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Polyelectrolyte multilayer films with pegylated polypeptides as a new type of anti-microbial protection for biomaterials

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Abstract

Adhesion of bacteria at the surface of implanted materials is the first step in microbial infection, leading to post-surgical complications. In order to reduce this adhesion, we show that poly(L-lysine)/poly(L-glutamic acid) (PLL/PGA) multilayers ending by several PLL/PGA-g-PEG bilayers can be used, PGA-g-PEG corresponding to PGA grafted by poly(ethylene glycol). Streaming potential and quartz crystal microbalance-dissipation measurements were used to characterize the buildup of these films. The multilayer films terminated by PGA and PGA-g-PEG were found to adsorb an extremely small amount of serum proteins as compared to a bare silica surface but the PGA ending films do not reduce bacterial adhesion. On the other hand, the adhesion of *Escherichia coli* bacteria is reduced by 72% on films ending by one (PLL/PGA-g-PEG) bilayer and by 92% for films ending by three (PLL/PGA-g-PEG) bilayers compared to bare substrate. Thus, our results show the ability of PGA-g-PEG to be inserted into multilayer films and to drastically reduce both protein adsorption and bacterial adhesion. This kind of anti-adhesive films represents a new and very simple method to coat any type of biomaterials for protection against bacterial adhesion and therefore limiting its pathological consequences.

Keywords: Polyelectrolyte multilayers; Poly(ethylene glycol); Protein adsorption; Bacterial adhesion; Surface modification

1. Introduction

The formation of bacterial slime or of biofilms at the surface of biomaterials represents a major medical problem [1] leading, if untreated, to chronic microbial infection, inflammation, tissue necrosis and eventually to death [2,3]. These infections are mostly due to *Staphylococcus epidermidis*, especially in the case of intravascular catheter-associated infections [4], and also to other gram positive strains such as *Staphylococcus aureus* in the case of metallic implants [5]. Adhesion of gram negative *Escherichia coli* has also been reported on catheters [6]. In all these cases, the formation of microbial biofilms is mostly linked to the bacterial ability to adhere onto material surfaces [7]. The adhesion of bacteria at the surface of the implant results from non-specific interactions such as electrostatic, hydrophobic, Van der Waals forces and from specific interactions, i.e. receptor–adhesion interactions [8]. The adhesion is initially reversible and becomes irreversible later during the infectious process. This emphasizes the necessity to reduce bacterial adhesion at the surface of the implanted materials [9].

From an overview of the literature, it comes out that poly(ethylene glycol) (PEG) adsorbed or grafted on substrates reduces protein adsorption [10–14], platelet adhesion [13] and lowers bacterial adhesion [15,16]. It also allows one to control cellular adhesion [14,17–19] and to create cell patterned surfaces [20]. This non-ionic biocompatible polymer [21] presents both a hydrophilic and a hydrophobic character. The repulsive properties

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of PEG against many macromolecules are explained by its strong affinity for water molecules [22,23]. When PEG molecules are adsorbed on a surface, a heavily hydrated polymer layer is formed, rendering adsorption of proteins entropically and enthalpically unfavorable [11]. In order to cover different types of surfaces with PEG, the molecule was directly grafted onto the substrate [15,24,25] or coupled to polyelectrolytes, such as poly(L-lysine) (PLL) [26] or poly(acrylic acid) [19], which were subsequently adsorbed as a monolayer.

The layer-by-layer self-assembly of polyelectrolytes on charged surfaces offers another possibility to deposit polyelectrolytes with grafted PEG onto substrates [27]. It consists in the adsorption of oppositely charged polyelectrolytes to form multilayered supramolecular assemblies. There is a growing interest in the use of polyelectrolyte multilayer buildups in various fields, such as microcapsules [28,29], micro- and nano-containers for storage, transport and release [30], polystyrene microspheres for application in immunoassays [31], post-surgical tissue regeneration [32] and anti-coagulant coatings [33]. As proteins play a crucial role in the initial adhesion events between a cell and a biomaterial surface [34], several studies have investigated the adsorption of individual proteins, such as human serum albumin [35], bovine serum albumin [36], or immunoglobulins [37] on the polyelectrolyte multilayers. For strong polyelectrolytes, it was shown that protein adsorption is mainly governed by electrostatic interactions: multilayer films ending by polyelectrolytes of opposite charge with proteins, present the highest adsorption [37–39]. For weak polyelectrolytes, the adsorption depends on the pH and ionic strength conditions [40].

Applied to avidin and biotin, the layer-by-layer technique has proven to enhance the binding ability as compared to covalently immobilized antibody monolayers [41]. This technique also allows one to build up multilayer films on various substrates like glass [42], metals [43,44] and non-ionic polymers [45,46]. Moreover, these films are of special interest to control particular properties of the surface like wettability [47] or cellular adhesion [32]. As PEG can potentially be coupled to any type of polyelectrolytes, its insertion in polyelectrolyte multilayer films can easily be achieved. To our knowledge, there is no study dealing with the insertion of PEG in a multilayer film in order to enhance its anti-adhesive properties.

The purpose of this work was to build up anti-adhesive films able to cover any type of surface and containing PEG. This type of films is expected to reduce the non-specific adsorption of proteins as well as the bacterial colonization of implanted materials. As (PLL/PGA) multilayer films ending with PGA have already proven a weak adsorption for serum proteins [48], the binding of PEG to PGA and the combination of the layer-by-layer technique may allow one to create an extremely non-adhesive surface. To this aim, we used poly(L-glutamic acid)-grafted-poly(ethylene glycol) (PGA-g-PEG) obtained by modifying the PGA backbone by a PEG. Quartz crystal microbalance-dissipation (QCM-D) and streaming potential measurements were used to characterize the multilayer buildup. The adsorption properties of these films with respect to serum proteins and their bacterial adhesion behavior were their investigated.

2. Materials and method

2.1. Polyelectrolytes and serum

PGA sodium salt (MW = 74 400), PLL hydrobromide salt (MW = 32 600) and 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide (EDC) were purchased from Sigma (St Quentin Fallavier, France). Poly(ethyleneimine) (PEI, MW = 750 000) was purchased from Aldrich (St Quentin Fallavier, France). Methoxypoly(ethylene glycol) carboxylic acid (MePEGCOOH, MW = 2000) was synthesized following the method of Zalipsky and Barany [49]. Methoxypoly(ethylene glycol) amine ($MePEGNH_2$, MW = 2000) and *N*-hydroxysulfo succinimide sodium salt (NHS) are available at Fluka (St Quentin Fallavier, France).

Polyelectrolytes were dissolved in a solution of 0.15 M of NaCl adjusted at pH 7.4 by the addition of NaOH solution. This solution was prepared using Millipore filtered water (Milli Q-plus system). The concentrations of the polyelectrolyte solutions were 5 mg/ml for PEI and 1 mg/ml for PLL and PGA. The PGA-g-PEG, and PLL-g-PEG were diluted at 0.5 mg/ml. For all experiments, a precursor film of PEI-$(PGA/PLL)_2$-PGA (noted Pre) was built in order to cover the surface of the substrate. The multilayers studied were terminated by three (PLL/PGA) or (PLL/PGA-g-PEG) bilayers, noted, respectively, Pre-$(PLL/PGA)_3$ and Pre-$(PLL/PGA\text{-}g\text{-}PEG)_3$.

Fetal bovine serum (FBS, 10270-106, Gibco BRL, Cergy Pontoise, France) was used at a concentration of 10% v/v in 0.15 M NaCl medium at pH 7.4. The total concentration of proteins in FBS is about 3.9 mg/ml (2.5 mg/ml of albumin and 1.4 mg/ml of globulins). In such conditions, both proteins are negatively charged (pI = 4.9 for albumin and pI = 6.3−7.3 for globulins).

2.2. Synthesis of pegylated polypeptides

2.2.1. Synthesis of PLL-g-PEG

The synthesis of PLL-g-PEG copolymer was performed according to the protocol described by Elbert et al. [32]. In total, 50 mg (0.24 mmol of amino group) of PLL, 192 mg (0.096 mmol acid group, in order to obtain a grafting ratio of 40%) of MePEGCOOH and 2 mg

(0.009 mmol) of NHS were dissolved in 1 ml of buffer, 50 mM of sodium tetraborate at pH 8.5. EDC (29.5 mg) (0.154 mmol) was either dissolved in the mixture with stirring. The reaction was allowed to proceed for 6 h at room temperature. After filtration, the reaction mixture was dialyzed (Spectra/Por, cut off at MW 10 000) for 24 h, first against phosphate buffer (2 l, 0.1 M, pH = 7.4, Na_2HPO_4/NaH_2PO_4) and subsequently against deionized water (2 l). The product was dried by Speed Vac and stored at –20°C. Mass of product: 62.3 mg. PLL-g-PEG ^{1}H NMR (200 MHz, D_2O, reference PEG 3.55 ppm) 1.31 (m, 2H, CH-$\underline{CH_2}$-CH_2-CH_2), 1.56 (m, 4H, CH-CH_2-$\underline{CH_2}$-$\underline{CH_2}$), 2.85 (m, 2H, CH_2-$\underline{CH_2}$-NH_2), 3.23 (s, 0.71H, $\underline{CH_3O}$-), 3.55 (sl, 42.69H, $\underline{CH_2O\bar{C}H_2}$), 4.14 (m, 1H, NH-$\underline{CH}$-CO). By ^{1}H-NMR, the areas of the lysine side-chain peaks, representing in this case one monomer of lysine, were compared to the PEG area to determine the graft ratio of the copolymer, provided that one molecule of PEG (MW = 2000) possesses 184 H. Finally, the grafting percentage is given by the ratio 42.69/184, i.e. 23 PEG for 100 monomer of lysine acid (graft ratio 23%).

2.2.2. Synthesis of PGA-g-PEG

In all, 20 mg (0.132 mmol in acid function) of PGA, 47.6 mg (0.024 mmol of amine function, in order to obtain a grafting ratio of 18%) of $MePEGNH_2$ and 1 mg (0.005 mmol) of NHS were dissolved in 1 ml of buffer, 50 mM of sodium tetraborate pH 8.5. EDC (7.3 mg) (0.038 mmol) was dissolved in the mixture with stirring. The reaction was allowed to proceed for 6 h at room temperature. After filtration, the reaction mixture was dialyzed (Spectra/Por, cut off at MW 10 000) for 24 h, first against phosphate buffer (2 l, 0.1 M, pH = 7.4, Na_2HPO_4/NaH_2PO_4) and subsequently against deionized water (2 l). The product was dried by Speed Vac and stored at −20°C. Mass of product: 24.7 mg. PGA-g-PEG ^{1}H NMR (200 MHz, D_2O, reference PEG 3.55 ppm) 1.87 (m, 2H, CH-$\underline{CH_2}$-CH_2), 2.13 (m, 2H, CH_2-$\underline{CH_2}$-CO), 3.23 (s, 0.49H, $\underline{C\bar{H}_3O}$-), 3.55 (sl, 29.79H, $\underline{CH_2O\bar{C}H_2}$), 4.16 (m, ^{1}H, NH-$\underline{C\bar{H}}$-CO). By ^{1}H-NMR, the areas of the glutamic side-chain peaks, representing in this case one monomer of glutamic acid, were compared to the PEG area to determine the graft ratio of the copolymer, provided that one molecule of PEG (MW = 2000) possesses 184 H. Finally, the grafting percentage is given by the ratio 29.79/184, i.e. 16 PEG for 100 monomer of glutamic acid (graft ratio16%).

2.3. Quartz crystal microbalance-dissipation

The measurements were performed using the QCM-D system from Q-Sense (Götenborg, Sweden). QCM-D consists in measuring the changes in the resonance frequency Δf of a quartz crystal [50,51] when material is brought from solution [52]. The crystal is excited at its fundamental frequency (about 5 MHz) and observation takes place at the third ($v = 3$), fifth ($v = 5$) and seventh ($v = 7$) overtones corresponding, respectively, to 15, 25 and 35 MHz. A decrease in $\Delta f/v$ is usually associated, in a first approximation, to an increase of the mass coupled to the quartz. The details of the experimental setup have been presented previously [53]. The crystal used here is coated with a $\approx$100 nm thick SiO_2 film deposited by active sputter-coating. The polyelectrolytes were injected into the measurement cell (internal volume of 100 μL), during 5 min at a flow rate of 0.13 ml/min using a peristaltic pump. After stabilization of the signals, 0.5 ml of the PEI solution is injected during 5 min and rinsed during 5 min with a 0.15 M NaCl solution. During these steps, the shifts in Δf are continuously recorded. The same procedure is used for the deposition of PGA by introducing 0.5 ml of the PGA solution. A PEI-(PGA/PLL)$_2$-PGA precursor film was always built to ensure that the substrate was totally covered. Then either a (PLL/PGA)$_3$ (three pairs of layers adsorbed) or (PLL/PGA-g-PEG)$_3$ was deposited on top of it. After the buildup of the multilayered film, the FBS solution was put into contact with it during 45 min, followed by a 45 min rinsing step with a 0.15 M NaCl solution at a flow rate of 0.13 ml/min.

2.4. Streaming potential measurements

Streaming potential measurements were carried out in order to determine the ζ potential of the multilayer architecture. The experiments were performed on a homemade apparatus developed by Zembala and Déjardin [54], which has also been described previously [53]. The principle is to measure the pressure and the potential differences on both ends of a 530 μm radius capillary made of fused silica via two flasks containing four electrodes. The capillary is first filled and rinsed several times with Tris-HCl buffer (Tris-(hydroxymethy-laminomethane), Tris, 5×10^{-4} M adjusted at pH 7.4 by concentrated HCl) and left at rest overnight for equilibration. Second, the streaming potential of the bare capillary filled with Tris-HCl buffer is measured. Then, 8 ml of the PEI solution is injected through the capillary with a syringe and left at rest for a 20 min adsorption time. The capillary is rinsed and equilibrated by an injection of 50 ml of Tris-HCl buffer before the streaming potential is measured. The PGA solution (8 ml) is then injected through the capillary in a similar way and the streaming potential is measured by following the same procedure. PLL and PGA adsorption are then alternately performed, and after each deposition step, the streaming potential is measured.

2.5. Preparation of polyelectrolyte multilayer films for bacterial adhesion tests

Polyelectrolyte multilayer films were built up on 12 mm diameter glass slides (CML, France) cleaned

with 10^{-2} M sodium dodecyl sulfate (SDS)/0.12 M HCl for 15 min at 100°C and extensively rinsed with Milli-Q water and were placed into culture plates (Nunc). For all the experiments, the multilayer films were directly deposited on the slides placed in the wells. To this end, the polycation (PEI) was first adsorbed by the deposition of 0.3 ml in a well and left at rest for 15 min. PEI solution was removed and three rinsing steps were performed by the deposition of 1 ml of 0.15 M NaCl at each time. PGA and PLL were then deposited and subsequently rinsed following the same procedure.

2.6. Bacterial adhesion assay

The *E. coli* gram negative strain used for the study has been kindly provided by Dr. Ph. Bocquet (Centre d'Etudes Nucléaires, Saclay, France). In order to obtain fluorescent bacteria, the *E. coli* were transformed using a plasmide bearing the GFPmut3 (Green Fluorescent protein mutant 3) gene under the control of the *Salmonella typhimurium* ribosomal protein promoter (generous gift of Dr. B. Lemaître, CNRS-CGM, Gif-sur-Yvette, France). The transformed bacteria expressing GFP were grown aerobically in Luria Broth (LB) and selected with ampicillin. For the adhesion tests, the bacteria were harvested in mid-exponential phase, diluted in LB to an optical density of 0.1 at a wavelength of 600 nm and plated on the polyelectrolyte multilayers. After 30 min, the cells were rinsed three times with PBS. The bacteria adsorbed on the substrate were visualized using an inverted fluorescence microscope (Nikon, Eclipse TE200) and photographed using a digital camera (DXM-1200, Nikon).

For each condition, i.e. bare glass substrate, PLL-g-PEG (monolayer), $(PLL/PGA\text{-}g\text{-}PEG)_1$, Pre-$(PLL/PGA)_2$-PLL, Pre-$(PLL\text{-}g\text{-}PEG/PGA)_2$-PLL-g-PEG, Pre-$(PLL/PGA)_3$, Pre-$(PLL/PGA\text{-}g\text{-}PEG)_1$ and Pre-$(PLL/PGA\text{-}g\text{-}PEG)_3$ multilayer films, the experiment was performed three times. For each experiment, three different slides were prepared. Fifteen randomly chosen fields were taken per slide at a magnification of $\times 400$ and the mean number of bacteria per counting area was derived for each slide. Later on, the mean number and the standard deviation (SD) were obtained for each condition. With this method, each condition was generating at least $3 \times 3 \times 10 = 90$ counting areas.

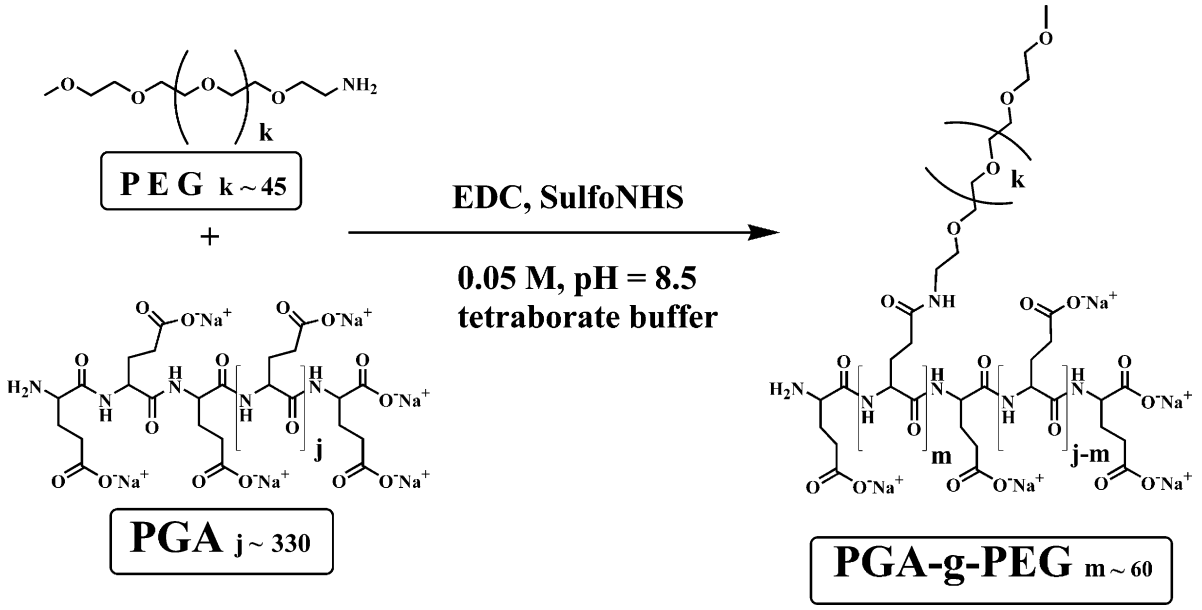
3. Results and discussion

3.1. Synthesis of pegylated poly(L-glutamic acid)

$MePEGNH_2$ was covalently coupled to the PGA backbone (Scheme 1) through an amide bridge in the presence of NHS and EDC. The PGA-g-PEG conjugate was purified by dialysis and then lyophilized. A coupling rate of 16% was determined by ^{1}H-NMR, corresponding to approximately 16 molecules of PEG for 100 molecules of glutamic acid. The calculated molar mass of the copolymer is about 230 000 g/mol.

3.2. Insertion of pegylated polyelectrolytes into multilayers

The stepwise buildup process of the multilayer film was monitored by QCM-D (Fig. 1). The (PLL/PGA) or



Scheme 1. Preparation of PGA-g-PEG polypeptide. Graft ratio was evaluated by ^{1}H-NMR.

(PLL/PGA-g-PEG) bilayers were always adsorbed onto a precursor film of PEI-$(PGA/PLL)_2$-PGA to ensure that the substrate was totally covered as confirmed by AFM observations [55]. Fig. 1a represents the evolution of $\Delta f/v$ at 15 MHz during the buildup of the Pre-$(PLL/PGA\text{-}g\text{-}PEG)_3$ film. The evolution of $\Delta f/v$ for $v = 5$ and 7 can almost be superposed to the one corresponding to $v = 3$. In the case of rigid and thin films, $\Delta f/v$ is proportional to the deposited mass according to Sauerbrey [56] relation. For hydrated films such as (PGA/PLL), this equation may not be valid due to the viscous dissipation in the film and was therefore not used to deduce quantitative data. The $\Delta f/v$ decrease observed after each new PLL, PGA and PGA-g-PEG injection is representative of the film buildup and is associated to an increase in the adsorbed mass. To our knowledge, this is the first time that a pegylated polyelectrolyte, PGA-g-PEG, is inserted at different steps in a multilayer film. A slight increase in $\Delta f/v$ is observed during the rinsing with a 0.15 M NaCl solution and may correspond to a small desorption of polyelectrolyte molecules. The decrease of $\Delta f/v$ accompanying each (PLL/PGA-g-PEG) deposition shows that these multilayers build up despite the lateral PEG chains on the polyanions. Qualitatively, the $\Delta f/v$ decreases measured for PGA and PGA-g-PEG deposition are also different. It appears that the adsorbed mass of PGA-g-PEG is higher than the adsorbed mass of PGA. This could be explained by the difference of molar mass between the two polymers 74 400 g/mol for PGA and 230 000 g/mol for PGA-g-PEG. On the other hand, when both PLL-g-PEG and PGA-g-PEG are used, respectively, as polycation and polyanion, the multilayers can no longer be constructed: the adsorption of PLL-g-PEG onto PGA-g-PEG being insignificant (Fig. 1b).

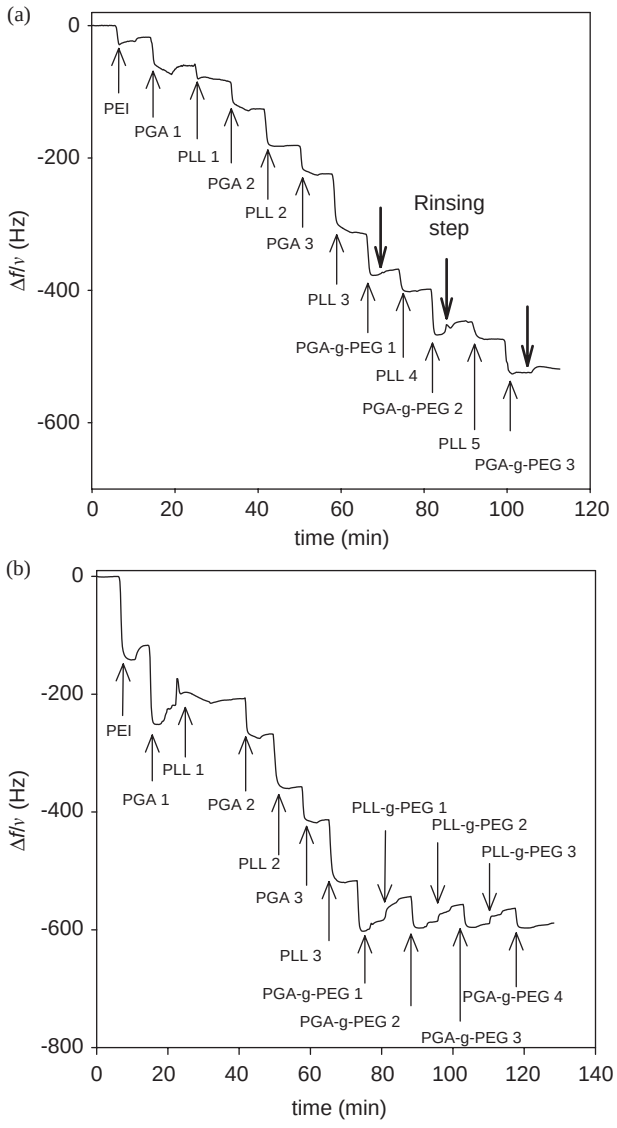


Fig. 1. Frequency shifts ($\Delta f/v$), corresponding to a 15 MHz frequency, as a function of time obtained by QCM-D during the buildup of (a) the multilayer PEI-$(PGA/PLL)_2$-PGA-$(PLL/PGA\text{-}g\text{-}PEG)_3$ and (b) the multilayer PEI-$(PGA/PLL)_3$-$(PGA\text{-}g\text{-}PEG/PLL\text{-}g\text{-}PEG)_3$-PGA-g-PEG.

The streaming potential technique was employed to determine the ζ potential of the film after each new deposition step during the buildup of a Pre-$(PLL/PGA\text{-}g\text{-}PEG)_3$ film (Fig. 2). After the deposition of the first PEI layer, the surface becomes positively charged (+58 mV). Deposition of the first PGA layer changes the ζ potential to a negative value (−42 mV). Then, the ζ potential alternates from positive to negative values for the PLL and PGA layers, respectively, as it was already shown [55]. This indicates that each newly deposited layer leads to an overcompensation of the previous charge excess. When the first PGA-g-PEG layer is added on top of a PLL layer, the ζ potential becomes negative

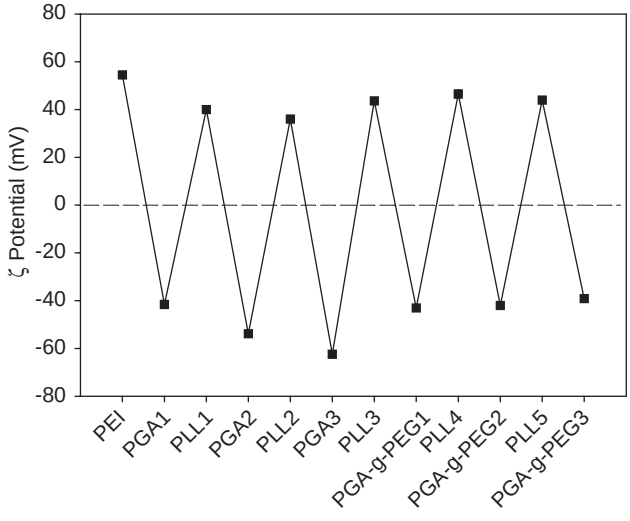


Fig. 2. Evolution of the ζ potential (mV) during the buildup of a PEI-$(PGA/PLL)_2$-PGA-$(PLL/PGA\text{-}g\text{-}PEG)_3$ film. Each data point represents the mean value of three successive measurements. Standard deviations are smaller than the symbol size. The line is only drawn to guide the eye. The ζ potential of silica substrate was -120.9 ± 1.5 mV.

(−42 mV). Further PLL and PGA-g-PEG adsorptions also lead to a total reversal of the ζ potential. This result shows that the buildup of the $(PLL/PGA\text{-}g\text{-}PEG)_3$ film also leads to a charge overcompensation as for non-pegylated architectures. Therefore, the grafting of PEG on PGA, even if the negative charge of the polymer is reduced by about 16%, does not modify its anionic properties or its ability to adsorb onto a positive PLL layer.

3.3. Protein adsorption

When a surface is brought into contact with a biological fluid, the first process that takes place is protein adsorption. We thus investigated serum proteins deposition on top of films ending by PLL, PGA or PGA-g-PEG. All the experiments were performed at the physiological pH of 7.4. Fig. 3 represents the increases of $(-\Delta f/v)$ obtained by QCM-D after the deposition of FBS directly on the SiO_2 surface of the QCM-D crystal and on the different multilayer films. Qualitatively, it appears that adsorption of proteins is maximum on top of the PLL-ending film as was already observed by optical waveguide lightmode spectroscopy for several PEI-$(PLL/PGA)_i$ films, where i is the number of layer pairs [48]. On the other hand, both PGA and PGA-g-PEG ending films lead to a low frequency shift compared to a bare silica crystal, which indicates a reduction of protein adsorption on both films. These results suggest that the adsorption of proteins from serum, which are for most of them negatively charged at pH 7.4, is mainly driven by electrostatic forces. This is confirmed by ζ potential measurements. When serum is put into contact with a Pre-$(PLL/PGA)_2$-PLL film, the ζ potential changes typically from +54 to −32 mV. On the other hand, it decreases from −96 to −55 mV, but remains negative, for a film of Pre-$(PLL/PGA)_3$ and does not change (−39 mV) for a Pre-$(PLL/PGA\text{-}g\text{-}PEG)_3$ film. This last result could be explained either by the fact that there is no more protein adsorption on the PGA-g-PEG ending film or by the fact that the ζ potential of this film is close to the protein-covered surface. As shown by QCM experiments, the amount of adsorbed proteins on a PGA-g-PEG ending films is equivalent to PGA ending films. This result would therefore work in favor of the second explanation.

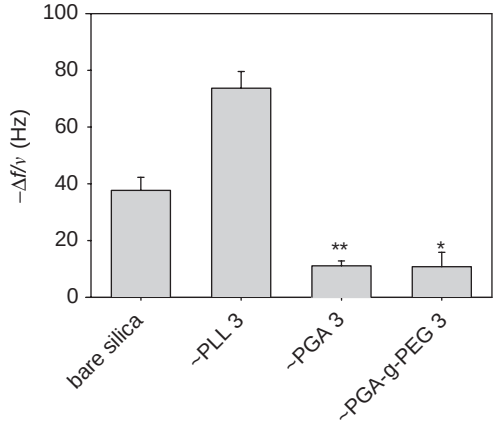


Fig. 3. Frequency shifts measured by QCM-D after contact of bare silica (SiO_2) crystal and the multilayer films with FBS. The PEI-$(PLL/PGA)_2$-PGA precursor film, noted Pre, is followed by multilayer films ∼PLL 3 = Pre-$(PLL/PGA)_2$-*PLL*, ∼PGA 3 = Pre-$(PLL/PGA)_3$, and ∼PGA-g-PEG 3 = Pre-$(PLL/PGA\text{-}g\text{-}PEG)_3$. The numbers represent the mean ± SD of at least three experiments. The Student's test, as compared to bare silica, $^*P<0.05$,$^{**}P<0.01$.

3.4. Bacterial adhesion

Polyelectrolyte multilayer films were tested with respect to the bacterial adhesion (Fig. 4). The fluorescent bacteria were seeded on top of the different films and the adhesion was measured after 30 min of contact. The adhesion is reduced by 72% for a Pre-(PLL/PGA-g-PEG) film compared to a glass substrate taken as control (control: 118.7 ± 20.2 bact/mm^2; ∼PGA-g-PEG 1: 33.7 ± 6.8 bact/mm^2). The bacteriophobicity of the film is increased when three bilayers of (PLL/PGA-g-PEG) are adsorbed on top of the precursor film. In this case, the number of bacteria is reduced by 92% as compared to glass (control: 118.7 ± 20.2 bact/mm^2; ∼PGA-g-PEG 3: 10.1 ± 6.8 bact/mm^2). This result can also be compared to the bacterial adhesion obtained on a monolayer of PLL-g-PEG and PLL/PGA-g-PEG bilayer directly adsorbed on the substrate. The adhesion

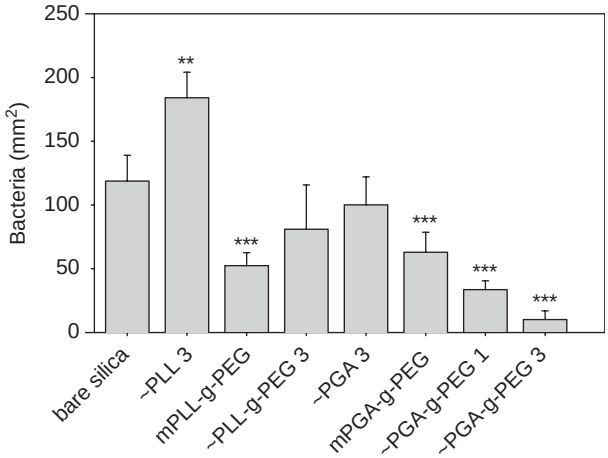


Fig. 4. Bacterial adhesion per mm^2 on bare silica, mPLL-g-PEG = PLL-g-PEG monolayer, mPGA-g-PEG = $(PLL/PGA\text{-}g\text{-}PEG)_1$ and multilayer films ∼PLL 3 = Pre-$(PLL/PGA)_2$-*PLL*, ∼PLL-g-PEG 3 = Pre-PLL-$(PGA/PLL\text{-}g\text{-}PEG)_3$, ∼PGA 3 = Pre-$(PLL/PGA)_3$, ∼PGA-g-PEG 1 = Pre-$(PLL/PGA\text{-}g\text{-}PEG)_1$ and ∼PGA-g-PEG 3 = Pre-$(PLL/PGA\text{-}g\text{-}PEG)_3$ with Pre PEI-$(PLL/PGA)_2$-PGA precursor film. The images were obtained after 30 min contact with the bacteria and gentle rinse with PBS. Each multilayer films was generating at least 90 counting area. The numbers represent the mean ± SD of at least three experiments. As compared to bare silica the Student's test, $^*P<0.05$,$^{**}P<0.01$,$^{***}P<0.001$.

is reduced by 68% on a monolayer of PLL-g-PEG (mPLL-g-PEG: 80.9 ± 34.7 bact/mm^2) and by 53% on a bilayer PLL/PGA-g-PEG (mPGA-g-PEG: 62.9 ± 15.7 bact/mm^2) compared to bare silica (control: 118.7 ± 20.2 bact/mm^2). Besides, statistical analysis showed significant differences ($P < 0.001$ by Student's test) in the adhered bacteria between control and modified surfaces by PGA-g-PEG. This result indicates that the use of several layers of the pegylated polyelectrolyte, through a layer-by-layer buildup, allows the non-adhesive properties of the PEG to be enhanced. Another possibility to increase the anti-adhesive effect on bacteria would be to build a multilayer film with both pegylated polycations and polyanions. For instance, PLL-g-PEG, whose chemical synthesis has already been described in the literature [12], could replace PLL and then may be lead to a total non-adhesive film. However, according to our results, the multiplication of pegylated layers leads to a destabilization of the film due to the repulsive properties of the PEG. Indeed, the adsorption of PLL-g-PEG onto PGA-g-PEG ending film is not significant, as the buildup of (PLL-g-PEG/PGA-g-PEG) bilayers in QCM-D shows no changes in frequency (Fig. 1b).

Bacteria were adsorbed on the films in the presence of Luria Broth medium, which contains nutrients for bacteria growth and among them many proteins. We tested also the bacterial adhesion on the pegylated films in the presence of serum, which contains mainly albumin. If we pretreated the pegylated multilayers 45 min with 10% bovine serum or if we put the bacteria on the pegylated multilayers with 10% bovine serum in the medium, no difference is seen with the control (control: $\sim$PGA-g-PEG 3: 10.1 ± 6.8 bact/mm^2; 45 min serum pretreated $\sim$PGA-g-PEG 3: 12.6 ± 6.0 bact/mm^2; serum in the bacterial medium on $\sim$PGA-g-PEG 3: 9.0 ± 1.6 bact/mm^2). Our experiments show that the presence of serum does not change the reduction of bacterial adhesion.

A reduction in bacterial adhesion of *E. coli* is obtained by using a multilayer ending by three bilayers of PLL/PGA-g-PEG as compared to Pre-(PLL/PGA)$_3$ film (Fig. 4). This reduction is not correlated with a reduction in adsorption of proteins (Fig. 3). We find for example that a similar amount of serum proteins is adsorbed on Pre-(PLL/PGA-g-PEG)$_3$ and Pre-(PLL/PGA)$_3$ films even if the former film is bacteriophobic. Other authors already found an absence of correlation between the ability of a surface to adsorb protein and to help bacterial [57] or cells [58] adherence. Most bacterial surfaces present hydrophobic areas [59,60]. Moreover, hydrophobic forces appear to be the most important physicochemical interactions for bacterial adhesion [16]; therefore, the strong association of water around PEG chains sterically should inhibit bacterial attachment as it has been shown for cell [58]. In accordance with Park et al. [61], our observation of a low adhesion of bacteria on pegylated multilayer films could be explained by the high hydration of PLL/PGA-g-PEG multilayers.

4. Conclusions

The pegylated polyelectrolyte multilayer technique described in this article represents a new and powerful tool to create anti-adhesive surfaces aimed at protecting implanted biomaterials against bacterial infection. A multilayer of PLL/PGA-g-PEG significantly improves the anti-microbial protection. This technique offers several advantages over conventional surface protection by PEG. First of all, it is an inexpensive technique, easy to use and therefore accessible for the commercial development of many applications. Second, because the buildup process of pegylated multilayers is based on the same mechanisms as for conventional polyelectrolyte multilayer films, the pegylated multilayers could in principle cover almost any type of biomaterial surface. This simple method would therefore replace the use of any covalent biomaterial surface modification.

The possibility of inserting in films several layers of pegylated polyelectrolytes by the layer-by-layer technique offers a striking advantage over the adsorption of a PEG monolayer. Moreover, polyelectrolyte multilayer films can also be used to functionalize the surface by incorporating peptides or therapeutic molecules giving to anti-microbial film other biological properties [62,63]. We are now investigating the effect of these films in vivo and also evaluating their stability with respect to different biological fluids and enzymes.

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