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Nisin adsorption to hydrophobic surfaces coated with the PEO–PPO–PEO triblock surfactant Pluronic® F108

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Abstract

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

Keywords

Nisin adsorption kinetics; *In situ* ellipsometry; Pluronic® F108; PEO-PPO-PEO triblock surfactants

1. Introduction

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

Implant-associated infections can result in systemic infections that in the worst-case scenario can lead to multiple organ failure and death, despite successful resolution of the original medical condition. The cost of such infections is high. For example, a single episode of central venous catheter-related bacteremia has been estimated to cost up to \$50,000 with an attributable

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mortality rate between 4 and 35% [1]. Infection is also a major problem for dialysis patients. Over 450,000 people in the US alone have end stage kidney failure and require chronic hemodialysis. For these patients, vascular access procedures are a major cause of morbidity and mortality. AV grafts are used in about 42% of patients with an infection rate between 11 and 20%. The mortality rate due to infection of these grafts is between 12 and 22%.

A number of antimicrobial coatings have been evaluated for their ability to reduce the incidence of implant-related sepsis. But in general, the prophylactic use of antibiotic-coated implants increases the risk of producing resistant strains of bacteria, while the use of other kinds of antibacterial compounds (antiseptics) provides inferior results in comparison to the use of clinical antibiotics. Antiseptic-based coatings have also been associated with reports of anaphylactic shock [2–4]. Lantibiotics are antibiotic compounds that include one or more lanthionine rings. The unique physical structure of lantibiotics makes them different in mode-of-action from traditional antibiotics, suggesting that they offer a means for preventing the rise of resistant microorganisms [5–8]. Lantibiotics such as nisin can adsorb to surfaces, maintain activity, and kill cells that have adhered *in vitro* [9–11]. The structure of nisin, by far the most extensively investigated lantibiotic with reference to biomaterials applications, is shown schematically in Fig. 1. *Staphylococcus aureus* and *Staphylococcus epidermidis* are the most frequently encountered biomaterial-associated pathogens [12–14], and both are Gram-positive bacteria. Nisin kills Gram-positive bacteria through a multi-step process that destabilizes the phospholipid bilayer of the cell and creates transient pores.

Application of nisin-coated implants *in vivo* was reported by Bower et al. [11], but the duration of coating activity was short. The nisin was bound by non-specific adsorption to hydrophobic catheter materials in that work, and the loss in activity could be attributed to nisin exchange with blood proteins at the surface. Since that time considerable effort in our laboratory has been placed on “tethering” nisin to solid surfaces for long-term antibacterial activity, in a manner allowing the level of solvent accessibility and molecular mobility needed for membrane binding, insertion, and pore formation to be preserved [15]. In particular we have synthesized thiol-modified nisin derivatives, by chemical modification of the primary amine group at the N-terminal residue. These were then chemically coupled to end-group activated poly(ethylene oxide)–poly(propylene oxide)–poly(ethylene oxide) (PEO–PPO–PEO) triblock polymers to form nisin-containing block copolymers. End-group activation involved replacing the terminal hydroxyl groups of the PEO chains with pyridyl disulfide moieties. Nisin was secured to the PEO chains in an “end-on” orientation, through a disulfide linkage.

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

It is well known that PEO resists protein interactions and F108 coated surfaces in particular have been shown to prevent protein adsorption [16–18]. It was therefore believed that nisin would not interact with the PEO–PPO–PEO triblocks, other than at the reactive site on the end-

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

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

2. Materials and methods

2.1. Protein and surfactant solutions

Nisin (MW 3510 Da) was obtained from Prime Pharma (Batch No. 20050116-1, Gordons Bay, South Africa) and was dissolved in filtered, 10 mM monobasic sodium phosphate solution to ensure complete solubilization. Filtered, 10 mM dibasic sodium phosphate was then added to bring the pH to 7.0. Nisin has an isoelectric point in the alkaline range (above 8.5) and is therefore positively charged at neutral pH. Bovine serum albumin (BSA; Sigma–Aldrich) was dissolved in filtered, 10 mM sodium phosphate buffer at pH 7.0. Solutions of nisin and BSA (each at 5.0 mg/mL) were aliquoted into 0.5 mL samples and stored at -80°C ; they were thawed immediately before use. The Pluronic® surfactant F108 (MW 14,600, with two end blocks of PEO that are 129 monomer units in length and a center block of PPO that is 56 monomer units in length, i.e., $\text{HO}-(\text{CH}_2-\text{CH}_2-\text{O})_{129}-(\text{CHCH}_3-\text{CH}_2-\text{O})_{56}-(\text{CH}_2-\text{CH}_2-\text{O})_{129}-\text{H}$) was purchased from BASF and was dissolved in 10 mM phosphate buffer (pH 7) as needed.

2.2. Surface modification

Silica wafers (Type P, DOP B, thickness $525 \pm 18 \mu\text{m}$, resistivity $0.01\text{--}0.02 \Omega \text{ cm}$; WaferNet Inc.) were cut into $1 \times 5 \text{ cm}$ strips using a tungsten pen. The silica strips were rinsed with acetone to remove dust and organic residues, washed in a solution of $\text{NH}_4\text{OH}:\text{H}_2\text{O}_2:\text{H}_2\text{O}$ (1:1:5 volume ratio) at 80°C for 10 min, then washed in a solution of $\text{HCl}:\text{H}_2\text{O}_2:\text{H}_2\text{O}$ (1:1:5 volume ratio) at 80°C for 10 min. They were then rinsed with deionized water and pure ethanol, and blown dry with a nitrogen stream. From this point, two methods were used to render the silica surfaces sufficiently hydrophobic for F108 coating. In one method, the dried silica strips were placed in a glass dish and transferred to a vacuum oven (200°C) housing an uncovered, 2 mL volume of hexamethyldisilazane (HMDS, Product number H7300, United Chemical Technologies). HMDS vapor deposition was allowed to occur for 90 min, at 20–25 in. Hg vacuum and 200°C . In another method, the cleaned, dried silica strips were transferred to a solution of 5% (V/V) PS200 (“Glassclad 18,” Product No. PS200, United Chemical Technologies) in water and agitated for 15–20 s. The samples were then rinsed thoroughly with deionized water and cured at 100°C for 1 h. The hydrophobicities of surfaces coated in these ways were measured by contact angle analysis. The contact angle was typically between 85° and 95° following HMDS treatment, and between 65° and 75° following PS200 treatment. Silanized silica surfaces were stored in an electronic desiccator until needed.

2.3. Surface coating with F108

HMDS- or PS200-treated samples were coated with F108 triblocks by incubation with 0.50% F108 in 10 mM sodium phosphate buffer. Incubation took place overnight (at least 16 h), in

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

PEO coatings were also prepared by covalent attachment of the F108 triblocks. McPherson et al. [19] and Park et al. [20] described pretreatment with a silane and subsequent γ -irradiation to covalently bind PEO–PPO–PEO triblocks (via the PPO block) to glass, metal and pyrolytic carbon surfaces. In short, through absorption of radiation or interaction with water-derived radicals, surface-bound free radicals are formed. These free radicals attack the adsorbed PPO block, forming new covalent bonds between the surface and polymer [19]. In this work, the F108 triblocks were covalently attached according to methods similar to those cited above. In brief, the silica samples were silanized with octadecyltrimethoxysilane, and the surface-polymer covalent attachment induced by UV radiation [21]. After UV treatment, samples were washed with PBS. F108 tri-block coatings stabilized in this way 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 [21].

2.4. In situ ellipsometry

A silica sample (coated with F108 or uncoated) was placed into a fused quartz, trapezoid cuvette (Hellma Cells, Germany) which was secured on the sample stage of an automatic *in situ* ellipsometer (Model L-104 SA, Gaertner Scientific Corp.) modified to allow for stirring and flow. After 4.5 mL of 10 mM phosphate buffer (pH 7.0) was injected into the cuvette, the ellipsometer stage was adjusted to obtain a maximum in reflected light intensity and steady optical properties. Surface optical properties were recorded every 15 s for 30 min before 0.5 mL of protein or F108 solution was injected into the cuvette to yield a final protein concentration of 0.50 mg/mL, or final F108 concentration of 0.5% (w/v). Adsorption was allowed to occur for a desired period of time, after which the surface was rinsed *in situ* with 10 mM sodium phosphate buffer at a flow rate of 31.6 mL/min for 5 min. Film properties were then monitored for a desired “incubation” period. Any additional protein adsorption and rinsing-incubation steps carried out in a given experiment were performed in the same manner described above.

A one-film-model ellipsometry program [22] was used to determine the adsorbed mass of protein from the ellipsometrically determined values of film thickness and refractive index (these optical properties being determined simultaneously at each measurement) according to Cuypers et al. [23]. A partial specific volume of 3.837 mL/g and a molecular weight to molar refractivity ratio of 0.729 g/mL were used. Each experiment was performed at least twice on each type of surface, with an average deviation from the mean of about 0.005 $\mu\text{g}/\text{cm}^2$.

3. Results and discussion

3.1. F108 adsorption at hydrophobic silica

Fig. 2 provides representative results for adsorption of F108 on a (PS200) silanized silica surface, rinsed sequentially at 60 and 90 min. The surface concentration of F108 reached a plateau immediately after its introduction to the cuvette, indicative of a high affinity adsorption at the hydrophobic surface, and consistent with adsorption patterns recorded for a number of small-molecule surfactants [24]. The adsorbed mass decreased somewhat after the first rinse, and this can be attributed to the elution of loosely bound triblocks. No such decrease in adsorbed mass was recorded after the second rinse step. (We note here that the PS200-treated silica samples supported a more stable F108 coating for monitoring protein adsorption and elution kinetics. Unless otherwise indicated, all F108 coatings represented by the data presented here were prepared from PS200-treated substrates.)

3.2. Nisin adsorption and elution kinetics

Fig. 3 provides a representative comparison of nisin adsorption and elution kinetics at bare hydrophobic and F108-coated surfaces. Nisin adsorption did not reach a plateau in either case, and based on dimensions of nisin in solution (if modeled as a cylinder, about 2×5 nm) a monolayer of molecules adsorbed “side-on” (i.e., in an area equal to $2 \times 5 = 10$ nm²) and “end-on” (i.e., in an area equal to $2 \times 2 = 4$ nm²) would result in an adsorbed mass of 0.058 and 0.145 µg/cm², respectively. Thus the patterns shown in Fig. 3 are consistent with multi-layer adsorption in each case. Nisin adsorption to the F108-coated surface was generally slower than adsorption to the bare hydrophobic surface, likely owing to steric inhibition by the pendant PEO chains. Nisin elution in protein-free buffer was similar at each surface initially, but elution was observed to continue only at the bare hydrophobic surface. Presumably, this is due to greater solvent accessibility to nisin that is loosely held in the outer layers in the case of adsorption to the uncoated surface, while nisin integrated into the PEO “brush” would show greater resistance to elution.

The effect of the PEO chains in adsorption and elution can be further revealed by analysis of nisin adsorption–elution data with reference to a “history-dependent” adsorption mechanism [25,26]. A number of macromolecular species, including proteins, exhibit history-dependent adsorption behavior owing to the slow relaxation of non-equilibrium structures at the interface. That is, for a given protein at a given surface loading, the rate of adsorption depends on the formation history of the adsorbed layer. This is particularly relevant near monolayer surface coverage when protein–protein interactions can influence the availability of surface area suitable for adsorption. Tie et al. [26] studied the adsorption of fibronectin, cytochrome *c* and lysozyme using optical waveguide lightmode spectroscopy in multi-step mode, where an adsorbing surface is alternately exposed to a protein solution and a solution free of protein. In general, they observed the initial adsorption rate during the second step exceeded that observed at the same surface coverage during the first step. They postulated that, for a given mass density at an interface, if proteins were arranged in “clusters” or aggregates, more “cleared” surface area would be available for further adsorption relative to proteins being randomly distributed. On the other hand, if the adsorbed protein films were at equilibrium, we would expect the same adsorption rates during each cycle, since the proteins would have identical structural characteristics.

Adsorption rate data can thus provide important information relevant to adsorbed layer structure [25,26], and we used the kind of sequential adsorption kinetic data presented in Fig. 4 for this purpose. Fig. 4 shows results of a two, nisin adsorption–elution steps performed in sequence at a bare hydrophobic surface and an F108-coated surface. For each surface, the initial slope of the second adsorption step was compared to the slope at the point in the first adsorption cycle with the same initial mass density. At both the coated and uncoated surface, the initial adsorption rate during the second step exceeded that observed at the same surface coverage during the first step. But the increase in slope was substantial in the case of uncoated silica as compared to F108-coated silica. That is, the first and second step adsorption rates were not as different for the F108-coated surface, indicating that less post-adsorptive rearrangement (or less “clustering”) of nisin occurred in this case. We suggest the lateral mobility needed for cluster formation and the generation of unoccupied surface area was inhibited by the PEO chains.

3.3. Functional evaluation of the F108 layer

Fig. 5 shows two representative plots of nisin adsorption and elution kinetics recorded on F108-coated surfaces. In one case the F108 triblocks were coated as described here (i.e., incubation of the silanized hydrophobic surface overnight with 0.5% F108, followed by buffer rinse to remove loosely bound F108 from the surface and replacement of the F108 solution with buffer),

and in the other case the F108 triblocks were coated by covalent attachment via the PPO chains. As shown in Fig. 5, the nisin adsorption and elution kinetics were quite similar at each surface. In regard to nisin adsorption, this indicates that the F108 layers prepared by hydrophobic association between the PPO block and the silanized surface functioned in a manner substantially similar to the F108 layers prepared by covalent attachment between the PPO block and silanized surface. Importantly, the similar kinetic patterns in Fig. 5 indicate that nisin adsorption was not likely associated with elution and replacement of F108 triblocks. If replacement of F108 was necessary for nisin adsorption, we would expect nisin adsorption at the UV-treated surface to be inhibited if not absent.

While nisin was clearly observed to adsorb in multi-layer quantities at the F108-coated surfaces, these surfaces retained their “large” protein rejecting properties as shown in the case of BSA adsorption in Fig. 6. Fig. 6a shows no change in adsorbed mass recorded upon introduction of BSA (MW 66 kDa) to an F108 layer prepared by 22 h incubation of the surface with F108 solution, and no change in adsorbed mass recorded upon rinsing and incubation in protein-free buffer. This indicates that BSA was rejected by the F108-coated surface. Fig. 6b shows results of a similar experiment, but where F108 was incubated with the surface for only 30 min prior to rinsing. Again, no change in adsorbed mass was recorded upon introduction of BSA to the F108 layer, suggesting that the triblocks adopted their energetically favorable molecular orientation, with PPO block associating with the surface and pendant PEO chains extending away from the surface, in a fairly short period of time.

Figs. 7 and 8 show representative results of sequential adsorption experiments performed with nisin and BSA at F108-coated surfaces (adsorption of BSA at a bare, hydrophobic surface is also included for comparison, in Fig. 7). As shown in Fig. 7, when BSA is introduced first, scattered data show adsorption of BSA to be of very low affinity. (Adsorption data recorded for BSA at F108-coated surfaces, interpreted with reference to optical constants of the coated surface—as opposed to optical constants of the bare, silanized surface as done in Fig. 6—were not easily reproducible, due to what we conclude are very low values of adsorbed mass. In particular, when the change in optical properties of a film (relative to the reference substrate optical properties) is below a certain threshold, the mathematical routine used here to determine film thickness and refractive index does not yield reliable results, instead often reporting negative values accompanied by noise. The data shown in Fig. 7 for BSA adsorption should therefore be taken only as an indication that the affinity of BSA for the F108-coated surface is very low.) Following a rinse and 30 min incubation of the surface in protein-free buffer, adsorbed mass increased immediately upon introduction of nisin. Fig. 8 shows that when nisin is introduced first, an increase in adsorbed mass is evident upon introduction of BSA. This may be attributed to electrostatic attraction between the cationic peptide nisin and the negatively charged BSA. This effect must be further explored in terms of extent of adsorption and tightness of serum protein binding. But it is important to note that these tests were conducted at a low ionic strength relative to human blood, where electrostatic charges would be more effectively masked, reducing electrostatic attractions like that suggested in Fig. 8.

4. Summary

The adsorption of the antimicrobial peptide nisin to stable, adsorbed layers of the PEO–PPO–PEO triblock surfactant Pluronic® F108 was quantified by *in situ* ellipsometry. While it is well understood that such layers inhibit protein adsorption, nisin, presumably due in part to its relatively small size, was able to adsorb in multilayer quantities. Comparison of nisin adsorption and elution kinetics at uncoated and F108-coated surfaces suggested that the rate of nisin adsorption and elution, and its lateral mobility at the interface, were generally greater at uncoated surfaces. This would be consistent with nisin adsorption or “entrapment” within the PEO brush layer, as opposed to adsorption to the PEO chains in a non-penetrating manner.

F108-coated layers rejected adsorption of the protein BSA. However, sequential adsorption experiments carried out with nisin and BSA showed measurable adsorption of BSA if nisin was present at the interface. These phenomena, along with evaluation of the structure and biological activity of nisin in F108 layers, are currently under study and will contribute to the subject of a future report.

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Fig. 1.
Schematic of nisin A. Abu: 2-aminobutyric acid; Dha: dehydroalanine; Dhb: dehydrobutyrine.

Fig. 2. Adsorption and elution of the Pluronic® surfactant F108 on a hydrophobic surface.

Fig. 3.
Comparison of nisin adsorption and elution kinetics at bare hydrophobic and F108-coated surfaces.

Fig. 4. Sequential adsorption and elution kinetics exhibited by nisin at bare hydrophobic and F108-coated surfaces. Data recorded during the first adsorption cycle are overlaid on the second adsorption cycle in order to compare adsorption rates recorded at the same initial mass density, but after different formation histories.

Fig. 5. Nisin adsorption and elution kinetics recorded at hydrophobic surfaces coated with F108 triblocks by hydrophobic association, and by covalent attachment.

(a)

(b)

Fig. 6. BSA adsorption and elution on an F108-coated surface prepared by (a) incubation for 22 h followed by 30 min incubation in surfactant-free phosphate buffer, and (b) incubation for 30 min followed by 30 min incubation in surfactant-free phosphate buffer.

Fig. 7. Sequential adsorption of BSA and nisin on an F108-coated surface. The adsorption of BSA at a bare hydrophobic surface is included for comparison.

Fig. 8.
Sequential adsorption of nisin and BSA on an F108-coated surface.