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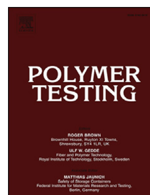

Material Properties

In vitro and *in vivo* evaluation of the antibacterial properties of a nisin-grafted hydrated mucin multilayer film

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ABSTRACT

Microbial infection is one of the most serious problems in the field of medical devices, particularly in implants. Herein, we have designed and constructed a (mucin/poly(ethyleneimine))$_n$ ((mucin/PEI)$_n$) multilayer film using a layer-by-layer self-assembly method and the grafting of antimicrobial peptides to enhance the bactericidal efficacy. Water contact angle measurements and atomic force microscopy images revealed that the hydrated multilayer film created a highly hydrophilic surface with a low roughness. The functionalized polydimethylsiloxane (PDMS) surfaces were shown to be effective in preventing bovine serum albumin (BSA) adsorption and in reducing bacterial adhesion. Bactericidal activity of the (mucin/PEI)$_n$-nisin coatings, measured by scanning electron microscopy and a LIVE/DEAD bacterial viability kit, was remarkably effective against *S. aureus* owing to the grafting of nisin. *In vivo* subcutaneous incisions were made in New Zealand white rabbits and were implanted with multilayer-film-modified and uncoated PDMS. Both the evaluation of the appearance of the wound and the histopathology analysis demonstrated that implantation of the antibacterial-coating-modified PDMS promoted wound healing and showed an anti-inflammatory effect. The multilayer film proved to be nontoxic towards human lens epithelial cells, which can potentially be widely used to modify biomedical implants.
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1. Introduction

Bacterial infection as an arising medical and public concern has led to significant mortality and morbidity worldwide [1–3]. Approximately more than half of all nosocomial infections are attributed to implant-associated infections, leading to the death of at least 1 million persons per year in the USA [4–6]. The adhesion of bacteria on the surface of the implants is the first and key step for the formation of bacterial colonization and a biofilm, which is a big threat to the long-term success of the implants [7–9]. The adhesion and proliferation of bacteria on various artificial surfaces affects the functionality of these specific interfaces. Bacteria will attach to various surfaces and subsequently multiply to form dense aggregations or biofilms with thicknesses ranging from a few micrometers to half a meter [10,11]. To overcome the problems caused by bacterial growth on these surfaces, various antibacterial coatings have been developed [12–15]. In general, antibacterial surfaces should be able to kill (bactericidal) or prevent the adhesion of bacteria (antiadhesive). However, antiadhesive surfaces do not possess long-term stability, owing to the degradation and detachment of the coating before the formation periimplant tissue. A contact-killing surface with desirable bactericidal activity will always be contaminated by the remaining dead bacteria, which may trigger immune responses or inflammation [3,16–18]. As a result, there is an urgent need to build a biologically functional coating that can both reduce adhesion of bacteria and have strong bactericidal properties.

The layer-by-layer (LBL) technique, which was first introduced by Decher et al., in 1992 [19], was first widely applied to charged polymers, whose deposition on surfaces is largely controlled by charge compensation mechanisms. This versatile method does not

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require any elaborate instrumentation and is independent of substrate shape, which makes it promising for the fabrication of thin films with integrated functions. Numerous components such as linear polymers, biomacromolecules, nanoparticles, block copolymers, metal ions, and micelles have been successfully employed to fabricate multilayer films using the LBL self-assembly method [20,21]. The method has recently been reviewed by Loh et al. LBL assemblies have widely been used for antibacterial applications [14]. On the one hand, bactericidal LBL assemblies that incorporate various biocides including heavy metals, antibiotics, cationic molecules, antimicrobial peptides, and enzymes are able to kill surrounding or contacted bacteria. On the other hand, bacterial adhesion resistant LBL films have been fabricated to adjust the substrate surface properties such as the surface free energy (or wettability), roughness, and surface charge, which may affect the adhesion of bacteria.

The interest in mucins and mucin coatings stems from their biological importance. Mucins are high molecular weight (200 kDa–200 MDa) glycoproteins that form the mucus gel on epithelial cell surfaces [20]. The mucus gel is a barrier against pathogens and lubricates the underlying cell surface. Adsorption of mucins has been found to lubricate surfaces, cause significant changes in contact angle, and repel the adhesion of microorganism and mammalian cells [22–24]. Because they are negatively charged, they can be paired with positively charged polymers for LBL assembly [20,23,25]. Various complementary polymer pairs such as positively charged chitosan, lactoperoxidase, and sugar-binding lectin wheat germ agglutinin have been used previously to form LBL multilayer films with bovine submaxillary mucin (BSM) and porcine gastric mucin (PGM). Poly(ethyleneimine) (PEI) has a hydrophilic backbone owing to its heteroatomic backbone, which is a hyper–branched polymer that contains primary-amine-functionalized end groups [26–28]. Because antimicrobial peptides (AMPs) are potentially useful for the treatment of multidrug-resistant infections, more attention has been paid to the structural modification and structure–function relationship of both naturally occurring and synthetic AMPs [29–31]. Nisin, a natural antimicrobial peptide produced by strains of *Lactococcuslactis subsp. Lactis*, was selected as a model peptide because of its high activity against a broad range of Gram-positive bacteria, its wide use as a food preservative, its lack of toxicity to humans, and its stability when used in coatings [32,33]. However, the immobilization of antimicrobial peptides on the biomaterials surface is not trivial owing to the absence of functional groups, and the final antibacterial properties are related to the functional surface group density. Hence, the deposition of thin films presenting different functionalities (such as carboxylic, primary amino, and protonated amino groups) has been exploited for electrostatic, hydrophobic, or covalent-binding-based immobilization.

The aim of the present study was to develop LBL multilayer films as hydrated multilayer films to resist bacteria and protein adhesion and to present different functionalities (primary amino groups) through grafting of nisin. The $(mucin/PEI)_n$ multilayer film was constructed using electrostatic interactions between mucin and PEI through a self-assembly method. To enhance the bactericidal efficacy, nisin was grafted onto PEI using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide/N-hydroxysulfosuccinimide (EDC/NHSS) activation. Specifically, the bactericidal activity against Gram-positive (*S. aureus*) bacteria was measured by shake-flask culture and LIVE/DEAD bacterial viability kit staining methods. Morphology and activity evaluation of human lens epithelial cells (HLECs) were used to explore the cytotoxicity and antiadhesive properties of the AMPs-grafted multilayer film. This approach represents a generalized strategy for the immobilization of antibacterial functional groups on LBL films, expanding the range of functionality of these versatile thin films.

2. Experimental

2.1. Materials

Commercially available bovine submaxillary mucin (BSM, SigmaAldrich) was used after being purified to remove some of the serumal bumin contanimants. For the purification, BSM was dissolved in water at 30 mg/mL and dialyzed in a 100 kDa molecular weight cut off membrane (Spectrum Laboratories) against water for 7 days. The mucin was then lyophilized for storage. Since commercial BSM solutions are known to form aggregates (larger than 400 nm depending on the batch) in solution, additional filtration was carried out using bottle top filters with a 0.45 mm PES membrane (VWR, 500 mL) when it is needed. Nisin (from LactococcuslactisVetec™ reagent grade, 2.5% (balance sodium chloride and denatured milk solids),*Mw*: 3354.07), poly(ethyleneimine) (branched PEI, *Mw*: 25 kDa), EDC and NHSS were purchased from Sigma-Aldrich. *Staphylococcus aureus* (*S. aureus*, ATCC 6538) was kindly provided by Prof. Jian Ji (Zhejiang University, Hangzhou, China). Hydrophobic silicone foldable lens were purchased from 66 Vision-tech CO., LTD with optical diameter at 6.0 mm (Suzhou, China). Polydimethylsiloxane (PDMS) was prepared from Sylgard®184 from Dow Corning, according to the manufacturer's instructions, using 10:1 ratio of elastomer base to curing agent. Ultrapure distilled water was obtained after purification using a Millipore Milli-Q system (USA).

2.2. Construction of the $(mucin/PEI)_n$ multilayer films

Silicon wafers and PDMS used as substrates were successively cleaned in ethanol, acetone and water for 10 min respectively and then dried with N_2. Substrates were first immersed into PEI solution (5 mg/mL) for 30 min to form a precursor layer. For the underlying $(mucin/PEI)_n$ multilayer film, the substrates were alternately dipped in mucin (pH 3.5, 0.2 mg/mL)and PEI (pH 10.8, 0.2 mg/mL). The substrates were first immersed in the mucin solution for 10 min, and then rinsed three times in buffer solution. The films were dried under a gentle stream of nitrogen gas. Next, the substrate was immersed in PEI solution for 10 min and then also rinsed with buffer solution. This dipping cycle corresponds to the deposition of one bilayer. The cycle was repeated until the desired number of bilayers was reached.

2.3. Immobilization of nisin on the $(mucin/PEI)_n$ multilayer films

In the process of nisin immobilization, nisin (1 mg/mL), EDC (10 mmol) and NHS (20 mmol) were successively added into 2-morpholino-ethanesulfonic acid (MES) buffer (0.1 M, pH 5.5) with magnetic stirring and this process continued 2 h. The multilayer film coated PDMS was immersed in the above mixed solution for 24 h at room temperature to graft nisin. The PDMS sheets were washed with MES buffer to remove the physically adsorbed nisin. The treated samples were dried at room temperature for 24 h and then at 30 °C for 12 h under vacuum prior to further characterizations.

2.4. Characterization of the multilayer films

The thickness of the self-assembly multilayer films on silicon wafer was measured by spectroscopic ellipsometry (M-2000 DITM, J.A. Woollam). The basic process of thickness measurement was that: continuous wave length ranging from 124 to 1700 nm and angle of incidence of both 65° and 70° were chosen for the

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