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Enhanced antiadhesive properties of chitosan/hyaluronic acid polyelectrolyte multilayers driven by thermal annealing: Low adherence for mammalian cells and selective decrease in adhesion for Gram-positive bacteria

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ABSTRACT

The development of antifouling coatings with restricted cell and bacteria adherence is fundamental for many biomedical applications. A strategy for the fabrication of antifouling coatings based on the layer-by-layer assembly and thermal annealing is presented. Polyelectrolyte multilayers (PEMs) assembled from chitosan and hyaluronic acid were thermally annealed in an oven at 37 °C for 72 h. The effect of annealing on the PEM properties and topography was studied by atomic force microscopy, ζ-potential, circular dichroism and contact angle measurements. Cell adherence on PEMs before and after annealing was evaluated by measuring the cell spreading area and aspect ratio for the A549 epithelial, BHK kidney fibroblast, C2C12 myoblast and MC-3T3-E1 osteoblast cell lines. Chitosan/hyaluronic acid PEMs show a low cell adherence that decreases with the thermal annealing, as observed from the reduction in the average cell spreading area and more rounded cell morphology. The adhesion of *S. aureus* (Gram-positive) and *E. coli* (Gram-negative) bacteria strains was quantified by optical microscopy, counting the number of colony-forming units and measuring the light scattering of bacteria suspension after detachment from the PEM surface. A 20% decrease in bacteria adhesion was selectively observed in the *S. aureus* strain after annealing. The changes in mammalian cell and bacteria adhesion correlate with the changes in topography of the chitosan/hyaluronic PEMs from a rough fibrillar 3D structure to a smoother and planar surface after thermal annealing.

1. Introduction

Biological fouling, the deposition of proteins or other biomolecules and the formation of a biofilm are often a problem in the design of interfaces for biomedical devices in contact with biological fluids. The unspecific deposition of proteins and the formation of a biofilm can severely compromise the use of the coating or device for the intended biomedical applications as the presence of proteins or bacteria can lead to undesired biological responses or infections [1,2]. It is also often the case that the adherence of cells to medical device surfaces must be restricted, for example in surgery devices either during or post intervention [3,4]. Several approaches have been developed throughout the years for the functionalization of surfaces in order to make them antifouling, i.e., pegylation, use of amphiphilic surfactants, biopolymer coatings, atomic layer deposition- modified polymeric coating, etc. [5–9]. The effectiveness of each approach usually depends on the degree of coverage of the surfaces and the stability of the coating with time. The reason why these antifouling coatings are effective is not always fully understood. The LbL technique is based on the alternating assembly of polyelectrolytes bearing opposite charges to form a thin film. The LbL technique is performed in aqueous media, it is simple and robust, and basically it only requires a charged surface for the assembly. The LbL technique has been used to produce coatings with antibacterial applications, with the capacity of releasing antibacterial agents, or capable of killing bacteria by contact, as well as surfaces combining the capacity of releasing of antibacterials with a low bacteria adhesion [10,11]. For biomedical applications, the control of mammalian and bacteria cells adhesion is a challenging task and of uppermost importance for the successful implementation of new material to be in contact with biological environments [12]. Multifunctional and intelligent interfaces, with good

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adhesion to certain type of cells and but limited adhesion to other cell lines, as well as with an effective response to specific cell functionalities find increasing applications in the biomedical field [13,14]. In the LbL deposition, besides polyelectrolytes other molecules [15,16], nanoparticles [17,18], lipid vesicles [19], and even cells [20,21] can be assembled on top of multilayers or placed at selected positions in the PEMs, provided that they are charged or may exhibit other type of supramolecular interactions with adjacent layers. Thus, hybrid supramolecular biomaterials with potentially new properties can be developed. For instance, multilayered biomaterials, comprising high-molecular-weight biopolymers and oppositely charged low-molecular-weight peptide amphiphiles have been fabricated [22]. Furthermore, hybrid inorganic and organic materials that appear inspired by nature can be fabricated [23]. LbL based hybrid structures can include nanoparticles with multiple functions [24]. In particular hybrid organic / inorganic assembly can benefit from the multiple functionalities that can be carried by, inorganic nanoparticles with potential for gen delivery [25,26] imaging, different type of disease treatment [27,28], and many other biomedical applications [29–31].

Among biopolymer coatings, chitosan-based ones are reported to have antifouling properties with limited protein adherence [32,33]. The layer-by-layer technique (LbL) offers a simple way to assembly chitosan as a dense coating [34,35]. Chitosan, as polycation, can be assembled with other biopolymers of biological origin bearing a negative charge, i.e., alginate or hyaluronate, resulting in a stable antifouling surface. It has been shown that chitosan/alginate coatings have good antifouling properties, with a restricted protein deposition when assembled on planar or colloidal surfaces. The authors coated poly(lactic-*co*-glycolic) nanoparticles with chitosan/alginate with the objective of enhancing nanoparticle circulation for drug delivery applications [36].

Recently, Muzzio et al. have shown that by means of thermal annealing it is possible to increase cell adhesion to polyelectrolyte multilayers of poly-L-lysine (PLL) and alginate (Alg) [37]. Despite the fact that the annealing reduces the deposition of proteins on the multilayers, thus acquiring antifouling properties, cells adhere better to annealed PLL/Alg multilayers than to unannealed ones. Annealing increases contact angle notoriously for PLL/Alg multilayers, from approximately 30 to around 90°. This change in contact angle means that the multilayers become more hydrophobic, which can result in not only a decrease in the amount of deposited proteins but also in an interaction of the proteins with the surface that is more controlled by the hydrophobic regions of the proteins than by electrostatic interactions [38]. A different arrangement of proteins on the annealed PEMs could explain why with lower protein deposition cell adherence increases. This particular response to annealing is specific to PLL/Alg PEMs. Other PEMs based on biopolymers show different behavior in relation to protein and cell adherence when they are annealed, as we will show here.

In this work we place particular emphasis on the impact of thermal annealing on the antifouling properties of chitosan/hyaluronic acid (Chi/HA) multilayers for proteins, eukaryotic and bacteria cells. We show that Chi/HA multilayers behave as largely antifouling materials and thermal annealing enhances the antifouling characteristics of the PEMs. For these studies, four different eukaryotic cell lines and two bacteria strains were tested. Annealed Chi/HA PEMs were characterized by atomic force microscopy, contact angle, ζ – potential measurements, the quartz crystal microbalance technique and circular dichroism. Smoother, highly negative hydrophilic surfaces with very low protein deposition and enhanced resistance to both eukaryotic and bacteria cells were produced after annealing. Adhesion data from the tested eukaryotic cells show that the epithelial cell line studied exhibit nonadherent characteristics neither on the unannealed nor on the annealed PEM, while for the other cell lines there is a significant decrease in adhesion to the PEMs after annealing. In the case of bacteria, there is a specific decrease of adhesion for Gram-positive bacteria, which is not observed with Gram-negative ones. Thermal annealing modifies cell-film interactions to an extent that depends on the cell line and may be used as an alternative friendly method for modifying surface properties for biological applications.

2. Materials and methods

2.1. Materials and reagents

Chitosan (Chi, Mw 100–300 kDa, Cat. No. 349051000) and hyaluronic acid (HA, Mw 1500–2200 kDa, Cat. No. 251770010) were acquired from Acros Organics. HEPES sodium salt (H7006), phosphate buffered saline (PBS, D1408), sodium acetate trihydrate (AcNa, S8625), acetic acid (AcH, 33,209), bovine serum albumin (BSA, A7906), fibronectin from human plasma (F1056), sodium dodecyl sulfate (SDS, L6026) and Minimum Essential Medium (MEM, M4526) were purchased from Sigma-Aldrich. Roswell Park Memorial Institute medium (RPMI, 12-702F) was acquired from Lonza.

2.1.1. Multilayer film preparation via layer-by-layer (LbL) assembly

PEMs were assembled on cover glasses entailing 15 layers of polyelectrolytes, the first and the last layer always being the polycation, $(Chi/HA)_7Chi$. Both polyelectrolytes were alternately assembled by manually dipping the cover glasses in the polyelectrolyte solutions for 15 min at 24 °C. Cover glasses were cleaned by immersing them in 10 mM SDS for 3 h, rinsed in sterile water three times, treated with 0.1 M HCl overnight, and thoroughly rinsed in water.

Both polyelectrolyte solutions were prepared at a concentration of 1 mg mL^{-1} in a 150 mM NaCl, 10 mM sodium acetate buffer (pH 5) (ACETATE Buffer) and filtered through a 0.45 μm filter. After each layer deposition, films were rinsed 3 times with ACETATE Buffer.

2.1.2. Multilayer film annealing

PEMs prepared as described in previous section (unannealed PEM) were UV-sterilized for 1 h in the laminar flow hood and left in a Memmert UNE 200–300 oven at 37 °C for 72 h for thermal annealing.

2.2. Quartz crystal microbalance with dissipation (QCM-D)

A QCM-D E4 device from Q-Sense was used to trace the assembly profile of the Chi/HA film on top of a SiO_2 (50 nm) coated quartz crystal (5 MHz, Q-Sense). All experiments were performed at 24 °C. For each polyelectrolyte deposition, a 1 mg mL^{-1} polyelectrolyte solution in ACETATE buffer was flown through a 4-sensor chamber with the help of a peristaltic pump and left under incubation for 10 min. Every deposition step was always followed by 10 min rinsing with the buffer solution. Experiments were also conducted to study the stability of the film in 10 mM HEPES buffer containing 150 mM NaCl (HEPES buffer), and the adsorption of bovine serum albumin (BSA) and fibronectin (FN) proteins on the film before and after annealing on the quartz crystals. HEPES buffer was chosen for these experiments because it mimics cell growth conditions better.

2.3. Protein adsorption

BSA and FN were absorbed on PEMs at 24 °C. Annealed and unannealed PEMs were immersed for 45 min in either 1 mg mL^{-1} BSA or 0.05 mg mL^{-1} FN in HEPES buffer. The difference in concentration for the two proteins takes into account their relative abundance in cell media.

2.4. Atomic force microscopy (AFM)

The morphology of air-dried PEMs was studied with an AFM from Nanovizard II AFM (JPK, Berlin, Germany). Images were collected in tapping mode with TESP-V2 cantilever (Bruker, AFM Probes) with a nominal spring constant of 40 N m^{-1} oscillating near a resonant frequency in the range 280 to 320 kHz.