV. This value increases to approximately -15 mV with the introduction of F108 or F68, another tri-block polymer (**Figure 11**). It may be concluded that tri-block polymers successfully cover to a hydrophobic surface.



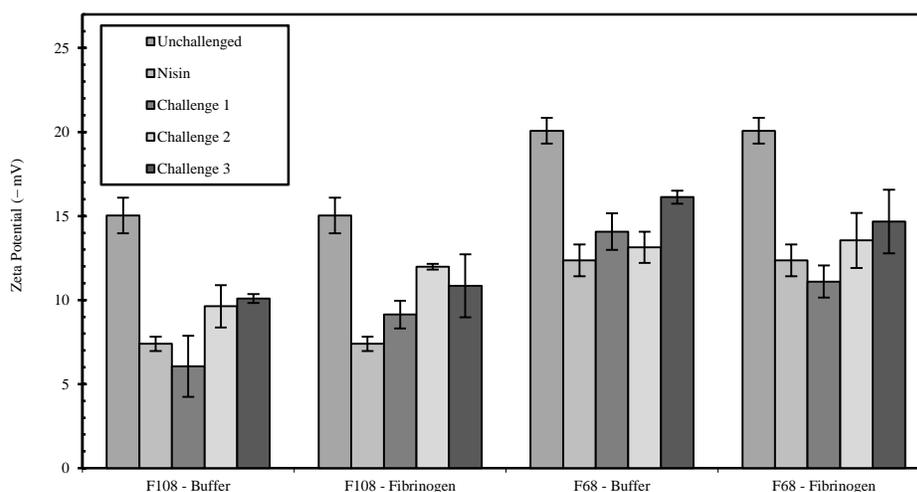
**Figure 11:** The zeta potential of a hydrophobic, silanized surface decreases when contacted with a tri-block polymer, either F108 or F68. The presence of the F108 tri-blocks increases the charge from -35 mV to -15 mV. The brush layer was successfully constructed.

Zeta potential measurements show a notable increase in zeta potential when nisin is introduced to an F108 or an F68 coated surface (**Figure 12**). The zeta potential value difference is consistent for both F108 and F68. This increase is consistent with nisin integration into the brush.

The buffer and fibrinogen challenges marginally decreased the surface charge with each challenge. The data suggests a slight trend of decreasing zeta potential value with each challenge that may suggest nisin elution from the brush layer. Notably, the changes observed in the buffer challenges are very similar to the changes observed in the fibrinogen challenges.

The predominantly constant charge indicates the presence of nisin does not significantly increase the layer's susceptibility to protein adsorption. However it is unclear whether fibrinogen simply displaces nisin, which would also result in limited zeta potential change. Because of fibrinogen's size, it is more likely to have a higher

attraction to the surface and so displace the nisin. It may be concluded that fibrinogen contact does not encourage nisin removal from the layer any more than normal buffer rinsing.



**Figure 12:** The negative zeta potential of test surfaces is presented for a series of protein and buffer challenges. Zeta potential directly correlates to microsphere surface charge and so is indicative of molecules present at a surface. Zeta potential values for F108 and F68 coated surfaces increase with nisin introduction but marginally decrease with subsequent fibrinogen or buffer challenges. Notably there is no significant difference between the buffer and the protein challenges. Multiple protein adsorption/desorption scenarios may explain this observation.

Zeta potential only measures at surface charge and not composition. Multiple situations may give rise to the same zeta potential change. Therefore, zeta potential results yield two possible scenarios: 1) fibrinogen does not adsorb to the surface with repeated challenges and nisin elutes from the layer over time or, 2) fibrinogen adsorbs to the surface and displaces nisin from the surface.

## **Limitations of tests**

Each of the methods applied yield semi-quantitative results. One test alone could not completely address the question at hand. The trends observed in the three tests cumulatively answer the posed question. Each test contained unavoidable experimental error. The ELISA and FITC-labeled fibrinogen tests sought to identify loosely bound protein on a theoretically non-fouling surface. Both tests required rigorous washings that may have removed fibrinogen from the surface. It was assumed that the amount of incidental fibrinogen removal was uniform across all test surfaces thereby permitting relative adsorbed amount comparisons. As previously mentioned, the zeta potential test yields a surface charge that may have multiple explanations for the same observed change.

More definitive tests could not be employed due to unavailability of technology and due to the constraints of the system: necessity to identify the protein on an opaque and solid surface, imperfections in coating synthesis that may have caused excessive background signal, and potentially fragile fibrinogen-surface association from the tri-block mobile character.

## **Conclusions and Recommendations**

Fibrinogen was expected in each test to associate more strongly to the surface of wafers without a brush layer. In the ELISA tests, lower signal, therefore less fibrinogen adsorption, was observed in surfaces with the brush layer. Nisin alone on a silica surface

decreased fibrinogen adsorption; this observation is logical when considering the decreased adsorption area available and any kinetic or thermodynamic barriers to exchange reactions between fibrinogen and adsorbed nisin. The presence of nisin in a brush layer marginally increased fibrinogen adsorption. In the FITC-labeled fibrinogen tests, lower signals were observed again in surfaces with the brush layer. Here, the addition of nisin to either PEO-coated or non-coated surfaces did not substantially change fibrinogen adsorption from the nisin-free counterparts. The combination of these tests suggests the PEO brush layer effectively inhibits fibrinogen adsorption and the presence of nisin in the brush layer does not significantly increase fibrinogen adsorption. The tests strongly suggest nisin does not increase the likelihood of preferential fibrinogen location at the surface.

Further testing will be carried out with a view towards formalizing this conclusion and determine the extent of nisin displacement and activity loss. Optical waveguide lightmode spectroscopy (OWLS) will directly quantify tri-block, nisin, and fibrinogen adsorption at the surface of the coating. The coating will be constructed on the OWLS sensor and then challenged with fibrinogen solution multiple times.

Nisin displacement and activity loss due to degradation and irreversible adsorption both result in coating functionality loss. These effects remain largely not quantified, and in future work nisin activity within the PEO layer will be evaluated by bioactivity assays. The assay will evaluate coatings challenged and not challenged with fibrinogen. The surface will then be incubated with *Staphylococcus epidermis* and bacterial kill zone quantified. The size of the bacterial kill zone over time will indicate the level of nisin activity. A comparison of the surfaces challenged and not challenged

with fibrinogen will indicate the extent of nisin displacement from the PEO layer. This work will be contribute to the subject of future reports.

## Bibliography

- [1] McGuire, J. *Biomaterials & Biointerfaces Class Notes*. Oregon State University. Spring 2011.
- [2] Halperin, A. "Polymer Brushes that Resist Adsorption of Model Proteins: Design Parameters." *Langmuir* **15**, 7 (1999) 2525-2533.
- [3] Li, J.-T., J. Carlsson, and K.D. Caldwell. Surface properties of poly[ethylene oxide]-containing copolymers on colloids. In: *Polymeric Materials Science and Engineering*, vol. 62. G.R. Pilcjer (ed), Am. Chem. Soc., Washington D.C., page 62, 1993.
- [4] Tai, Yuan-Ching, Joseph McGuire, Jennifer A. Neff. "Nisin antimicrobial activity and structural characteristics at hydrophobic surfaces coated with the PEO-PPO-PEO triblock surfactant Pluronic® F108." *Journal of Colloid and Interface Science* **332** (2008) 104-111.
- [5] McPherson, T.B., H.S. Shim, and K. Park. Grafting of PEO to glass, nitinol, and pyrolytic carbon surfaces by gamma irradiation. *J. Biomed. Mater. Res.* **38** (1997) 289-302.
- [6] Kuwano, Koichi, Noriko Tanaka, Takashi Shimizu, Kohei Nagatoshi, Shinsuke Nou, Kenji Sonomoto. "Dual antibacterial mechanisms of nisin Z against Gram-positive and Gram-negative bacteria." *International Journal of Antimicrobial Agents* **26** (2005) 396-402.
- [7] Breukink E, Kruijff B. "The lantibiotic nisin, a special case or not?" *Biochim Biophys Acta* **1492** (1999) 223-234.
- [8] Carr FJ, Chill D, Maida N. "The lactic acid bacteria: a literature survey." *Crit Rev Microbiol* **28** (2002) 281-370.
- [9] Gross E, Morell JL. "The structure of nisin." *J Am Chem Soc* **93** (1971) 4634-4635.
- [10] Buchman GW, Banerjee S, Hansen jN. "Structure, expression, and evolution of a gene encoding the precursor of nisin, a small protein antibiotic." *J Biol Chem* **263** (1988) 16260-16266.
- [11] Liu, Wei. "Some Chemical and Physical Properties of Nisin, a Small-Protein Antibiotic Produced by *Lactococcus lactis*." *Applied and Environmental Microbiology* (1990) 2551-2558.

- [12] Morris, S. L., R. C. Walsh, and J. N. Hansen. "Identification and characterization of some bacterial membrane sulfhydryl groups which are targets of bacteriostatic and antibiotic action." *J. Biol. Chem.* **259** (1984) 13590-13594.
- [13] van Heusden HE, Breukink E. "Lipid II induces a transmembrane orientation of the pore-forming peptide lantibiotic nisin." *Biochemistry* **41** (2002) 12171-12178.
- [14] Breukink E, Weidemann I, van Kraaij C, Kuipers OP, Sahl H, de Kruijff B. "Use of the cell wall precursor lipid II by a pore-forming peptide antibiotic." *Science* **268** (1999) 2361-2364.
- [15] Sahl HG, Bierbaum G. "Lantibiotics: biosynthesis and biological activities of uniquely modified peptides from Gram-positive bacteria." *Annu Rev Microbiol* **52** (1998) 41-79.
- [16] Hurst AH, Hoover DG. Nisin. In: Davidson PM, Sofos JN, Granen AL, editors. *Antimicrobials in foods*. New York, NY: Marcel Dekker; 1993.
- [17] Hurst, A. "Nisin." *Adv. Appl. Microbiol.* **27** (1981) 85-123.
- [18] Chan, Weng C, Barrie W Bycroft, Lu-Yun Lian, Gordon C.K. Roberts. "Isolation and characterization of two degradation product derived from the peptide antibiotic nisin." *Elsevier Science Publishers* **252** (1989) 29-36.
- [19] Tai, Yuan-Ching, Pranav Joshi, Joseph McGuire, Jennifer A. Neff. "Nisin adsorption to hydrophobic surfaces coated with the PEO-PPO-PEO triblock surfactant Pluronic® F108." *Journal of Colloid and Interface Science* **332** (2008) 112-118.
- [20] Matt P Ryder, Karl F Schilke, Julie A Auxier, Joseph McGuire, Jennifer A Neff. "Nisin adsorption to polyethylene oxide layers and its resistance to elution in the presence of fibrinogen." *J. Colloid Interface Sci.* Vol 350, Is 1, Pg 194-199. Oct 2010.
- [21] Cierniewski, C.S., T. Krajewski. "Interaction of fibrinogen with fluorescein isothiocyanate." *Thrombosis Research* **13** (1978) 615-623.
- [22] Jung-He Wu, Scott L Diamond. "A Fluorescence Quench and Dequench Assay of Fibrinogen Polymerization, Fibrinogenolysis, or Fibrinolysis." *Analytical Biochemistry* **224** (1995) 83-91.



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