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Achieving low-fouling surfaces with oppositely charged polysaccharides via LBL assembly[☆]

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ABSTRACT

The aim of this work is to understand and achieve low fouling surfaces by mixing two oppositely charged polysaccharides through layer-by-layer (LBL) assembly. Diethylaminoethyl-dextran hydrochloride and alginate were employed as a model system to build LBL films. A surface plasmon resonance (SPR) biosensor was used to measure quantitatively the adsorption behavior of charged macromolecules during LBL buildup and the protein adsorption behavior of each deposited bilayer *in situ* in real time accordingly. Results show that LBL films have lower protein adsorption as the films are constructed above the substrate surface. These LBL films eventually reach very low fouling when they are sufficiently far from the substrate surface, where the substrate surface effect is minimized and bilayers consisting of positively and negatively charged macromolecules are uniformly mixed. Single proteins, undiluted human blood serum and plasma and cells were tested for adsorption to LBL films with similar trends. To verify the generality of these findings, alginates of low and high molecular weights and carboxymethylcellulose as a substitute to alginate were studied with similar trends observed. These results demonstrate that oppositely charged polymers, when uniformly mixed, are able to achieve low fouling properties. Findings from this work will provide a fundamental understanding of and design principles on how to build nonfouling LBL films.

Statement of Significance

We demonstrate that protein adsorption decreases with the increase of bilayer numbers. Results indicate that oppositely charged components tend to be uniformly mixed and distinct change layers in classical layer-by-layer (LBL) theories no longer exist as LBL films are sufficiently far from the substrate surface. Findings from this work provide a fundamental understanding of and design principles on how to build nonfouling LBL films.

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1. Introduction

Nonfouling surfaces are of great interest in many applications, such as tissue engineering, drug delivery, biosensing, and marine protection [1]. Nonfouling surfaces can be created with certain hydrophilic polymers via “grafting from” or “grafting to” surface coating techniques. Poly(ethylene glycol) (PEG) polymers have

been widely used over the last 40 years [2–4]. Unfortunately, PEG polymers are subject to oxidation in biochemically relevant solutions [5,6]. PEG polymer resistance to protein adsorption is diminished above 35 °C due to weakened hydrogen bonding [7]. Another family of nonfouling materials employed is based on zwitterionic polymers, which contain a positively charged and a negatively charged moiety within a single pendant group. Carboxybetaine (CB), sulfobetaine (SB), and phosphorylcholine (PC) are three typical examples of zwitterionic materials [8–10] and possess ultra-low fouling properties. CB-based polymers have additional features of charge-switching and functionalization [10]. Furthermore, it has been demonstrated that self-assembled

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monolayers (SAMs), copolymers or hydrogels including peptides containing a certain ratio of mixed positively and negatively charged monomer subunits exhibit nonfouling properties [11–16]. Based on these results and the mechanism of zwitterionic materials, it is possible to prepare a new nonfouling surface by balancing the net charge and dipole of a mixture of oppositely charged polyelectrolytes deposited on a surface.

Layer-by-layer (LBL) assembly is a popular surface functionalization approach to construct polyelectrolyte multilayer (PEM) films by the alternative deposition of polycation and polyanion species driven by electrostatic interactions [17]. It is generally accepted that surface charge reversal is the driving force for the multilayer buildup, but layer assembly is not as simple as the accumulation of two alternatively charged layers, as demonstrated in two recent publications. Asymmetric growth behavior was observed when the stoichiometry of two polyelectrolytes was close to 1:1 [18]. Another example showed that the zeta potential of the multilayer was not dependent on the polyelectrolyte deposited as the top layer [19]. Furthermore, many LBL films target biomedical applications [20,21]. However, protein adsorption on these LBL films has been one major issue for practical applications of these systems. Here, we demonstrate that protein adsorption on LBL films gradually decreases as the films are increasingly far from the substrate surface and the effect of the substrate surface on LBL assembly vanishes. Thus, although LBL films have distinct positively and negatively charged layers when they are closer to the substrate surface, the bilayer (one positive and one negative layer) will be more uniformly mixed and the net charge and dipole of the whole film gradually tends to be minimized after a certain number of bilayers have been deposited. The point at which the outer layer becomes nonfouling depends on the molecular weight, charge density and concentration of the two oppositely charged polyelectrolytes used to build the LBL film.

Among the polyelectrolyte complexes used in LBL assembly, polysaccharide-based PEMs have been paid increasing attention due to their inherent biocompatibility and have been identified as potential biomaterials for drug delivery [22,23], antibacterial coatings [24,25] and other therapeutic applications [26–31]. Despite these promising properties, however, protein adsorption on the surface is still a difficult issue that needs to be resolved [32,33]. Herein, in this work, two polysaccharides of cationic dextran/alginate are used to build the multilayer. A surface plasmon resonance sensor (SPR) was employed to quantitatively measure the adsorption behavior of charged macromolecules during LBL buildup and the protein adsorption behavior of each deposited bilayer *in situ* in real time. Thus, the relationship between the number of bilayers and the amount of adsorbed protein can be established. Findings from this work will provide a fundamental understanding of and design principles on how to build nonfouling LBL films.

2. Experimental section

2.1. Materials

Diethylaminoethyl-dextran hydrochloride (500 kDa MW, 3.2% nitrogen), low molecular weight alginate (12–80 kDa MW), high molecular weight alginate (80–120 kDa MW), carboxymethyl cellulose sodium salt (250 kDa MW and degree of substitution (DS) of 1.2), fibrinogen (from bovine), lysozyme (from egg white), and phosphate buffered saline (PBS, 0.01 M phosphate, 138 mM sodium chloride, 2.7 mM potassium chloride, pH 7.4) were purchased from Sigma–Aldrich (Milwaukee, WI). Pooled human serum and plasma were purchased from Biochemed Services (Winchester, VA). Cellulose acetate membrane filters (0.25 μ m) were purchased from VWR International, Visalia, CA.

2.2. Preparation of polysaccharide solutions

Solutions of diethylaminoethyl-dextran (CD) hydrochloride, alginate (ALG) and carboxymethyl cellulose (CMC) were used for the construction of multilayer films. Solutions were prepared by direct dissolution of polysaccharides in 0.15 M NaCl and the pH value was then adjusted to 5.5 using concentrated NaOH or HCl solutions. All polysaccharide solutions were filtered through a 0.25 μ m cellulose acetate membrane filter prior to use.

2.3. Surface plasmon resonance (SPR) biosensor

SPR measurements were conducted with a custom-built surface SPR sensor from the Institute of Photonics and Electronics, Academy of Sciences (Prague, Czech Republic) as described previously [34]. It is equipped with a four-channel flow-cell, temperature control, and associated with a peristaltic pump for delivering samples. The SPR sensor is based on the attenuated total reflection method and wavelength modulation. Its sensitivity is dependent on the distance from the SPR active surface. Therefore, the sensor response must be calibrated after polymer films are deposited. Since SPR sensitivity depends on the distance of the binding event from the SPR active surface, the sensor response is calibrated based on the thickness of the polymer film using previously described methods [34]. When the film is closer to the SPR surface, a 1 nm SPR wavelength shift is equivalent to a surface coverage of ~ 17 ng/cm² adsorbed proteins. To account for the film thickness, the conversion of SPR wavelength shift to adsorbed protein is adjusted using a calibration factor between 1.023 and 1.232 [34]. Therefore, the conversion of SPR wavelength shift (nm) to adsorbed protein (ng/cm²) on films with different thicknesses is calculated as the product of 17, SPR wavelength shift and calibration factor. SPR chips used in experiments were glass slides coated with an adhesion promoting titanium film (~ 2 nm) followed by a gold film (~ 48 nm) deposited via an electron beam evaporator.

2.4. Formation of multilayer films

The growth of a CD/ALG multilayer film was monitored *in situ* with SPR. CD and ALG were deposited alternatively on the gold coated SPR chip surface at a flow rate of 40 μ L/min for 10 min, with 5 min 0.15 M NaCl solution rinses between depositions. In this work, the number of bilayers was constructed as needed under experimental conditions. The term “bilayer” refers to the assembly created by a pair of oppositely charged polysaccharides. The thickness of the dry multilayer film was measured using a α -SE ellipsometer (J.A. Woollam Co., Lincoln, NE).

2.5. Protein adsorption

The amount of protein adsorption for multilayer films on a SPR chip was tested. PBS buffer was flowed for 20 min at a rate of 40 μ L/min to establish a baseline first. Solutions of fibrinogen and lysozyme in PBS (1.0 mg/mL), 100% human serum, and 100% human plasma were then flowed for 10 min in four separate channels followed by PBS for 10 min to remove unbound proteins. The difference in SPR response in PBS buffer solutions before and after a protein solution is introduced is the amount of adsorbed protein.

2.6. Cell adhesion

Prior to cell adhesion experiments, samples were soaked and rinsed with copious amounts of sterilized PBS. NIH-3T3 fibroblasts (ATCC, Manassas, VA) were plated at 4×10^4 cells/mL in 3 mL

Dulbecco's Modified Eagle Medium (DMEM) with 10% (v/v) fetal bovine serum and 1% (v/v) penicillin–streptomycin (PS) (Invitrogen Corp, Carlsbad, CA) and incubated overnight at 37 °C in 5% CO₂ and 100% relative humidity. The cell seeding density was determined using a hemocytometer. Cell adhesion and morphology were observed under a phase-contrast microscope (Nikon Eclipse TE2000-U, Tokyo, Japan). The number of cells adhered was determined by visually counting cells present in at least three microscope images after an overnight incubation period (100× objective magnification).

2.7. AFM imaging of CD/ALG multilayers

The morphology of dried multilayers was observed with an atomic force microscope (AFM) in contact mode using a Dimension 3100 AFM (Digital Instruments/Veeco, Woodbury, NY) operated in air.

2.8. Statistical methods

Statistical analysis was performed using Excel. Mean values with standard deviation are reported and all experiments were performed in triplicate. The error bars correspond to the standard deviation.

3. Results and discussion

3.1. Characterization of CD/ALG multilayer buildup by SPR, ellipsometry and AFM

CD and ALG were selected as a model system. The growth of CD/ALG multilayers was monitored *in situ* by SPR. CD was first deposited onto the negatively charged gold-coated glass surface [35], followed by a 0.15 M NaCl rinse, and finally the adsorption of alginate as the outer layer. This process was repeated 4 times to construct 4 bilayers, which allows us to investigate the relationship between the number of bilayers and the degree of protein adsorption, and the change in fouling properties of a LBL film as the outer layer builds further from the substrate surface. CD is a polycationic derivative of Dextran. There are ~0.03 tertiary amine groups per saccharide unit with a pKa of 9.2. Each CD polymer has about 90 tertiary amine groups under experimental conditions (Null.18 tertiary amine groups per kDa MW). Alginate is a linear copolymer, consisting of (1,4)-linked β -D-mannuronic acid (M) and α -L-guluronic acid (G) residues in its backbone. There are two carboxylate groups (COOH) per disaccharide unit with a pKa of 3–4. The number of COOH groups is about 60–400 for each alginate polymer (12–80 kDa MW), which equals 5 COOH groups per kDa MW. Charge balance can be achieved between CD and ALG by mixing the two polysaccharides in dilute concentrations and adjusting the pH value and concentration.

Fig. 1 shows the SPR response of the buildup process of CD/ALG multilayers. The shift in wavenumber increased with the increase in layer number. Two alginates (12–80 kDa and 80–120 kDa) with different molecular weights were employed separately to construct CD/ALG multilayers. Both LBL films follow the same buildup behavior as shown in Fig. 1. However, the CD/ALG multilayer with a higher ALG molecular weight has thicker layers than the ALG with a lower molecular weight. The total thickness of the 4 bilayers for the dry CD/ALG (80–120 kDa) multilayer film is 12 nm, while it is 7 nm for the dry CD/ALG (12–80 kDa) multilayer film as measured by ellipsometry. Furthermore, we tested the nonfouling properties of the two CD/ALG multilayers with different molecular weights. Results show similar trends for the fibrinogen adsorption as a function of layer number. The nonfouling level of the 4 bilayers

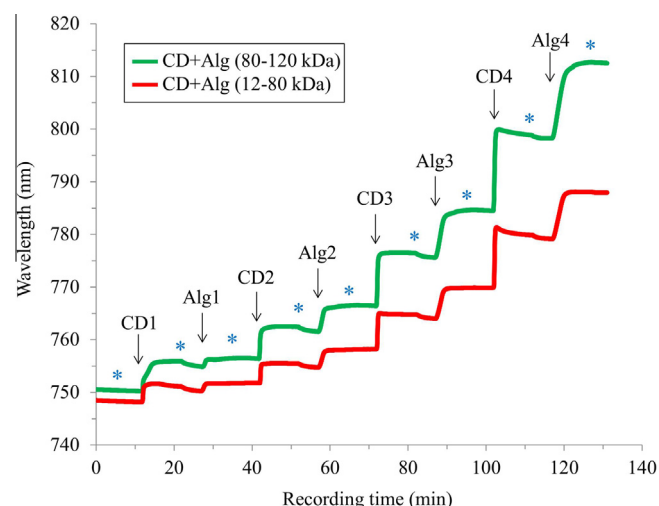


Fig. 1. The typical buildup of CD/ALG for 4 bilayers in SPR using alginate comprised of two molecular weights (red line for 12–80 kDa ALG and green line for 80–120 kDa, respectively). Black arrows indicate the injection of CD or ALG. The blue stars indicate the injection of aqueous NaCl (0.15 M, pH 5.5). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

was 8.0 ng/cm² adsorbed fibrinogen for the ALG molecular weight of 80–120 kDa and 7.3 ng/cm² adsorbed fibrinogen for the ALG molecular weight of 12–80 kDa, respectively. Thus, we chose alginate with a molecular weight of 12–80 kDa as a main model system to reduce the total thickness of the multilayer film to improve the sensitivity of SPR detection since SPR signal decreases with distance from the substrate surface. The CD/ALG (12–80 kDa) was further constructed up to 5 bilayers and 6 bilayers to study the fouling level of human serum and plasma. The dry thicknesses of the 5 bilayer CD/ALG film and the 6 bilayer CD/ALG film was measured as 9.2 nm and 19.6 nm, respectively. The surface roughness value (Rq) for the CD/ALG (12–80 kDa) multilayer film increased from 1.0 to 1.7 nm with the increase in bilayer number from 1 to 4 as determined by AFM (see Fig. S1).

3.2. Protein adsorption

Initially, fibrinogen (Fg) and lysozyme (Lyz) were employed as model proteins to test the fouling levels for LBL films. Fg (>200 kDa) is larger and negatively charged, while Lyz (<100 kDa) is smaller and positively charged in PBS buffer at a pH of 7.4. The fouling levels of three control surfaces decreased in the order of bare gold > alginate coating > dextran coating for both fibrinogen and lysozyme. CD displayed lower fouling since it is a hydrophilic and positively charged polysaccharide while alginate is a less hydrophilic and negatively charged polysaccharide under physiological conditions (See Fig. S2). CD displayed adsorbed protein levels of 180.2 ng/cm² and 5.1 ng/cm² for Fg and Lyz due to electrostatic attraction and repulsion, respectively. ALG displayed adsorbed protein levels of 281.3 ng/cm² and 132.5 ng/cm² for Fg and Lyz, respectively. Higher Fg adsorption on alginate is due to a Fg conformation change since Fg is more flexible and more likely to adhere to surfaces than Lyz [36]. Fouling levels on the bare gold control were very high, as expected for an uncoated surface. Protein adsorption levels depend on the hydrophilicity (or hydrophobicity), charge and conformation properties of both proteins and polysaccharide surfaces.

Prior to LBL assembly, several ALG (12–80 kDa) concentrations were screened to optimize the fouling properties of the first bilayer

as a proof of concept. As indicated in Fig. S3, the minimum fouling level corresponded to the combination of an ALG concentration of 2.0×10^{-2} g/L and a CD concentration of 1.0 g/L during assembly. Under the optimal conditions, 4 bilayers were built and the fouling properties were tested as shown in Fig. 2a. Interestingly, all LBL films exhibited low fouling behavior. Even for a film containing only 1 bilayer surface, Fg adsorption is diminished by 6 times compared with the best single layer coating surface (CD coating). As expected, protein adsorption dramatically dropped as the number of layers increased due to better mixing of charged layers and minimization of the net charge and dipole. The lowest adsorption occurred on a film containing 4 bilayers, with Fg adsorption at 7.3 ng/cm^2 and Lyz adsorption at 1.6 ng/cm^2 .

Furthermore, we tested the fouling level of undiluted human blood serum and plasma as shown in Fig. 2b. Both curves of the fouling levels for undiluted human blood serum and plasma have the same trends as those for single proteins. As expected, the fouling level decreases as the number of LBL bilayers increases. The final fouling level for a 4 bilayer film for human blood serum and plasma were 129.3 ng/cm^2 and 144.7 ng/cm^2 , respectively. For single protein fouling, 4 bilayers are enough to avoid non-specific adsorption to the surface. However, under much more challenging conditions such as undiluted human serum and plasma, there is room for improvement. Thus, we increased the bilayer numbers to 5 and 6. The fouling level of 5 bilayers and 6 bilayers is 5.1 ng/cm^2 and 3.4 ng/cm^2 for single Fg, respectively. The fouling values

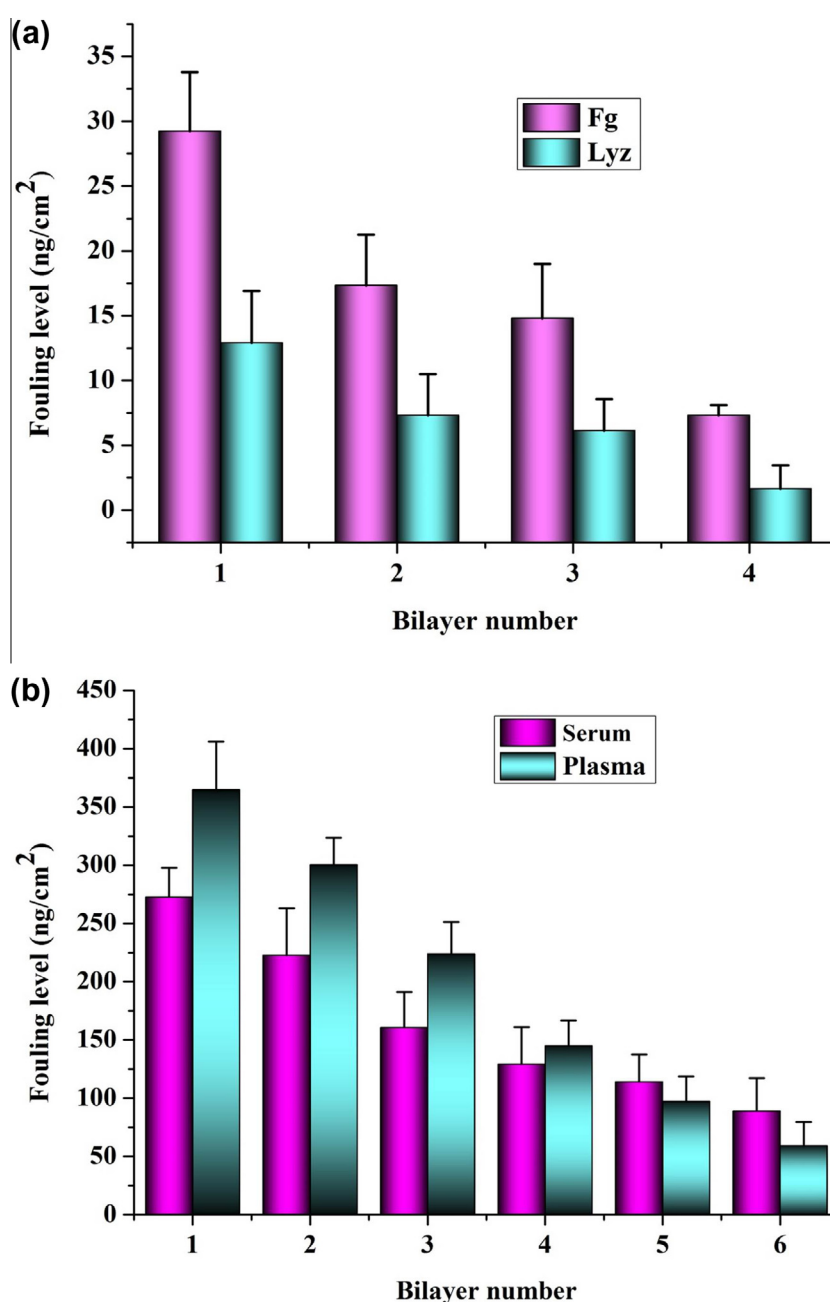


Fig. 2. Fouling level of CD/ALG (12–80 kDa) as a function of bilayer number a) for single protein fibrinogen (Fg) (magenta) and lysozyme (Lyz) (teal) for the upper figure and b) for undiluted human blood serum (magenta) and plasma (teal) for the lower figure. The concentrations used for LBL assembly were 1.0 g/L for CD and 2.0×10^{-2} g/L for Alg in 0.15 M NaCl with pH 5.5. The error bars correspond to the standard deviation of three replicates. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

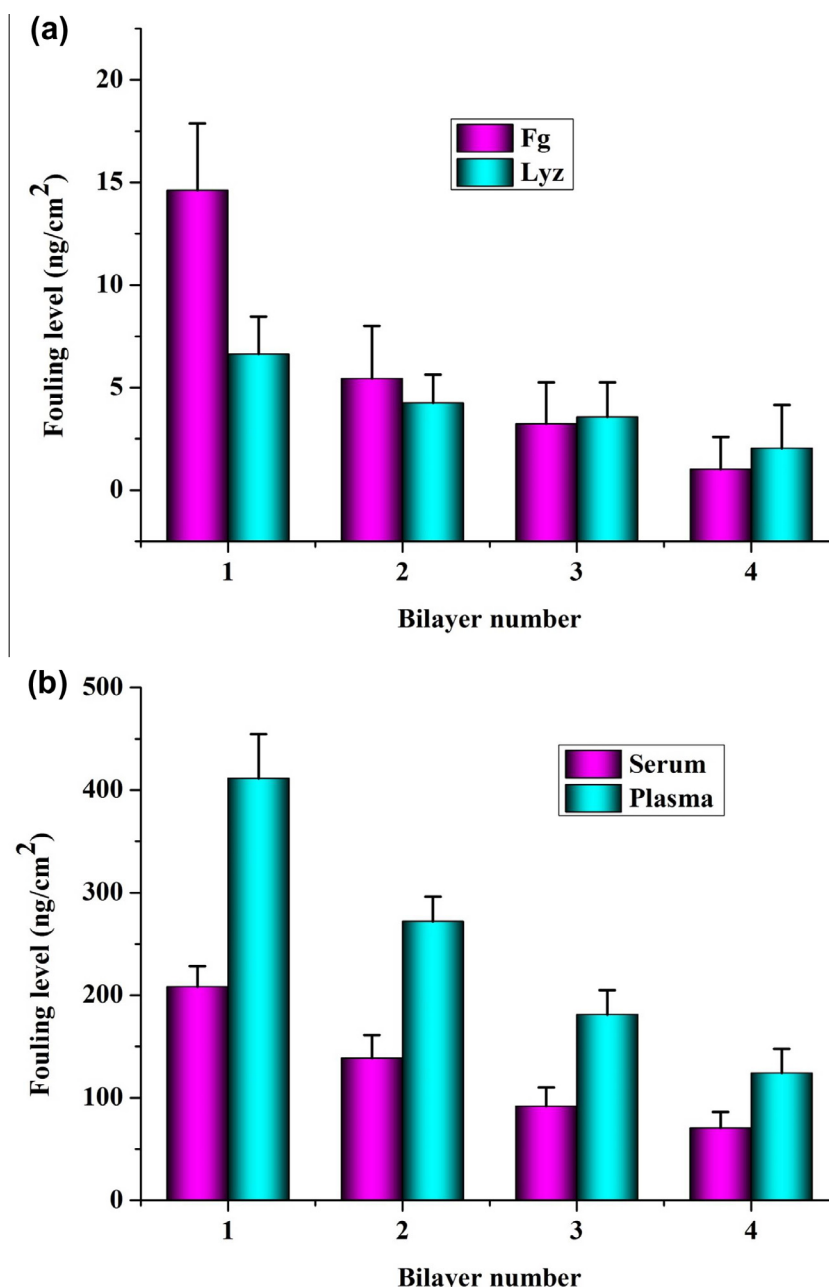


Fig. 3. Fouling level of CD/CMC as a function of bilayer number a) for single protein fibrinogen (Fg) (magenta) and lysozyme (Lyz) (teal) for the upper figure and b) for undiluted human blood serum (magenta) and plasma (teal) for the lower figure. The concentrations used for LBL assembly were 1.0 g/L for CD and 2.0×10^{-2} g/L for CMC in 0.15 M NaCl with pH 5.5. The error bars correspond to the standard deviation of three replicates. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

of 5 bilayers and 6 bilayers for undiluted human blood serum decreased to 114.1 ng/cm² and 88.9 ng/cm², respectively. Correspondingly, for undiluted blood plasma, fouling values were 97.2 ng/cm² and 59.3 ng/cm². After construction of the 5th bilayer, the film thickness increases rapidly as the two oppositely charged components are more uniformly mixed and distinct charged layers in the LBL film assembly disappear. As speculated, the net charge of the entire multilayer gradually balances out. Therefore, the fouling level decreases with the increase of bilayer numbers. To verify the generality of these findings, we substituted ALG with carboxymethyl cellulose (CMC) to build 4 bilayers and observed similar trends of the fouling level as a function of bilayer number, as can

be seen in Fig. 3. The CD/ALG and CD/CMC LBL films both yielded a total dry thickness of 7 nm after the construction of 4 bilayers.

3.3. Cell adhesion

We further evaluated cell adhesion behavior of CD/ALG films. NIH-3T3 fibroblast cells were seeded onto glass chips coated with different multilayers and incubated overnight. As expected, the number of adhered cells decreased with an increase in the number of bilayers as shown in Fig. 4. The 4-bilayer system had very low cell adhesion. These results show that CD/ALG multilayers can achieve low protein adsorption and minimal cell adhesion.

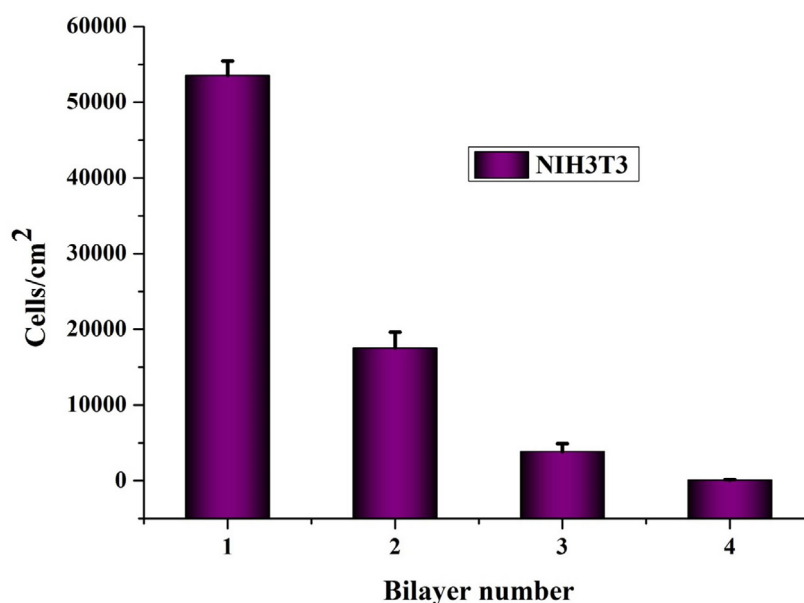
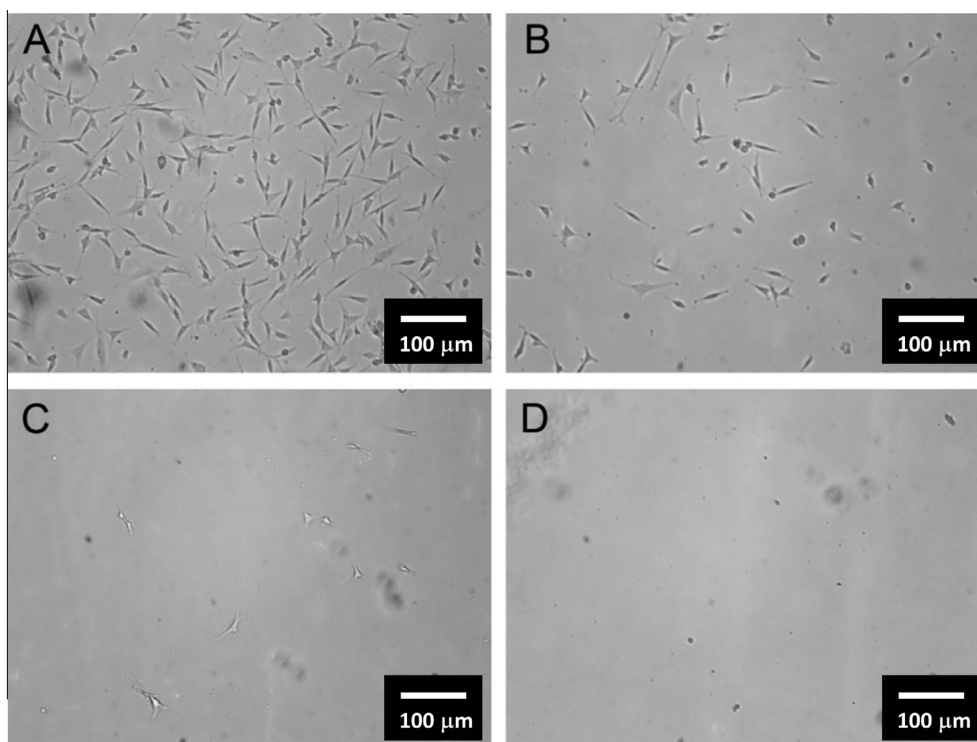


Fig. 4. Cell adhesion after incubation overnight for glass chips coated with CD/ALG films for (A) 1 bilayer, (B) 2 bilayers, (C) 3 bilayers and (D) 4 bilayers, respectively in the upper figure. The quantitative results are shown in the lower figure.

4. Conclusions

In this work, a new avenue for low fouling surfaces was explored by depositing alternating layers of oppositely charged polysaccharides through the LBL technique. Fouling properties decreased with the increase of bilayer numbers, for protein adsorption from single fibrinogen and lysozyme, for undiluted human serum and plasma, and for cell adhesion. The molecular weight, charge density, concentration of two oppositely charged polyelectrolytes, and the external conditions of pH values determine if the outer layer becomes a nonfouling surface. While 4 bilayers are sufficient to achieve very low fouling to single proteins, 6 bilayers or

more are needed to achieve low fouling to undiluted blood plasma and serum. These results indicate that nonfouling LBL films can be achieved when the substrate surface effect is minimized and when two mixed charged macromolecules are uniformly mixed throughout buildup.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.actbio.2016.04.013>.

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