Published in final edited form as:

J Colloid Interface Sci. 2008 June 1; 322(1): 104–111. doi:10.1016/j.jcis.2008.02.062.

Nisin antimicrobial activity and structural characteristics at hydrophobic surfaces coated with the PEO–PPO–PEO triblock surfactant Pluronic® F108

Yuan-Ching Tai^a, Joseph McGuire^{a,*}, and Jennifer A. Neff^b

^a Department of Chemical Engineering, Oregon State University, Corvallis, OR 97331, USA

^b Allvivo Vascular, Inc., Lake Forest, CA 92630, USA

Abstract

The antimicrobial peptide nisin has been observed to preferentially locate at surfaces coated with the poly[ethylene oxide]–poly[propylene oxide]–poly[ethylene oxide] (PEO–PPO–PEO) surfactant Pluronic® F108, to an extent similar to its adsorption at uncoated, hydrophobic surfaces. In order to evaluate nisin function following its adsorption to surfaces presenting pendant PEO chains, the antimicrobial activity of nisin-loaded, F108-coated polystyrene microspheres and F108-coated polyurethane catheter segments was evaluated against the Gram-positive indicator strain, *Pediococcus pentosaceus*. The retained biological activity of these nisin-loaded layers was evaluated after incubation in the presence and absence of blood proteins, for contact periods up to one week. While an increase in serum protein concentration reduced the retained activity on both bare hydrophobic and F108-coated materials, F108-coated surfaces retained more antimicrobial activity than the uncoated surfaces. Circular dichroism spectroscopy experiments conducted with nisin in the presence of F108-coated and uncoated, silanized silica nanoparticles suggested that nisin experienced conformational rearrangement at a greater rate and to a greater extent on bare hydrophobic surfaces relative to F108-coated surfaces. These results support the notion that immobilized, pendant PEO chains confer some degree of conformational stability to nisin while also inhibiting its exchange by blood proteins.

Keywords

PEO–PPO–PEO triblock surfactant; Pluronic® F108; Lantibiotics; Nisin; Antimicrobial activity; Circular dichroism

1. Introduction

In a previous paper we described the adsorption and elution behavior of nisin at silanized silica surfaces coated with the PEO–PPO–PEO triblock copolymer Pluronic® F108 [1]. Comparison of nisin adsorption and elution kinetics at uncoated and F108-coated surfaces suggested nisin “entrapment” within the PEO brush layer, as opposed to adsorption to the PEO chains in a non-penetrating manner. It is generally understood that PEO resists protein interactions and 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.

* Corresponding author. Fax: +1 541 737 4600., E-mail address: joseph.mcguire@oregonstate.edu (J. McGuire).

Nisin is a small (3510 Da) amphiphilic peptide that is cationic at neutral pH, having an isoelectric point above 8.5. It is an effective inhibitor of Gram-positive bacteria, and has been shown to adsorb to surfaces, maintain activity, and kill cells that have adhered *in vitro* [2]. Nisin kills susceptible bacteria through a multi-step process that destabilizes the phospholipid bilayer of the cell and creates transient pores. Previous work on nisin structure and function, its mechanism of antimicrobial action, surface activity and potential for use as an anti-infective agent in medical device coatings, was summarized earlier [1]. In this paper we describe the antimicrobial activity of nisin-loaded, F108-coated polystyrene microspheres and F108-coated polyurethane catheter segments after incubation in the presence and absence of blood proteins. In addition, circular dichroism spectra were recorded for nisin in the presence of F108-coated and uncoated, silanized silica nanoparticles in order to gain information on nisin conformational rearrangement on bare hydrophobic relative to F108-coated surfaces.

2. Materials and methods

Nisin was obtained from Prime Pharma (Batch number 20050116-1, Gordons Bay, South Africa) and was dissolved in filtered, 10 mM monobasic sodium phosphate solution to ensure complete solubilization. Filtered, 10 mM dibasic sodium phosphate was then added to bring the pH to 7.0. Nisin solutions were aliquoted into 0.5 mL samples, frozen at -80°C and thawed just before use. Fresh equine blood was obtained from the College of Veterinary Medicine, Oregon State University. Equine plasma was used because it has the same plasma proteins as human blood. Immediately upon its receipt, cells were separated from serum by centrifugation at 8000 rpm for 10 min. Serum was then stored at -80°C until needed. Pluronic[®] F108 (BASF) and polyethylene glycol (MW 6000, Product No. 81253, Sigma Aldrich) were each dissolved in 10 mM phosphate buffer (pH 7) as needed.

2.1. Measurement of antimicrobial activity of coated polystyrene microspheres

2.1.1. Preparation of F108-coated surfaces—Polystyrene microspheres (1.247 μm diameter, Part No. 81002497100290, Seradyn) were mixed with F108 (5 mg/mL) and incubated in phosphate buffer overnight on a rotator. The hydrophobic PPO block of the F108 molecule adsorbs on the polystyrene surface such that the hydrophilic PEO chains extend into the solution phase. Unbound F108 was removed from coated microspheres by repeated washing, including vortexing and sonication, centrifugation and re-suspension in phosphate buffer.

2.1.2. Nisin loading and incubation—The F108-coated and bare microsphere samples were independently mixed with 8×10^{-3} mg/mL nisin and incubated in phosphate buffer for 1 h on a tube rotator at room temperature. Unbound nisin was then removed by repeated washing (sonication, centrifugation and re-suspension in phosphate buffer). The absence of unbound nisin in the supernatant was verified by application of an agar plate diffusion assay [3,4] on a plate seeded with *Pediococcus pentosaceus*. The agar diffusion assay is the most common type of nisin activity assay. In brief, holes were aseptically punched in a nutrient agar plate seeded with *P. pentosaceus*, and samples of supernatant were placed into the wells. After incubation, zones of inhibition about each well were recorded. Microsphere suspensions were used only after detecting no nisin activity in the supernatant (i.e., no visible inhibition zones around the wells) after the final wash step. Nisin (8×10^{-3} mg/mL) was also incubated in microsphere-free phosphate buffer (10 mM) and F108 (5 mg/mL) solutions for controlled comparison. The nisin-loaded microsphere and control samples were then incubated in phosphate buffer or equine serum of desired dilution (10, 50 and 100% serum) for desired periods of time (0, 1, 4 and 7 days) at 37°C .

2.1.3. Cultivation of *P. pentosaceus* and measurement of antibacterial activity

—MRS broth was used for cultivation of the nisin-sensitive *P. pentosaceus* strain FBB 61-2. MRS (52.2 g, Cat. No. 1.10661, EMD Chemicals, Inc.) was dissolved in 1 L of DI water and autoclaved at 121 °C for 30 min. *P. pentosaceus* was incubated overnight (20 h) at 37 °C and placed on an orbital shaker at 220 rpm. The optical density (OD₆₀₀) of the overnight culture, and a 100-fold dilution of the overnight culture, was measured to ensure consistency of cell density.

After incubation of samples in either buffer or equine serum, microspheres were washed twice then mixed with a 100-fold dilution of overnight *P. pentosaceus* culture at 37 °C for 4 h. These were sampled and diluted 100-fold. Culture samples (0.5 mL) were then evenly dispersed with MRS-based melt agar (44 °C) on Petri dishes. The dishes were incubated at 37 °C for 48 h, until bacteria colonies became visible. The number of colonies recorded after 48 h was taken as an indication of the potency of the nisin coatings during the period of suspension with *P. pentosaceus*.

2.2. Measurement of antimicrobial activity of coated polyurethane catheter segments

Polyurethane catheter segments (22 GA, 1.0 in I.V. catheters, REF 381423, Becton Dickinson) were coated by incubation with F108 (5 mg/mL) in 10 mM phosphate buffer for 24 h in disposable test tubes. They were then rinsed with multiple test tube volumes of phosphate buffer to remove unbound F108.

F108-coated and bare segments were incubated in 0.5 mg/mL nisin for 1 h at room temperature. After 1 h, the catheter segments were rinsed with multiple test tube volumes of filtered 10 mM sodium phosphate buffer to remove unbound nisin. Nisin treated catheter segments were then incubated in 25% equine serum or 10 mM phosphate buffer for a desired period of time. The nisin-treated segments were then rinsed with copious phosphate buffer, in each case administered to the lumen through a syringe.

Nisin sensitive *P. pentosaceus* (cultivated as outlined above) were used to seed MRS agar dishes. Rinsed catheter segments were inserted onto the *P. pentosaceus*-seeded plates for an agar diffusion assay of antibacterial activity. Plates were incubated at 37 °C for 48 h, and the area of the kill zone was measured to provide an indication of nisin activity.

2.3. Evaluation of nisin structural characteristics

Silica nanoparticles, made hydrophobic by silanization with hexamethyldisilazane (Product R816, 190 m²/g, 10–12 nm diameter, Degussa), were coated with F108 by suspension in phosphate buffer overnight on a rotator. The amount of F108 used for this purpose (1.35 mg/mL) was selected as sufficient to cover the surface area presented by the nanoparticles in suspension, based on a specific coating density of F108 estimated to be 3.3 mg/m² [5]. F108-coated and bare hydrophobic silica nanoparticles were then incubated with nisin (0.5 mg/mL) for a desired period of time (4 h to 1 week) at room temperature. The amount of nanoparticles selected for combination with nisin (2.19 mg/mL nanoparticles) provided 1.25 times more surface area than that required to support a nisin coating of 0.15 µg/cm². Nisin adsorption was allowed to occur for 2 h. A nisin loading of 0.15 µg/cm² is consistent with monolayer adsorption (based on dimensions of nisin in solution, a monolayer of molecules adsorbed “end-on” would result in an adsorbed mass of about 0.145 µg/cm²), and earlier work with in situ ellipsometry indicated that 2 h would provide abundant time for adsorption to that level.

CD spectra of nisin-nanoparticle suspensions and control samples were recorded between 300 and 180 nm on a Jasco J-720 spectropolarimeter with a 0.2 mm path length and cylindrical cuvette at 25 °C. In each case six scans were recorded and averaged in order to increase the

signal-to-noise ratio. CD spectra of nisin-loaded nanoparticle suspensions and controls were recorded along with reference samples in each case (nanoparticles + buffer, nanoparticles + F108 + buffer, F108 + buffer, and buffer only) in order to subtract background signals and ensure the measurement of nisin structural properties only.

3. Results and discussion

3.1. Antimicrobial activity of coated polystyrene microspheres

Fig. 1 gives a comparison of the antimicrobial activity of F108-coated and uncoated microspheres after incubation in phosphate buffer at 37 °C for 1 and 4 days. These results show that the nisin activity retained by F108-coated microspheres was not significantly different from that retained by uncoated microspheres. In addition, microsphere activity did not decrease significantly over the time period tested. When nisin was incubated in the absence of microspheres, however, samples did lose activity during the period between 1 and 4 days. This may suggest some degree of stabilization against activity loss gained by nisin associated with the microspheres. This association is characterized by a high interfacial concentration of nisin consistent with its existence in multiple layers [1], and its “storage” in this form may have inhibited activity loss under solution conditions otherwise known to be unfavorable for nisin activity.

Fig. 2 gives a comparison of the antimicrobial activity of F108-coated and uncoated microspheres after incubation in 50% serum (i.e., whole serum diluted with phosphate buffer) at 37 °C for 1 and 4 days. In this case, while microsphere activity was lower than that observed in the absence of serum protein challenge (Fig. 1), nisin activity retention by F108-coated microspheres after 4 days was significantly greater than that retained by uncoated microspheres. This suggests that the protein repelling function of the pendant PEO chains of the F108 coating inhibited exchange of nisin by blood proteins. When nisin was incubated in the absence of microspheres, samples lost activity during the period between 1 and 4 days, independent of whether the buffer contained F108. This loss was observed in Fig. 1 as well, but is more substantial in the case of incubation with serum. However, nisin activity retention in microsphere-free samples was considerably greater in buffer containing F108. This may be due to a surfactant function of F108, in which its binding to hydrophobic components in the serum inhibits nisin association with such components, and in turn the loss in activity that would result from such unproductive associations.

Fig. 3 shows a comparison of antimicrobial activity between uncoated and F108-coated microspheres, after contact with nisin and incubation with equine serum at 37 °C for 7 days. While an increase in serum protein concentration reduced the activity of nisin retained on the microspheres in each case, the F108-coated microspheres clearly retained more nisin activity than the bare microspheres, when challenged by blood proteins. As above, these results indicate that the F108 coating inhibits exchange of nisin by blood serum proteins.

3.2. Antimicrobial activity of coated polyurethane catheter segments

Fig. 4 shows a comparison of the antimicrobial activity of F108-coated and uncoated catheter segments after incubation in phosphate buffer at 37 °C for 5 h, and for 1, 3, and 6 days. Note that in these tests, the area of the kill zone provides an indication of nisin activity such that large kill zone area is consistent with high activity. This is in contrast to the assay used with the microspheres (Figs. 1–3), where the number of colonies provided an indication of nisin activity such that a small number of colony forming units is consistent with high activity. Fig. 4 shows that while activity levels generally decreased with time, the nisin activity retained by F108-coated segments was significantly greater than that retained by uncoated segments after 6 days. This result may be due to an inhibition of nisin elution gained by entrapment in the

PEO chains. It is also possible that nisin may experience some structural alteration and concomitant loss of activity with prolonged surface contact, with such structure change being more pronounced at the uncoated polyurethane surface.

When the catheter segments were challenged by blood proteins, during incubation with 25% equine serum as shown in Fig. 5, a larger contrast was observed between the F108-coated and uncoated surface groups, especially with increasing incubation time. These results support the notion that the pendant PEO chains of the F108 coating inhibited the exchange of nisin by blood proteins. Both F108-coated polystyrene microspheres and F108-coated polyurethane catheter segments were generally observed to retain more nisin activity than their uncoated counterparts in the absence of blood proteins as well, but the difference in function of uncoated and F108-coated substrates was much more substantial in the presence of blood proteins.

3.3. Nisin structural characteristics

Fig. 6 shows CD spectra of nisin incubated in (nanoparticle-free) solutions containing F108 or polyethylene glycol (PEG) at selected concentrations, for 2 h and 1 week. The molecular weight of PEG (MW 6000) was selected to approximate that of a single PEO chain in the F108 triblock (MW about 5700). As shown in Fig. 6, the spectra for nisin in all groups are quite similar, implying that neither F108 nor PEG had any effect on nisin conformation. In addition, these spectra provide no evidence of structure change upon nisin incubation in solution, whether for 2 h or 1 week.

Fig. 7 shows CD spectra of nisin suspended with hydrophobic silica particles in sodium phosphate buffer (pH 7) at 25 °C, after incubation for 4 h (Fig. 7a) and 1 week (Fig. 7b). An upward shift in ellipticity between 190 and 215 nm is evident upon contact with the nanoparticles after 4 h. However, in comparison to the uncoated particle sample, nisin spectra recorded in suspension with F108-coated nanoparticles remained more similar to spectra recorded for nisin in the absence of particles. A more substantial upward shift in ellipticity between 190 and 215 nm is evident after contact with the nanoparticles for 1 week. Again, however, nisin spectra recorded in suspension with F108-coated nanoparticles were considerably less altered than those recorded for nisin in suspension with uncoated nanoparticles. Fig. 7b also shows that nisin spectra remained substantially similar for F108-coated nanoparticle suspensions and particle-free solutions beyond 220 nm, while spectra recorded for uncoated nanoparticles differed from the other two samples in this region. These results suggest that nisin experienced greater structural alteration in suspension with uncoated, hydrophobic silica than in suspension with F108-coated surfaces.

It is important to note that computer programs which use protein data sets for analyses of CD spectra such as CDSSTR [6], K2D [7] and CONTIN [8,9] are not designed for application to small proteins or polypeptides. In particular, it is difficult to fit spectra of small polypeptides having similar amounts of beta and random conformations, as these spectral contributions tend to cancel each other. If the protein has a low alpha helix content as well, the ellipticity will be dominated by the contributions of aromatic groups. While convex constraint analysis programs such as CCA+ [10] can give accurate results for small proteins, CCA+ and similar programs give secondary structure results only in the usual categories, e.g., α -helix and β -turns [11]. Nisin has a structure containing unusual amino acid residues and thioether bonds [12–14]. It is comprised of only 34 amino acid residues, and does not contain secondary structures such as helices and β -conformations. Therefore, a quantitative breakdown of nisin secondary structure is difficult to secure with any certainty. But nisin is an asymmetric bio-molecule, and conformational changes in its structure, at least in a qualitative sense, ought to be detectable using circular dichroism.

Fig. 8 shows the effects of increasing surface area on CD spectra of nisin suspended with uncoated (Fig. 8a) and F108-coated silica nanoparticles (Fig. 8b) after incubation for 4 h. An increase in particle surface area would provide more opportunity for nisin structural alteration if, at the particle concentrations used above, some nisin had remained unbound or otherwise not closely associated with the interface (e.g., residing in a diffuse outer layer). Fig. 8 shows that an upward shift in ellipticity between 190 and 215 nm is evident upon contact with a 5-fold increase in particle surface area in each case. Fig. 8 also shows that, upon a 5-fold increase in uncoated particle surface area, nisin spectra beyond 215 nm continued to deviate from that recorded at lower surface area, while nisin suspensions with high and low amounts of F108-coated particles showed substantially similar spectra beyond 215 nm. Analogous to the effects of incubation time presented in Fig. 7, these results show that relative to nisin spectra recorded in (particle-free) solution, nisin spectra recorded in suspension with F108-coated nanoparticles remained considerably less altered than those recorded for nisin in suspension with uncoated nanoparticles. These results suggest that nisin experienced greater structural alteration when adsorbed to bare, hydrophobic particles than when adsorbed to the same particles previously coated with F108.

4. Summary

The antimicrobial activity of nisin-loaded, F108-coated polystyrene microspheres and F108-coated polyurethane catheter segments was evaluated against *P. pentosaceus*. Both types of F108-coated substrates were generally observed to retain more nisin activity than uncoated control samples, and this difference in function was more pronounced in the presence of blood proteins. Circular dichroism conducted with nisin incubated with F108-coated nanoparticles suggested that nisin experienced less structural alteration than when incubated with uncoated control samples. While the mechanism of any nisin association with PEO remains undetailed, the results presented here would be consistent with enhanced conformational stability as well as enhanced resistance to elution by dissolved protein, gained as a result of nisin entrapment among immobilized, pendant PEO chains.

Acknowledgements

We are indebted to Dr. Norma J. Greenfield of the Department of Neuroscience and Cell Biology, Robert Wood Johnson Medical School (University of Medicine and Dentistry of New Jersey) for valuable assistance with interpretation of CD spectra and for providing the CCA+ program and instructions. We thank Dr. Mark Harder of the OSU Department of Biochemistry and Biophysics for assistance with CD measurement and helpful discussion. We are also grateful to Dr. Jill Parker of the OSU College of Veterinary Medicine for providing equine blood for our experiments. This work was supported in part by the National Institute of Diabetes and Digestive and Kidney Diseases (grant no. R43 DK 072560).

References

1. Tai Y-C, Joshi P, McGuire J, Neff JA. *J Colloid Interface Sci.* in press
2. Bower CK, McGuire J, Daeschel MA. *Appl Environ Microbiol* 1995;61:992. [PubMed: 7793927]
3. Rogers AM, Montville TJ. *Food Biotechnol* 1991;5:161.
4. Wolf CE, Gibbons WR. *J Appl Bacteriol* 1996;80:453. [PubMed: 8849648]
5. Neff JA, Tresco PA, Caldwell KD. *Biomaterials* 1999;20:2377. [PubMed: 10614943]
6. Manavalan P, Johnson WC Jr. *Anal Biochem* 1987;167:76. [PubMed: 3434802]
7. Böhm G, Muhr R, Jaenicke R. *Protein Eng* 1992;5:191. [PubMed: 1409538]
8. Andrade MA, Chacon P, Merolo JJ, Moran F. *Protein Eng* 1993;6:383. [PubMed: 8332596]
9. Merolo JJ, Andrade MA, Prieto A, Moran F. *Neurocomputing* 1994;6:443.
10. Perzcel A, Park K, Fasman GD. *Anal Biochem* 1992;203:83. [PubMed: 1524219]
11. Greenfield NJ. *Anal Chem* 1996;235:1.

12. Wiedemann I, Breukink E, van Kraaij C, Kuipers OP, Bierbaum G, de Kruijff B, Sahl HG. *J Biol Chem* 2001;276:1772. [PubMed: 11038353]
13. Hsu ST, Breukink E, de Kruijff B, Kaptein R, Bonvin AMJJ, van Nuland NAJ. *Biochemistry* 2002;41:7670. [PubMed: 12056898]
14. van Heusden HE, de Kruijff B, Breukink E. *Biochemistry* 2002;41:12171. [PubMed: 12356318]

Fig. 1. Antimicrobial activity of F108-coated and uncoated polystyrene microspheres contacted with nisin and incubated at 37 °C with 10 mM sodium phosphate buffer, pH 7.

Fig. 2. Antimicrobial activity of F108-coated and uncoated polystyrene microspheres contacted with nisin and incubated at 37 °C with 50% equine serum.

Fig. 3. Antimicrobial activity of F108-coated and uncoated polystyrene microspheres contacted with nisin and incubated at 37 °C with equine serum for 7 days.

Fig. 4. Antimicrobial activity of F108-coated and uncoated polyurethane catheter segments contacted with nisin and incubated at 37 °C with 10 mM sodium phosphate buffer, pH 7.

Fig. 5. Antimicrobial activity of F108-coated and uncoated polyurethane catheter segments contacted with nisin and incubated at 37 °C with 25% equine serum.

Fig. 6.

CD spectra of nisin incubated in nanoparticle-free solutions for 2 h and 1 week. Nisin concentration was 0.5 mg/mL. “1×” refers to a PEG concentration of 0.85 mg/mL or a F108 concentration of 2.08 mg/mL; “5×” refers to a PEG concentration of 4.25 mg/mL or a F108 concentration of 10.4 mg/mL.

Fig. 7. CD spectra of nisin contacted with F108-coated and uncoated hydrophobic silica nanoparticles and incubated in 10 mM sodium phosphate buffer (pH 7) at 25 °C, for (a) 4 h; and (b) 1 week. Nisin concentration was 0.5 mg/mL.

Fig. 8. CD spectra of nisin contacted with (a) uncoated hydrophobic silica nanoparticles; and (b) F108-coated silica nanoparticles. Incubation time was 4 h in each case. Nisin concentration was 0.5 mg/mL. “1×” refers to a nanoparticle concentration of 2.19 mg/mL and F108 concentration (b) of 1.35 mg/mL; “5×” refers to a nanoparticle concentration of 11.0 mg/mL and F108 concentration (b) of 6.75 mg/mL.