Biomolecule-based antibacterial coating on a stainless steel surface: multilayer film build-up optimization and stability study

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The goal of this paper was to establish the durability profile of antibacterial multilayer thin films under storage and usage conditions. Thin films were built on stainless steel (SS) by means of a layer-by-layer process alternating a negatively charged polyelectrolyte, polyacrylic acid, with a cationic antibacterial peptide, nisin. SS coupons coated with the antibacterial film were challenged under environmental and usage conditions likely to be encountered in real-world applications. The change in antibacterial activity elicited by the challenge was used as an indicator of multilayer film resistance. Antibacterial SS samples could be stored for several weeks at 4°C in ambient air and antibacterial films were resistant to dipping and mild wiping in water and neutral detergent. The multilayer coating showed some weaknesses, however, that need to be addressed.

Keywords: polyelectrolyte multilayer films; polyelectrolyte complexes; antibacterial peptides; stainless steel; durability; stability

Introduction

Attachment and growth of unwanted bacteria as sessile communities (biofilms) on surfaces contribute to the dispersal of pathogenic and non-pathogenic microorganisms. The detrimental consequences are extremely diverse, eg spread of infectious diseases, implant-related infections causing health problems in hospitals, consumer product spoilage in manufacturing, and reduced efficiency and production and maintenance problems in industry (Hall-Stoodley et al. 2004). One approach to preventing biofilm growth is to create antibacterial surface coatings (Tiller et al. 2001; Klibanov 2007; Vasilev et al. 2009).

Layer-by-layer (LbL) assembly of polyelectrolyte films is a widely used technology (Ariga et al. 2007; Schlenoff 2009) based on a simple, inexpensive, environmentally friendly process that can easily be scaled up provided the film contains few layers. Major research groups acknowledge multilayer film technology as a promising approach to creating multifunctional coatings. In particular, it appears to be a good way to produce antibacterial coatings (Lichter et al. 2009), notably by incorporating either reservoirs of silver ions (Malcher et al. 2008), or antibacterial proteins (Rudra et al. 2006) or peptides (Etienne et al. 2004; Guyomard et al. 2008; Vreuls et al. 2010). Moreover, biomolecules preventing bacterial adhesion can be incorporated into LbL-assembled films (Boulmedais et al. 2004; Vreuls et al. 2010), and it is possible to alter the interactions of cells or bacteria with the surface by loading the multilayer thin films with pharmaceuticals (Vodouhe et al. 2006) or by controlling the stiffness and compliance of the films (Thompson et al. 2006; Lichter et al. 2008). Yet few published studies have focused on the stability of multilayered films functionalized with biomolecules under usage conditions close to those prevailing in real-world applications.

Intrinsically, films made with water-soluble polyelectrolytes are expected to be fragile when used in an aqueous environment. This is an advantage when the aim is quick delivery of an active ingredient, but it can be a problem when persistence of longer-term activity is intended (Schlenoff 2009). Su and Li (2008) describe highly stable gold nanoparticle/lysozyme multilayer thin films resistant to immersion in 0.1 M HCl, NaOH, and anionic surfactant solutions. There are reports of chemical (Schneider et al. 2007; Nuraje et al. 2011) or thermal crosslinking (Dai et al. 2005), temperature curing, or even covalent grafting to confer durability to
multilayer thin films. Chemical crosslinking of polyelectrolyte multilayer films built from chitosan and hyaluronic acid is reported both to impart greater stability and to improve the resistance of antibacterial activity to immersion in phosphate buffer (Chua et al. 2008). Covalent linking of antibacterial biomolecules to the polyelectrolyte is another means of conferring longer-lasting antibacterial activity to the films (Chollet et al. 2007; Humblot et al. 2009).

Vreuls et al. (2010) reported the durability of multilayered films loaded with the antimicrobial peptide nisin, when subjected to mild cleaning. Five [PAA-nisin]-bilayer coatings built by LbL were shown to remain antibacterial after immersion in water or in a neutral nonionic household cleaner and after wiping with a water-soaked sponge. The antibacterial effect was lost, however, after scrubbing with detergent.

In the present study, the conditions of LbL film build-up on stainless steel (SS) were optimized before studying the resistance of antibacterial multilayer coatings under usage and storage conditions.

Materials and methods

Nutrient media

Bacto™Tryptone, Bacto™Yeast Extract, Tryptic Soy Agar (TSA) and Tryptic Soy Broth (TSB) were obtained from Becton Dickinson (USA). Agar-agar was purchased from Merck (USA). Luria-Bertani medium (LB) was prepared with 10 g of Bacto™ Tryptone, 5 g of Bacto™ Yeast Extract, and 10 g of NaCl l⁻¹ of deionized (DI) water. LB agar (LBA) medium was obtained by adding 15 g of agar-agar l⁻¹ of LB.

\[ P(DOPA)-co-P(DMAEMA^+) \] copolymer

Polyelectrolyte \( P(DOPA)-co-P(DMAEMA^+) \) copolymers were synthesized by one-step radical polymerization of 2-methacryloyloxyethyltrimethylammonium chloride (DMAEMA^+) and N-methacryloyl 3,4-dihydroxy-L-phenylalanine methyl ester monomers (Charlot et al. 2009), provided by the Center for Education and Research on Macromolecules (CERM), University of Liège (Belgium).

Polyelectrolytes

Polyallylamine hydrochloride (PAH, \( M_w = 50–60,000 \) g mol⁻¹, ref. 28, 322–3), polyethylene imine (PEI, \( M_w \approx 25,000 \) by LS, average \( M_n \approx 10,000 \) by GPC, branched, ref. 40,872–7), poly(sodium 4-styrene sulfonate (PSS, \( M_w \approx 70,000 \), ref. 243051), and polyacrylic acid (\( M_w = 1800 \), ref. 323667) were obtained from Sigma-Aldrich.

Antimicrobial peptide nisin

Nisin (Nisaplin®), provided by Danisco for research purposes, was used as received without further purification. The commercial sample contains 2.5% nisin, 77.5% NaCl, 12% milk proteins, 6% carbohydrates, and 2% moisture. The peptide has a pH > 8.5.

SS surface and cleaning procedure

304 2B SS foils supplied by the CRM Group AC&CS (Belgium) were cut to obtain \( 2 \times 2 \) cm² coupons. After removal of the protective film, the surfaces were cleaned by rubbing in acetone, then in ethanol, and dried under a gentle flush of N₂. The SS used in this study had a roughness of 0.8 μm.

Assembly of functionalized thin multilayered films

An LbL deposition process was used to assemble polyelectrolyte multilayers on clean SS coupons by hand dipping at room temperature (21°C ± 2°C). SS coupons were dipped into the P(DOPA)-co-P(DMAEMA^+) solution (1 g l⁻¹ in DI water) for 10 min and then rinsed by dipping successively in two different DI water baths for 1 min.

PAH and PSS polyelectrolyte solutions were prepared at 2 g l⁻¹ in 150 mM NaCl. The dipping time for each was 2 min, followed by two 1 min rinses with 150 mM NaCl. The cycle was repeated as necessary to obtain five bilayers of pre-coating. The PAH layer adjoining the functional multilayer film was further rinsed in two different DI water baths for 1 min.

Polyacrylic acid (PAA) and nisin solutions were prepared at 2 g l⁻¹ in DI water adjusted to the adequate pH with 0.1 M HCl or 0.1 M NaOH.

Five bilayers films made of PAA and nisin polyelectrolytes (PAA-nisin) were assembled by successive cycles of immersion of the SS coupons, coated or not with precursor layers, first in the solution of negatively charged PAA polyelectrolyte and then in the solution of positively charged nisin. After each immersion, two 1 min rinses were performed with DI water at the same pH as the immersion solution. The cycle was repeated as necessary to obtain the five bilayers.

PAH and PSS polyelectrolyte solutions were prepared at 2 g l⁻¹ in 150 mM NaCl. PAA and nisin solutions were prepared at 2 g l⁻¹ in DI water adjusted to pH 4.5. A suspension of 0.1 g l⁻¹ Laponite RD (Rockwood Additives Ltd, UK) in DI water was prepared by continuous and vigorous stirring of the dispersion during clay addition, after which the dispersion was left overnight on a magnetic stirring plate.

These different solutions were applied in the following order: for each of the five precursor layers:
In vitro antibacterial test

The Japanese Industrial Standard JIS Z 2801 (identical to ISO 12296:2007) was used to measure the antibacterial efficacy of multilayer films against the Gram + bacterium *Bacillus subtilis* 168. The test involves quantifying the survival of one Gram + and/or one Gram − bacterial strain having been held in contact for 24 h at 35°C with the surface covered with the antibacterial agent or coating. The contact time and temperature can be varied.

A preculture of test bacteria (incubated at 37°C overnight in 3 ml of LB) was used to seed a fresh culture into 50 ml of LB. The bacterial concentration of the test inoculum was adjusted to about 2–4 × 10⁶ cells ml⁻¹. SS coupons (2 × 2 cm²) were placed in Petri dishes containing damp blotting paper and 200 µl of the test inoculum were pipetted onto each substratum. The inoculum was covered with a piece of polyethylene film cut from a sterile Stomacher bag (ref. B40542, Fisher Scientific) to ensure spreading of the inoculum onto the substratum and to avoid drop evaporation. The Petri dishes containing the inoculated coupons were incubated at 37°C for 24 h.

The SS substrata were placed, inoculated side downward, in glass jars (ø5 cm) containing 20 ml of 500-fold-diluted LB and about 20 g of 4-mm glass beads. The jars were shaken horizontally for 10 min and then their content was sonicated in a water bath (50–60 KHz) for 2 min. Viable bacteria were counted by plating 10-fold dilutions on LB agar and incubating the plates at 37°C overnight.

The antibacterial activity was measured by comparing the survival of bacteria on coated SS coupons with their survival on uncoated surfaces. The results are expressed as 'log₁₀ reduction' vs controls. The Japanese Industrial Standard suggests that this value should not be <2 for a coating to be considered antibacterial. At least three results were collected for each tested coating.

In vitro antibacterial suspension test

An overnight preculture of *B. subtilis* 168 was diluted to OD₆₀₀ = 0.6 in 3 g l⁻¹ of Bacto™ Tryptone and 1.5 g l⁻¹ of Bacto™ Yeast Extract in DI water. Ten microliters of this bacterial suspension were inoculated into a 100 µl Laponite RD suspension (1 or 0.1 g l⁻¹ in DI water) with 10, 25, 50, or 100 µl of a solution of nisin at 2 g l⁻¹ solution in DI water. The final assay volume was made up to 1 ml with DI water containing 3 g l⁻¹ of Bacto™ Tryptone and 1.5 g l⁻¹ of Bacto™ Yeast Extract. After shaking for 18 h at 37°C, the number of surviving colony-forming units (CFUs) was estimated by serial dilution, spreading on LBA, and colony counting after overnight incubation at 37°C.

Drip flow biofilm reactor

A drip flow reactor (DFR) made of SS was used to assess biofilm formation on coated and uncoated SS samples. The three chambers of the reactor (6 cm long, 3.6 cm wide and 2.5 cm deep) were fitted with removable polycarbonate plastic lids fixed with thumb-screws. Before each experiment, the reactor was wrapped in aluminum foil and autoclaved. Then, in a biological hood, sterile rubber tubes were attached to the inlet and effluent ports of the sterilized reactor.

With the reactor placed horizontally, each chamber holding two 2 × 2 cm² SS coupons was inoculated with 10 ml of *B. subtilis* 168 overnight culture diluted to 3 to 5 × 10⁴ CFU ml⁻¹ in 300 mg l⁻¹ of TSB. Bacteria were allowed to settle onto the samples for 15 min, then the peristaltic pump was started to deliver a continuous flow of 30 mg l⁻¹ of TSB at the rate of 6 ml min⁻¹ for 30 min. During the continuous flow period the reactor was placed on a stand holding the device inclined at an angle of 40° with respect to the horizontal. The experiment involved two inoculation/continuous flow steps followed by a 4 h rest period (no flow) and then by two inoculation/continuous flow steps and a 16 h rest period. This protocol was followed on three consecutive days. The whole experiment was carried out at room temperature (23°C ± 2°C). On day 4, the SS samples were rinsed with 30 mg l⁻¹ of TSB at the rate of 6 ml min⁻¹ for 30 min before collection for biofilm quantification. The biofilm-covered side of each coupon was scraped with a sterile wooden stick for approximately 15 s. The scraper was then rinsed by stirring in a glass jar (ø5 cm) containing 10 ml of 30 mg l⁻¹ TSB and about 20 g of 4-mm glass beads. The scraping and rinsing processes were repeated eight times to ensure full coverage of the coupon surface. The scraped biofilms were disaggregated and homogenized by sonication in a water bath (50–60 KHz) for 2 min and shaking the jars horizontally for 10 min. Viable cultivable bacteria were counted by plating 10-fold dilutions on TSA agar and incubating the plates at 37°C.
SS coated by plasma deposition with a 100 nm-thick coating of AgOx was used as a positive reference for antibacterial activity, and uncoated SS as a control.

Quartz crystal microbalance (QCM) monitoring of functionalized film assembly

Construction of polyelectrolyte films was monitored in situ with a quartz crystal microbalance (QCM, Q-Sense AB, Göteborg, Sweden). This instrument measures, as a function of time, shifts in resonance frequency (Δf, Hz) of a quartz crystal triggered by a mass change under flow. The flow rate of the polymer and antibacterial peptide solutions in the flow module was set at 100 μlm in 1 and the temperature stabilized at 24°C. SS-coated 5-MHz sensor crystals (QSX 304, Q-Sense) were used to assess (a) the deposition of an adherence layer made of PEI (8 g l⁻¹ in 150 mM NaCl) and (b) the adsorption of PAA and nisin involved in build-up of the antibacterial multilayered film, after stabilization of the signal under DI water flow. Before each PAA or nisin layer addition, excess material was rinsed off with DI water. The results are reported as negative frequency changes of the 11th overtone. The resonance frequency of the crystal in contact with water was used as reference value, so that ΔF was equal to 0 prior to deposition of the adherence layer.

PAA-nisin complex formation in solution as a function of pH and ionic strength

Blends of PAA and nisin were prepared in DI water or in 150 mM NaCl. Because of the intrinsic salt content of the nisin-containing material, the solution of peptide in DI water contained 0.013 mol l⁻¹ NaCl. Solutions of the polyanion PAA (2 g l⁻¹ in DI water) and of the polycation nisin (2 g l⁻¹ in DI water) were mixed at 1:1 volume ratio, at 21°C ± 2°C. Aggregation of the peptide with the polyanion, as a function of pH and ionic strength, was monitored by measuring the OD at 340 nm. The pH of the PAA/nisin mixture solution was modified under stirring with m volumes of 0.1 M HCl or NaOH to scan a pH range from 2.7 to 10.

Durability of antibacterial multilayered polyelectrolyte films

The strength of the P(DOPA)-co-P(DMAEMA⁺)-(PSS-PAH)₅-(PAA-nisin)₅ films was evaluated by subjecting coated SS samples to the following environmental and usage conditions: (a) variable storage periods in dry or humid closed containers or in ambient air, at 4, 25, and 60°C; (b) alternation of humid and dry storage periods; (c) a 1-week storage period in sunlight behind a glass; (d) a 2 h immersion in either DI water or 1% non-ionic household cleaner in DI water or 0.1 M HCl or 0.1 M NaOH; and (e) wiping with a sponge dipped in a neutral-pH, fatty-alcohol-ethoxylate-based household cleaner (1% in water) or in tap water. Antibacterial activity was measured according to JIS Z 2801 before and after each challenge (at least three replicates).

Results

Multilayer film construction: premises

In a first experiment, one-half of ~50 SS coupons coated with P(DOPA)-co-P(DMAEMA⁺)-(PAA-nisin)₅ showed antibacterial activity (>2 log₁₀ bacterial population reduction vs uncoated control). The control (PAA-PAH)₅ showed the same bacterial population as the uncoated control.

(PAA-nisin)₅ film construction: QCM study

Figure 1 shows the evolution of the crystal resonance frequency as a function of the number of layers deposited (PAA and nisin layers, starting with a PEI adherence layer). When PAA was used at pH 3 in NaCl and nisin at pH 6 in phosphate buffer containing NaCl (Vreuls et al. 2010), the frequency increased with layer number for the first two bilayers, indicating gradual polyelectrolyte deposition and film build-up on the SS-coated crystal. Then the frequency curve tended to dampen for the three remaining bilayers, indicating that film build-up leveled off.

![Figure 1. QCM measurements of the successive adsorption of PAA and nisin layers onto a coated sensor made of 304 SS. The shift in resonance frequency (overtone n = 11) is plotted as a function of layer number. Five PAA-nisin bilayers (PAA and nisin at 2 g l⁻¹) are recorded under four sets of conditions. □ = PAA in 150 mM NaCl (pH not adjusted, measured pH = 3), nisin in 0.1 M phosphate buffer pH 6, 150 mM NaCl; ▲ = PAA and nisin in DI water, pH 4.5; ● = PAA and nisin in DI water, pH 3.5; • = PAA and nisin in DI water, pH 6.](image-url)
Improvement of the PAA-nisin multilayer assembly conditions

Correlation between PAA-nisin complex formation in solution and film build-up

In an initial screening study, the aggregation of nisin with PAA was estimated under various conditions by measuring the OD_{340} due to light scattering. In this experiment, the pH of the PAA and nisin solutions was varied, and the solutions were prepared in DI water or in 150 mM NaCl. Figure 2 shows that in the absence of added salt, aggregation was maximal between pH 3.3 and 3.8. Almost no aggregation occurred when the polyelectrolytes were prepared in 150 mM NaCl.

Film construction: QCM study

On the basis of the above screening study (Figure 2), a QCM study of film assembly was conducted. Deposition was monitored under conditions of good complex formation, at three different pH values in DI water. Figure 1 shows the evolution of the crystal resonance frequency measured with a QCM during assembly of the five PAA-nisin bilayers as a function of layer number and composition, with PAA and nisin aqueous solutions adjusted to pH 3.5, 4.5, and 6. Before each PAA or nisin layer addition, excess material was rinsed off with DI water. The frequency increased as the layer number increased, indicating sequential deposition of the anionic polyelectrolyte and cationic antibacterial peptide and regular build-up and linear growth of the PAA-nisin films at the three studied pH values. From the second PAA-nisin bilayer onward, the slope of the frequency versus layer number was steeper at pH 4.5 than at pH 3.5 or at pH 6, indicating that the amount of material deposited on the crystal was greater. This trend was confirmed by the $-\Delta F$ values corresponding to nisin layer deposition, measured at pH 3.5, 4.5, and 6 and listed in Table 2: $-\Delta F$ was greater at pH 4.5, throughout deposition of the five peptide layers. The use of PAA and nisin solutions prepared at pH 4.5 in DI water thus emerged as optimal for PAA-nisin bilayer assembly.

Antibacterial activity

The antibacterial activity of ~50 coated coupons assessed against *B. subtilis* according to JIS Z 2801 (Table 1, line 2) shows that building the PAA-nisin films at pH 4.5 in DI water increased the proportion of samples demonstrating antibacterial activity ($\geq 2 \log_{10}$ of reduction of the bacterial population vs the uncoated control) and reduced the standard deviation (SD) of the measurement set as compared to film assembly under the conditions tested previously (Table 1, line 1).

Influence of precursor layers on multilayer film assembly reproducibility

The antibacterial activity of ~40 coupons prepared by depositing five PAA-nisin bilayers onto a precursor coating made of five (PSS-PAH) bilayers was measured against *B. subtilis* according to JIS Z 2801. Table 1, line 3 shows that depositing the antibacterial multilayer film on a stack of precursor layers led to the highest proportion of antibacterial samples and the smallest SD, indicating further improvement in the reproducibility of film construction.

Durability/stability of the antibacterial multilayered polyelectrolyte films

Test runs were carried out to study the stability/durability of the antibacterial multilayered films assembled on SS under the best process conditions determined above, viz. 5 (PSS-PAH) precursor bilayers, PAA and nisin dissolved in DI water at pH 4.5. The film-coated SS coupons were subjected to various storage conditions and durability challenges relevant to post-manufacturing and final usage conditions. The JIS Z 2801 test was used to measure the antibacterial activity of the samples before and after each challenge (Table 3). Coated SS could be stored in ambient air at 4°C for 2 months or at room temperature for 1 month without losing antibacterial activity. Storage in closed containers with humidity proved detrimental to activity retention, probably because of water condensation and stagnation on the film. In the absence of excess humidity, samples were successfully stored for at least 2 weeks at room temperature or in the refrigerator, but the antibacterial activity was lost...
after 24 h in an oven at 60°C. Coated surfaces exposed to sunlight or subjected to alternating humid/dry periods for 1 week at room temperature did not retain their antibacterial activity.

The bactericidal properties of the coatings remained unchanged after dipping in water or detergent, but the films did not retain their activity after immersion in dilute acid or base.

Brief wiping of the multilayer films had no detrimental effect on the antibacterial activity, but when a film was subjected to more than 10 back-and-forth sponge strokes; it lost its antibacterial activity. These preliminary results suggest that one of the weaknesses of multilayer films is their poor resistance to rubbing.

Drip-flow biofilm reactor test

SS coupons precoated and coated under optimal conditions as described above were also challenged in a drip flow reactor (DFR). The DFR protocol implemented here involved repeated 3-day-long batch inoculations interrupted by intervals of continuous nutrient flow and surface drying, simulating situations where the coated surface is alternately and repeatedly soiled, wetted/sprayed, or not in use. The anti-biofilm properties of multilayer antibacterial films were assessed by measuring biofilm accumulation on coated as compared to uncoated SS coupons. SS coated with silver oxide was used as a reference antibacterial surface.

Viable plate counts obtained after biofilm harvesting from the coupons (Table 4) showed that the optimally produced five-(PAA-nisin) bilayer film failed to confer any anti-biofilm properties to SS. This may be due to a lack of resistance of the antibacterial film to the flow, to a weakness of the antibacterial peptide towards repeated microbial insults, or to leaching of nisin out of the film.

A hybrid antibacterial multilayer film

The results (Table 3) demonstrate a weakness of PAA-nisin multilayer films, ie their lack of resistance to abrasion, observable notably in case of rubbing with a sponge. As hybrid assemblies of organic polyelectrolytes with inorganic particles such as clay platelets commonly exhibit better mechanical resistance (Srivastava and Kotov 2008; Podsiadlo et al. 2009), the authors sought to improve the resistance of the films to the flow, to a weakness of the antibacterial peptide towards repeated microbial insults, or to leaching of nisin out of the film.

| Multilayer film assembly parameters | Total samples n<sub>tot</sub> | Antibacterial samples n<sub>AB</sub> (≥2 Log<sub>10</sub> bact. pop. red. vs control) | Ratio n<sub>tot</sub>/n<sub>AB</sub> | Log<sub>10</sub> bact. pop. red. vs control | ± SD |
|-----------------------------------|-----------------------------|-----------------------------------------------|----------------------------|-----------------------------------------|------|
| Line 1 (1) Adherence layer + 5 (PAA-nisin) bilayers PAA in 150 mM NaCl, pH 3 nisin in PO₄ buffer with 150 mM NaCl, pH 6 | 49 | 25 | 1.96 | 3.82 ± 4.0 |
| Line 2 PAA and nisin in DI water pH 4.5 (2) Adherence layer + 5 precursor bilayers + 5 (PAA-nisin) bilayers | 46 | 37 | 1.24 | 5.47 ± 3.26 |
| Line 3 PAA and nisin in DI water pH 4.5 | 37 | 33 | 1.12 | 5.63 ± 2.83 |

Note: The antibacterial activity was measured by the JIS Z 2801 test.

Table 2. ∆f (Hz) corresponding to nisin layer deposition at pH 3.5, 4.5, and 6 (without NaCl) throughout the deposition of five peptide layers.

| Nisin layer n | −∆f (Hz) at pH 3.5 | −∆f (Hz) at pH 4.5 | −∆f (Hz) at pH 6 |
|---------------|-------------------|-------------------|-----------------|
| 1             | 22                | 34                | 31              |
| 2             | 20                | 66                | 39              |
| 3             | 35                | 62                | 35              |
| 4             | 39                | 54                | 33              |
| 5             | 38                | 60                | 36              |

Note: The JIS Z 2801 test was used to measure the antibacterial activity before (START) and after the durability challenge. Coatings are rated “antibacterial” if they delivered at least 2 Log<sub>10</sub> reduction vs the control.
were defined step by step, identifying first the best polyelectrolyte pair complexation conditions in solution and then confirming proper film assembly on the QCM. (Supplementary data Figures 1 and 2 [Supplementary material is available via a multimedia link on the online article webpage].) The antibacterial activity of a set of SS coupons coated with the clay-containing film was then compared with that of a set of coupons coated with the optimized clay-free film (Table 5). In this experiment, the clay-containing film displayed no antibacterial activity against *B. subtilis* 168. A QCM study (Supplementary Data Figure 2) [Supplementary material is available via a multimedia link on the online article webpage] showed that this film did build up properly. This suggests that in the presence of clay, nisin might not diffuse out of the film or/and might lose its antibacterial activity in contact with the clay.

Table 6 shows the results of an experiment where the antibacterial activity of nisin in solution, at various concentrations, was measured against a suspension of Gram+ bacteria in the presence of various concentrations of clay platelets. Surviving bacteria were counted after incubation of the suspensions for 18 h at 37°C. At the highest Laponite concentration tested, nisin, whatever its concentration showed no antibacterial activity.
although it retained its antibacterial activity when the ratio of nisin to clay was higher. These results indicate that there could be a range of values of the clay-to-nisin ratio at which the peptide still exerts its antibacterial activity. A similar test was carried out at the nisin/clay ratio of the Laponite-containing film by building both this film and the clay-free film onto SS, scraping the films from the coupons and measuring the antibacterial activities of collected films against a *B. subtilis* suspension (see Methods).

Table 4. Viable plate counts obtained after biofilm harvesting from P(DOPA)-co-(DMAEMA +)-(PSS-PAH)₅-(PAA-nisin)₅ film coated SS subjected to low fluid shear in a drip flow reactor.

| Samples                        | Harvested biofilm Log₁₀ CFUs 4 cm⁻² |
|--------------------------------|-------------------------------------|
| Uncoated SS                    | Sample 1: 5.4 Sample 2: 5.2          |
| Coated SS adherence layer      | Sample 1: 5.6 Sample 2: 5.0          |
| (PSS-PAH)₅-(PAA-nisin)₅        |                                     |
| Plasma-coated SS 100 nm AgOₓ   | No CFU No CFU                         |

Table 5. Antibacterial activity of a set of PAA-nisin-coated SS coupons prepared with or without Laponite clay platelets in the precursor layers and/or the antibacterial layers.

| Laponite clay | In precursor layers | In antibacterial film | Reduction of bacterial population vs control (Log₁₀) Av (n = 3) ± SD |
|---------------|---------------------|-----------------------|---------------------------------------------------------------------|
| No            | No                  | 7.9 ± 0               |
| Yes           | No                  | 7.9 ± 0               |
| Yes           | Yes                 | 0.9 ± 0               |

Note: The antibacterial activity was measured by the JIS Z 2801 test.

Table 6. Antibacterial activity of nisin against a suspension of *B. subtilis*, in the presence of various concentrations of Laponite.

| CFU ml⁻¹ | Nisin (2 g l⁻¹ in phosphate buffer pH 6) | Volume (µl) |
|----------|------------------------------------------|-------------|
|          |                                          |             |
| Laponite RD (g l⁻¹ in DI water) | 100 | 50 | 25 | 10 |
| 1        | 3.4 × 10⁷ | 3.4 × 10⁷ | 3.4 × 10⁷ | 3.4 × 10⁷ |
| 0.1      | 0       | 0       | 0       | 4.7 × 10⁷ |
| 0        | 0       | 0       | 0       | 0       |

Table 7. Antibacterial activity against a suspension of *B. subtilis* of a scraped multilayer coating containing Laponite and control (without Laponite).

| Coating scraped from SS coupons: | Log₁₀ CFUs |
|----------------------------------|------------|
| (PSS/PAH)₅-(PAA-nisin)₅         | No CFU     |
| (PSS/PAH/Clay)₃-(PAA/nisin/PAA/PAH)₅ | 9.2       |
| Uncoated SS                     | 9.2        |

Discussion

Polyelectrolyte film build-up can be predicted from the ability of the polyanion and polycation to form a complex (Laugel et al. 2006; Sukhishvili et al. 2006; Mjahed et al. 2010). Vreuls et al. (2010) reported preliminary results on the durability of nisin-loaded multilayer films built on SS surfaces and subjected to cleaning operations. (PAA-nisin)₅ coatings remained antibacterial after immersion in water or in a neutral nonionic household cleaner and after wiping with a sponge dipped in water, but their antibacterial effect was lost when they were scrubbed with detergent. Poor resistance to rubbing thus emerged from these initial results as a weakness of such films.

The goal of the present paper was to obtain a more detailed strengths vs weaknesses profile of PAA-nisin antibacterial multilayered films and to investigate several strategies for enhancing the structural stability of these coatings and preserving their functional benefits.

First the conditions for film construction were improved by optimizing the pH and ionic force of the polyelectrolyte-containing medium. Working at pH 4.5 in water allowed linear growth of the film and improved the reproducibility of the deposition process. Deposition of a five (PSS-PAH) bilayer precursor further improved the reproducibility of film construction.

In experiments where sets of SS surfaces coated under optimal conditions with multilayered films were subjected to storage conditions and durability challenges, it was demonstrated that such antibacterial SS samples were ideally stored at 4°C in ambient air, and that they were resistant to dipping in water/detergent and mild wiping with a sponge. Yet the coating showed weaknesses: it did not resist sunlight, humidity, heat, treatment with an acid or base, fluid shear, or tougher mechanical stress.

In recent years, Podsiadlo et al. (2005, 2008) conducted research on the preparation of strong functional hybrid materials by LbL assembly of polyelectrolytes and clay platelets. Build-up of a base made of at least three precursor bilayers is reported to eliminate the influence of the substratum, to improve
the organization of the multilayer and its anchoring, and to lead to a more homogeneous final coating (Yoo 1998; Etienne 2005). The present attempt to improve the mechanical resistance of the coating by inserting Laponite platelets into the (PAA-nisin) multilayer films was unsuccessful, because the antibacterial peptide in the hybrid multilayer film lost its activity.

In the current state of this research, the results suggest that LbL films deposited from aqueous solutions of polyelectrolytes should be stored and used in protected environments. Overall, the results reported here confirm Schlenoff’s assertion (2009) that polyelectrolyte multilayer film technology may be more appropriate where quick active ingredient delivery is intended rather than where lasting activity is required. Chemical polyelectrolyte cross-linking combined with covalent grafting of the antibacterial biomolecules is currently being investigated to enhance the structural stability of multilayer coatings and to preserve their functional benefits.

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