Understanding the Effect of Hydrophobic Protecting Blocks on the Stability and Biopassivity of Polymer Brushes in Aqueous Environments: A Tiramisù for Cell-Culture Applications

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ABSTRACT.

Despite the wide range and versatility of polymer-grafting methods to produce biopassive interfaces, commonly applied polymer brushes suffer from an intrinsic lack of long-term stability when incubated in aqueous media under physiological conditions. The robustness of brush films in such environments can be greatly improved by applying a hydrophobic, “protecting” layer

between the supporting substrate and the interfacial, biopassive brush. Block-copolymer brushes synthesized by sequential surface-initiated atom transfer radical polymerization (SI-ATRP) forming well-defined bilayered architectures can produce coatings with tunable interfacial properties and improved stability when subjected to cell-culture conditions. Three poly(n-alkyl methacrylates) (PAMA) exhibiting different mechanical properties, have been tested as protecting layers. A biopassive, hydrophilic brush is chosen to constitute the interfacial layer in all three cases. On the one hand, the structure of the interfacial layer clearly regulates film biopassivity, as well as its nanomechanical and nanotribological properties, as determined by atomic force microscopy (AFM) techniques. On the other hand, the composition and the mechanical properties of the polymer constituting the substrate-bound layer govern the stability of the entire bilayered film in the aqueous cell-culture environment. Precise control of bilayered brush architecture thus determines the film's applicability for cell manipulation or biomaterials surface engineering.

INTRODUCTION

Biomaterials surface modification by polymer grafting has been increasingly applied as an effective method to provide passivation against non-specific biological contamination (e.g. preventing adhesion of proteins and microorganisms)¹⁻⁶. Furthermore, polymer grafting has been applied, in order to introduce specific functions for the immobilization of biomolecules, while also modulating the interfacial physico-chemical properties of the underlying supports⁷⁻¹¹. This versatile strategy has been successfully applied to enhance the sensitivity and selectivity of bio-sensors¹², to direct stem cell settlement on scaffolds for tissue engineering¹³ and to improve the integration of synthetic implants within the body^{14,15}. Despite the wide range of polymer compositions and diverse grafting protocols that have been applied, such “brush” layers¹⁶ often suffer from an intrinsic lack of long-term stability when they are applied within “real” biological media. Depending on the composition of the brushes and the nature of the functionalized substrates, failure of the polymer coating can occur either due to polymer-brush degradation¹⁷ or detachment of entire chains by cleavage of the anchoring groups to the underlying substrate¹⁸⁻²⁰. This last shortcoming is more difficult to tackle, due the limited chemistries available for the functionalization of most inorganic and polymeric supports. In the case of polymer brushes fabricated by means of surface-initiated polymerization (SIP) from inorganic surfaces, self-assembled monolayers (SAMs)²¹ of silane-, catechol- or thiol-bearing initiators are initially fabricated on silicon dioxide²², metal oxide²³⁻²⁶ or gold surfaces²⁷, respectively. In all these cases, linkages to the grafting substrates are susceptible to oxidation in aqueous environments, and moreover, the initiator adsorbates often contain ester or amide functions that can be hydrolyzed under physiological conditions^{20,28-30}. In order to improve the durability of hydrophilic polymer brushes when immersed in physiological media, the most successful

approach has relied on shielding the linkages to the underlying substrate by introducing an additional, substrate-bound polymer layer that is insoluble in the medium. In other words, a hydrophobic, “protecting layer” can be introduced between the surface anchors and the interfacial, hydrophilic brush, in order to reduce solvent permeation to the grafting surface. This method was successfully exploited by Quintana et al.³¹, who fabricated block-copolymer brushes incorporating a substrate-bound, hydrophobic polystyrene (PS) or poly(methyl methacrylate) (PMMA) layer and an interfacial, hydrophilic poly(sulfobetainemethacrylamide) (PSBA) block, to produce biopassive coatings that could resist marine fouling for more than 14 weeks. Paripovic et al. also exploited a similar strategy to improve the stability of poly(methacrylic acid) (PMAA) brushes in cell-culture medium, through the introduction of a PMMA block as a substrate-bound, protecting layer¹⁹. Although in the aforementioned studies the presence of a hydrophobic underlayer improved the stability of hydrophilic brushes, a systematic investigation on the influence of the supporting layer properties on the physico-chemical characteristics of the bilayered films has never been undertaken. Alternative approaches to improve coating stability have involved the reduction of brush grafting density, thus depleting osmotic-pressure-driven brush desorption. Additionally, different anchor chemistries have been investigated to minimize initiator hydrolysis and the subsequent detachment of the grafted chains^{18,19,32}. Although both these strategies enhanced the endurance of the grafted films, in many cases they also triggered film interactions with proteins from the surrounding medium, thus reducing the antifouling performance of the entire coating. Aiming to systematically investigate how polymer-brush architecture can be modulated to improve the stability of anti-biofouling films, we report the synthesis and characterization of different block-copolymer brushes forming bilayered coatings, and displaying different structural stabilities

when incubated in cell-culture medium under physiological conditions. Block-copolymer brush coatings were synthesized by sequential, surface-initiated atom-transfer radical polymerization (SI-ATRP)³³ from initiator-functionalized silicon dioxide surfaces. These comprised three different protecting, substrate-bound poly(n-alkylmethacrylates) (PAMA) layers, and a biopassive, interfacial layer, based on poly(oligoethylene glycol methacrylate) (POEGMA)³⁴.

The interfacial properties, and the nanomechanical and nanotribological characteristics of the different bilayered films were studied by a variety of methods, including Fourier-transform infrared spectroscopy (FTIR), water contact angle (CA), variable angle spectroscopic ellipsometry (VASE), atomic force microscopy (AFM), nanoindentation^{35,36}, and lateral force microscopy (LFM)³⁷. These analyses highlighted how each particular bilayered brush architecture markedly determined the physico-chemical properties of the films. In particular, the composition of the substrate-bound layer was shown to influence the density of the interfacial POEGMA brush, consequently regulating swelling and biopassive performance of the whole film. Additionally, the nature of the protecting layer also affected the endurance of the bilayered films, which was systematically tested in cell-culture media at 37°C.

The results obtained from this study suggest how a precise modulation of brush composition, within stratified architectures, enables the fabrication of biopassive coatings, presenting a substantially improved stability in physiological media. Such an approach could potentially be applied to improve the stability of brushes grown from silicon dioxide, metal oxides or gold surfaces. These designed coatings could thus find numerous applications in the surface engineering of biomaterials intended to be applied as cell-culture platforms (such as scaffolds and implants), or for body-fluid contact.

EXPERIMENTAL

Materials

Dimethylchlorosilane (Sigma-Aldrich, 98%), 10-undecen-1-ol (Sigma-Aldrich, 98%), 2-bromo-2-methylpropionyl bromide (Acros Organics, 98%), and chloroplatinic acid hexahydrate (ABCR Deutschland 99.9%) were used as received for the synthesis of the SI-ATRP initiator. Methyl methacrylate (MMA) (Sigma-Aldrich, 98%), butyl methacrylate (BMA) (Sigma-Aldrich, 98%), lauryl methacrylate (LMA) (Acros Organics, 96%), and oligo ethylene glycol methacrylate (OEMGA) (Sigma-Aldrich average $M_n=360$) were purified from hydro-quinone monomethyl ether (MEHQ) inhibitor by passing them through a basic alumina column. 4,4'-dinonyl-2,2'-bipyridine (dNbpy) (Sigma-Aldrich, 97%), 2,2'-bipyridine (Sigma-Aldrich, 99%), anisole (Acros Organics, 99%), methanol (Sigma-Aldrich HPLC grade), Dulbecco's modified eagle's medium (DMEM)-low glucose (Sigma-Aldrich), Fibrinogen from human plasma (Alexa Fluor® 546) and copper(II) bromide (CuBr_2 , Sigma-Aldrich, 99%) were used as received. Copper(I) bromide and copper(I) chloride (CuCl and CuBr , Sigma-Aldrich) were purified by stirring in glacial acetic acid overnight, followed by filtration and washing with methanol and diethyl ether. 2-[4-(2-hydroxyethyl)piperazin-1-yl] ethane sulfonic acid (HEPES) (Sigma-Aldrich) was used as received to prepare buffer solutions ($\text{pH} = 7.4$) with the addition of 10 mM NaCl (Sigma-Aldrich) to yield "HEPES-2" mixtures for protein-adsorption studies.

Synthesis of bilayered, block-copolymer brush films. Piranha-cleaned, surface-oxidized silicon substrates (P/B <100>, Si-Mat Silicon Wafers, Germany) were functionalized via a previously described surface-deposition protocol³⁸, using a solution of 11-(2-bromo-2-methylpropionyl)-dimethylchlorosilane (BPCS) initiator, synthesized according to Sanjuan et al³⁹.

All polymerizations were carried out under a nitrogen atmosphere. In a typical SI-ATRP to obtain a substrate-bound brush layer, the alkyl methacrylate monomer (MMA, BMA or LMA) was mixed with anisole in the ratio of 9:1 v/v. Then the ligand (dNbpy) was added to the mixture and dissolved. The solution was degassed by three freeze-thaw cycles and was subsequently transferred through a cannula into a second flask containing CuBr and CuBr₂ (typical reactant ratio monomer:dNbpy:CuBr:CuBr₂ = 150:1.5:1:0.01). The mixture was subsequently heated to 90°C and kept under stirring until a homogenous dark brown solution was formed. Finally, the solution was transferred via a cannula to a flask containing the BPCS-functionalized substrates and SI-ATRP was carried out for the necessary time. The polymerization temperatures were varied as a function of the particular monomer, namely MMA, BMA and LMA were polymerized at 95, 100, 110°C, respectively. SI-ATRP of the substrate-bound layers was terminated by the addition of an excess of quenching solution, consisting of a degassed mixture of CuBr₂ and dNbpy dissolved in anisole (anisole:dNbpy:CuBr₂ = 150:1.5:1). Termination was carried out for 30 minutes, after which the substrates were removed from the flask, washed extensively with toluene and chloroform and finally dried under stream of nitrogen.

The grafting of interfacial POEGMA brushes by SI-ATRP from “quenched” PAMA layers was carried out following similar experimental procedures to the substrate-bound blocks with the following molar ratios of reactants: OEGMA:2,2' bipyridine:CuCl:CuBr₂ = 1200:18:4.5:1. In detail, OEGMA was dissolved into a water/methanol mixture (OEGMA:water:methanol = 6.5:4:1 v/v). To this solution, 2,2' bipyridine was added and the mixture was degassed by argon bubbling for 30 minutes. Later on, the solution was transferred via a cannula into a flask containing CuCl and CuBr₂ and kept under stirring until a homogenous solution was formed. Finally, the polymerization mixture was transferred to flasks containing the PAMA-coated and

Figure 1 illustrates the synthesis of the block copolymer brush. (a) Schematic of the SiO₂ surface with a silane monolayer and a brominated initiator. (b) Polymerization of alkylmethacrylate in a flask containing CuBr, CuBr₂, dNbpy, and anisole for 20-60 min. (c) Polymerization of CuBr₂, dNbpy, and anisole for 30 min. (d) Polymerization of OEGMA, CuCl, bpy, and water/methanol for 240-300 min. (e) Schematic of the final block copolymer brush structure with a SiO₂ core, a PMMA block (n), and a PEO block (m).

Methods

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$$n=A+B\cdot\lambda^{-2}$$

For dry measurements, the ambient was air, for which a refractive index $n = 1$ was taken. The film thickness (T) and the two Cauchy parameters A and B were set as fitting parameters. For measuring the thickness of bilayered films in water, the swollen POEGMA layer was fitted by a two-component effective medium approximation (EMA) model, consisting of a Cauchy and a water component. Also in this medium, a layered model (Si/SiO₂/PAMA(Cauchy)/EMA-POEGMA/water-ambient) was used to determine the swollen thickness of the POEGMA layer. In this case, the fitting was done by setting d , A and B values as constants for the PAMA (corresponding to the parameters measured prior to the polymerization of the POEGMA block), and considering T , and water content (w) of the EMA-POEGMA layer as fitting parameters. The values of A and B obtained in dry conditions for the corresponding POEGMA layer were assigned to the Cauchy component of EMA-POEGMA in the aqueous medium.

AFM nanoindentation and LFM measurements were performed using an AFM (MFP3D®, Asylum Research, Oxford Instruments, Santa Barbara, USA) equipped with a liquid cell. The normal spring constants of the cantilevers (tipless Mikromash, NSC-35, Estonia and Olympus OMCL-AC160TS-C3, Japan) were measured by the thermal-noise method ⁴⁰ and the torsional spring constant was measured according to Sader's method ⁴¹. The probes used for nanomechanical and nanotribological measurements consisted of a calibrated cantilever, onto which a silica micro-particle of 6 μm radius (EKA chemicals AB, Kromasil R, Sweden) was glued. These probes were fabricated by applying a micro droplet of UV-curable adhesive (Norland optical adhesive, New Jersey, USA) to the end of the tip-less cantilever using a custom-made micromanipulator.

AFM height micrographs of the films were taken using a Bruker Icon Dimension in air and ultrapure-water using tapping and peak-force tapping modes respectively. For tapping mode an Olympus-OMCL-AC160TS-C3 (Japan) with a spring constant of 26 N/m was used. For peak-force mode a Bruker-SNL-10 tip with a spring constant of 0.35 N/m was used.

FTIR spectra were recorded in vacuum with an infrared spectrometer (IFS 66 V, Bruker Optik GmbH, Germany) equipped with a liquid-nitrogen-cooled MCT detector. FTIR measurements were performed in transmission mode, while a background spectrum was collected from a freshly cleaned, bare silicon wafer.

Static water contact angles were measured with a RameHart goniometer (RameHart Instrument Co., Model-100, New Jersey, USA).

Protein-adsorption experiments. Polymer-brush-coated and bare silicon dioxide substrates were immersed in HEPES-2 buffer for 30 minutes, in order to hydrate the interfacial POEGMA layer before contacting protein-containing solutions. Subsequently, the hydrated films were immersed into a 50 $\mu\text{g/ml}$ solution of fibrinogen-Alexa Fluor 546 conjugate (Fgn) dispersed in HEPES-2 buffer. After 3 hours of incubation, the samples were removed from the protein solutions and immersed in HEPES-2 buffer for 15 minutes, then they were washed several times with ultra-pure water and dried under a stream of nitrogen.

The fluorescence intensity of the samples before and after exposure to protein solution was measured by a fluorescence microscope (Zeiss 200M with motorized excitation/emission filter wheels, LUDL) at a fixed exposure time of 1.5 seconds. The images were then processed by ImageJ (<http://imagej.nih.gov/ij>) to obtain grayscale-intensity values of the samples before and after exposure to protein solutions. To determine the relative protein adsorption, the intensity of

the image before exposure to protein was subtracted from the intensity of the image obtained after exposure to protein solutions.

Stability experiments. In order to evaluate the structural stability of the bilayered PAMA-b-POEGMA brush films under cell-culture conditions, the samples were immersed in DMEM cell-culture medium and incubated 37°C, 100% of relative humidity (RH) and 5.5% of CO₂ (Mettert, INCO 2, Mettert GmbH, Germany) for different times, varying from 12 hours to 7 days. Three samples for each homopolymer and bilayered film were analysed after each day of incubation. At each time point, each sample was washed extensively with ultra-pure water and finally dried under a stream of nitrogen. The surface of the treated films was subsequently imaged with an optical microscope (Zeiss Axioskop 2 Plus) to evaluate film deterioration. The optical micrographs after 0, 4, 6 and 7 days of incubation were reported to highlight the differences in film stability.

RESULT AND DISCUSSION

Sequential SI-ATRP of bilayered brushes. The synthesis of bilayered brush films was carried out by sequential SI-ATRP from initiator-functionalized silicon dioxide surfaces. Following overnight incubation in a 10mM solution of BCPS, the successful formation of initiator SAMs on silicon dioxide substrates was confirmed by static water contact angle (CA) and VASE measurements, which indicated a significant change from bare, piranha cleaned hydrophilic silicon dioxide substrate (CA <10 °) to a hydrophobic layer exhibiting CA = 78°±3 and average dry thickness of 0.8 ± 0.2 nm^{38,39}. Following the first SI-ATRP step, PAMA brush layers with average dry thicknesses of between 20 and 30 nm were obtained, as measured by VASE (Table 1).

Table 1. Dry thicknesses (measured by VASE) and static CA values of the different polymer brush films following each step of polymerization.

Sample	Block 1		Block 2		Total thickness[nm]
	Thickness [nm]	Contact angle	Thickness [nm]	Contact angle	
PLMA-b-POEGMA	21±3	105°±2	27±5	51°±2	48±5
PBMA-b-POEGMA	27±4	85°±2	36±6	50°±2	63±6
PMMA-b-POEGMA	23±3	75°±2	21±5	46°±3	44±5
POEGMA	49±2	45°±3	-	-	-

The composition and physico-chemical properties of the different substrate-bound layers were subsequently investigated. Static water CA measurements showed that the hydrophobicity of the PAMA brush layers increased with the length of the alkyl side-chains (n) in the monomer unit. PLMA ($n=12$) brush films showed the highest CA value, followed by PBMA ($n=4$) and PMMA ($n=1$) (Table 1). FTIR spectra of all the different PAMA brushes showed a strong band at 1740 cm^{-1} , which was attributed to the C=O stretching of the ester groups in the methacrylate units (Figure 1a). Additionally, distinct bands appeared between 2500 cm^{-1} and 3050 cm^{-1} , corresponding to the C-H stretching vibrations by the alkanes. In case of PMMA brush films, two strong bands in this region are attributed to the C-H stretching of the CH_2 units in the polymer backbone, and the CH_3 groups of the MMA monomer unit. PBMA and PLMA films both showed three bands in the same region, corresponding to the C-H stretching of the CH_2 in the polymer backbone and the CH_2 and CH_3 groups in the side chains (Figures 1b and 1c).

Figures 2a-d show the AFM height micrographs of the PAMA films. All the homopolymer brushes present a uniform surface with a root mean square roughness (R_q) ranging from 0.3 to 1.1 nm (calculated over $10 \times 10 \mu\text{m}^2$).

Figure 1. FTIR spectra of polymer coatings and their corresponding blocks. a) POEMGA, PMMA and PMMA-b-POEGMA. b) POEGMA, PBMA and PBMA-b-POEMGA. c) POEGMA, PLMA and PLMA-b-POEGMA.

Force-vs-indentation (FI) curves obtained by AFM nanoindentation in water revealed the contrast in the mechanical properties between the three different substrate-bound brush layers (Figure 3). The FI curve for PMMA showed a steeper slope after contact point, compared to the corresponding profiles recorded for PBMA and PLMA films, suggesting that this brush showed the highest modulus among the substrate-bound films. It should be noted that the glass transition temperatures (T_g) for bulk PMMA, PBMA and PLMA are centered approximately at 105°C, 20°C, -65°C, respectively⁴². Hence, the PMMA brush film is in its glassy state, whereas the PLMA and the PBMA brushes are rubbery layers. The reduced Young's modulus (E) of the films was estimated from the measured FI curves applying the Hertz⁴³ model:

$$F = (4\sqrt{R/3(1-\nu^2)})E\delta^{1.5}$$

Where F is the applied load, R is the radius of the colloid used as a probe and ν is the Poisson's ratio (considered to be 0.5). By averaging calculated E from 50 force curves on different points

of the sample, the modulus of the PMMA brush was estimated 2100 ± 300 MPa, more than twice that of the average E value for PBMA (886 ± 83 MPa) and two orders of magnitude higher than the average E measured for PLMA (60 ± 23 MPa). The estimated E values for the different PAMA films were found to be in good agreement with the Young's moduli measured for glassy and rubbery polymer thin-films and brushes with AFM^{35,44}.

Figure 2. AFM tapping mode height micrographs of homopolymer and block-copolymer brushes. a) PMMA, $R_q = 1.0$ nm; b) PBMA, $R_q = 1.1$ nm; c) PLMA, $R_q = 0.4$ nm; d) POEGMA, $R_q = 0.4$ nm; e) PMMA-b-POEGMA, $R_q = 1.7$ nm; f) PBMA-b-POEGMA, $R_q = 2.6$ nm; g) PLMA-b-POEGMA, $R_q = 1.8$ nm (all the R_q were calculated over $10 \times 10 \mu\text{m}^2$).

The molecular weight (M_w) of the PAMA coatings can be estimated by the following equation⁴⁵:

$$M_w = d\rho N_A / \sigma$$

Where d is the dry thickness, ρ is the density of the bulk polymer, N_A is Avogadro's number and σ is the grafting density. In a previous study in our group⁴⁶, the grafting densities and molecular weights of PAMA brushes synthesized by means of SI-ATRP from a silicon dioxide substrate were determined by employing a UV-cleavable initiator. UV-detached polymer chains were

analyzed by gel-permeation chromatography (GPC), and grafting densities of PMMA, PBMA and PLMA polymer brushes were found to be 0.36, 0.16 and 0.15 chains/nm² respectively. These values corresponded well with values estimated in the literature for PAMA brushes ^{26,47}. Assuming that our coatings have similar grafting densities and inputting these values and the corresponding dry thicknesses of our PAMA films into the above equation, we estimate the average molecular weights of the PMMA, PBMA and PLMA brushes to be 46kDa, 111kDa, and 93kDa, respectively.

Following the grafting of the first PAMA block for the desired reaction time, SI-ATRP was interrupted by injection of a degassed quenching solution, consisting of an excess of Cu(II)-dNbpy complex. In this way, a high concentration of active, bromide-bearing chain ends could be retained for the subsequent re-initiation during the second SI-ATRP step ^{48,49}. In order to avoid the synthesis of a mixed-brush structure within the substrate-bound block ^{50,51}, SI-ATRP of the interfacial, POEGMA brush layers was conducted in a water/methanol mixture, which is a poor solvent for the substrate-bound PAMA brush layers. In this way, the collapsed hydrophobic brushes can act as resist films, inhibiting the diffusion of the polymerization medium to the unreacted initiator molecules at the grafting substrate, and confining the growth of the POEGMA brush at the film interface ⁵².

Following SI-ATRP of OEGMA from the PAMA brushes, the presence of each targeted bilayered architecture was confirmed by a substantial decrease of the measured CA, and by a near doubling of the dry film thickness, as measured by VASE (Table 1). Additionally, for all the different bilayered films, the recorded CA values were found to be very similar to that measured for a homopolymer POEGMA brush film, confirming the fabrication of purely POEGMA-containing outer interfaces. The FTIR spectra collected for PMMA-, PBMA- and PLMA-b-

POEGMA films also verified the presence of an additional POEGMA brush layer by the appearance of a broad band centered at 3450 cm^{-1} , and attributed to OH stretching of the terminal hydroxyl groups of OEG side chains, and by an increase in the relative intensity of the C=O signals at 1740 cm^{-1} (Figure 1a-c).

Furthermore, the addition of the POEGMA blocks on the PAMAs resulted in a slight increase of R_q on all samples, with values ranging from 1.7 to 2.6 nm ($10 \times 10\text{ }\mu\text{m}^2$) (Figures 2e-g), when recorded in air, and from 0.8 to 2.8 nm ($10 \times 10\text{ }\mu\text{m}^2$) in ultra-pure water (Figure S1). The relatively low R_q values obtained for all the PAMA-b-POEGMA films and their uniform morphology confirmed the homogeneity of the bilayered films.

Figure 3. Force-vs-indentation profiles recorded on the different PAMA blocks in water (spring constant: 21 N/m, tip diameter: 15 nm).

Protein adsorption. The antifouling properties of the different brush architectures were evaluated by monitoring the adsorption of fluorescently-labelled fibrinogen (Fgn). This particular protein was especially chosen as it is very sensitive to surface defects on antifouling films, as demonstrated by Pei et al ⁵³. Among all the tested bilayered films, the thickness of POEGMA brush layers was very similar. Additionally, protein adsorption was also tested on a

homopolymer POEGMA brush film, presenting a comparable thickness value to that of the POEGMA component in each bilayered film (Table 1). As expected, the “pure” POEGMA brush showed very low protein adsorption compared to a bare silicon dioxide substrate used as control^{18,19,34,54,55} (Figure 4). In contrast, Fgn adsorption on the different bilayered films was found to be strongly dependent on the nature of the particular substrate-bound PAMA layer. PMMA-b-POEGMA showed a marked reduction in protein adsorption compared to the silicon dioxide surface, with similar biopassive properties to those of the POEGMA homopolymer brush.

Figure 4. Relative fluorescence intensity from a fibrinogen-Alexa Fluor 564 conjugate adsorbed on a silicon dioxide substrate, a POEGMA homopolymer brush film and the different bilayered coatings. The relative fluorescence intensity was considered as a measurement for the amount of unspecifically physisorbed proteins (the original fluorescence micrographs are provided in the Supporting Information).

In contrast, Fgn adsorption progressively increased on PBMA-b-POEGMA and PLMA-b-POEGMA bilayered films. Raw fluorescence microscope images (Figure S2) after exposure to proteins confirmed the results reported in Figure 3, where PLMA-b-POEGMA showed

considerably higher protein adsorption compared to other PAMA-b-POEGMAs and bare silicon dioxide.

We believe that this could be due to the lower grafting density of POEGMA interfacial brushes when grafted from PBMA and PLMA substrate-bound layers, when compared to PMMA films. As previously mentioned, the recent study by Kang et al. highlighted how PMMA, PBMA and PLMA brush films synthesized by SI-ATRP and presenting similar dry thicknesses, displayed a gradual decrease of brush grafting densities from the less bulky PMMA to the more bulky PLMA, as determined by gel-permeation chromatography of detached polymer chains ⁴⁶. Densely packed PMMA grafts thus allowed the formation of a denser and a more biopassive POEGMA brush interface, which efficiently inhibited both primary and secondary protein adsorption ⁵⁶. A similar dependence of nonspecific protein adsorption on brush grafting density has also been observed in other studies ^{50,51}, especially in the case of POEGMA homopolymer brush films ⁵⁷.

In order to further confirm the structural representation provided for the PAMA-b-POEGMA bilayered films, VASE was performed in aqueous medium, to estimate the swelling ratio ($T_{\text{swollen}}/T_{\text{dry}}$) of the interfacial POEGMA layers in the different samples (Figure 4). For this purpose, we assumed that the main contribution to the swelling of the bilayered films was from the POEGMA layer, and the swelling of the hydrophobic PAMA block is negligible to that of the POEGMA block. This assumption was backed by the fact that including the thickness of the PAMA layer as a fitting parameter in the model did not improve the fit.

For a given grafted-chain length (and thus polymerization time), a variation of swelling ratio by POEGMA brushes represents an indirect but reliable indication of a change in grafting density ⁵⁷⁻⁵⁹. As highlighted in Figure 5 the lowest value of $T_{\text{swollen}}/T_{\text{dry}}$ was recorded for POEGMA

homopolymer brushes, indicating that homopolymerization from initiator-functionalized silicon dioxide substrates assured the formation of dense brushes by SI-ATRP. PMMA-b-POEGMA bilayered films showed an increase of around 10% of POEGMA swelling ratio, while a more pronounced increment of $T_{\text{swollen}}/T_{\text{dry}}$ values by POEGMA was measured for PBMA- and PLMA-b-POEGMA, which displayed around 18 and 27% increases of water content within the interfacial layer. These experimental results corroborated the progressive decrease of POEGMA grafting density with the increasing bulkiness and thus with the decreasing coverage of the substrate-bound grafts. Notably, the fluorescence-microscopy measurements reported in Figure 4 evidenced a remarkable, nearly threefold increase in the intensity of adsorbed Fgn on PLMA-b-POEGMA films, when compared to PBMA-b-POEGMA. This increase of protein adsorption was presumably due to presence of defects on the low-grafting-density POEGMA interfacial brush when grafted from PLMA and PBMA. Hence the higher hydrophobicity of the PLMA underlayer ($CA=105^\circ$) enhanced the affinity for the adsorbing proteins when compared to PBMA ($CA=85^\circ$). In addition, this observation confirmed the importance of the substrate chemical composition (in these cases considered as the substrate-bound, “resist” layer) to determine primary protein adsorption, also for a relatively big protein like Fgn, which usually undergoes mainly secondary adsorption on surface-grafted polymer layers^{56,60–62}

Figure 5. Swelling ratio of the POEGMA layer of polymer coatings measured by VASE

Nanomechanical and nanotribological properties of bilayered brush films. AFM nanoindentation and LFM in HEPES-2 buffer were subsequently applied to probe the nanomechanical and nanotribological properties of the different bilayered brush films synthesized. Figure 6 shows typical AFM approaching and retracting profiles obtained for the bilayered films and a homopolymer POEGMA brush film. Hertz fitting of the first 10% of the approaching profiles allowed an estimation of the apparent Young's moduli of the interfacial, POEGMA layers on each film (Figure 6a). From these measurements, the resulting E values showed a maximum for the POEGMA homopolymer brush and a gradual decrease from PMMA- to PBMA- and PLMA-b-POEGMA (Figure 7a). This trend suggested that POEGMA grafting density was directly correlated to the interfacial stiffness of the layer, i.e. denser POEGMA grafts formed more rigid and elastic interfaces, while loosely grafted brushes incorporating higher amount of water displayed a more compliant character. It should be noted that by taking into consideration just the top 10% of the indentation profiles to estimate the film stiffness, possible substrate effects on the measured E values are expected to be reduced. Nonetheless,

given the different mechanical properties of the underlying PAMA layers and the expected stress field propagation within the whole bilayered films, a certain dependence of the recorded stiffness on each particular bilayered architecture and on the supporting silicon dioxide substrate cannot be *a priori* ruled out. The average adhesion force on each brush architecture studied was recorded from the retracting profiles of the FI curves (representative profiles are reported in Figure 6b for each film tested).

Figure 6. a) Force vs. indentation (approach). b) Force vs. distance (retraction) curves of the bilayered polymer brushes obtained with AFM colloidal probe microscopy (CPM) (spring constant: 0.9 N/m, diameter of silica probe: 12 μm) in HEPES-2 buffer.

Figure 7. Apparent a) modulus and b) adhesion of stratified polymer films measured by AFM colloidal probe microscopy (CPM) (spring constant: 0.9 N/m, diameter of probe: 12 μm) in HEPES-2 buffer. Each data point is averaged over 50 force curves.

The recorded adhesion values were found to be similar for the different films (Figure 7b), although the bilayered brushes showed slightly higher pull-off force than the POEGMA

homopolymer. This result could again be related to the decreasing interfacial grafting density by POEGMA grafts between homopolymer and block-copolymer films⁵⁸. More loosely grafted POEGMA brushes can be more easily stretched away from the surface by an interacting AFM probe when compared to more densely packed grafts^{60, 61}, as is suggested by the long-range, multiple detachment events observed in the typical retracting profiles recorded for PBMA-b-POEGMA and PLMA-b-POEGMA (Figure 6b). In addition, the lower POEGMA coverage on the bilayered films could also lead the AFM probe to interact with the underlying, hydrophobic PAMA layers, thus producing higher recorded pull-off forces. Hence POEGMA interfacial density strongly determines the nanomechanical properties of the brush surfaces. In order to investigate whether this parameter also influenced the nanotribological properties of the diverse synthesized films, LFM was subsequently performed. Figure 8 shows the friction-vs-load (FL) plots recorded on the POEGMA homopolymer brush and the bilayered films. The slope of the FL profiles provides the coefficient of friction (COF) of each coating. As evidenced by the different FL profiles, friction gradually increased from POEGMA homopolymer brush, to PMMA-b-POEGMA, PBMA-b-POEGMA and PLMA-b-POEGMA, which showed the highest friction among the brush films tested. As was already observed in a previous report from our group⁵⁰ the nanotribological characteristics of multilayered films are commonly determined by the chemical composition and the properties of the interfacial layer alone. Accordingly, the POEGMA brush governs the nanotribological properties of bilayered films. The difference in COF can thus be attributed to the variation of the grafting density of the POEGMA layer among the different brush architectures studied. In line with this finding, Yan et al. and Bhairamadiet al. investigated the relationship between brush grafting density and friction also observing how lower brush coverages led to higher COF^{59,63}

Figure 8. Friction vs. load plots of polymer coatings, measured by CPM (torsional spring constant: $3\text{E-}7$ N.m, diameter of silica probe: $12\text{ }\mu\text{m}$, stroke length: $2\text{ }\mu\text{m}$, stroke speed: $4\text{ }\mu\text{m/s}$ in HEPES-2 buffer

Stability of brush films in cell culture media. Although POEGMA homopolymer brushes have been reported to display outstanding biopassive properties⁶⁴ they suffer from an intrinsic instability when incubated in aqueous media under physiological conditions. An improvement in brush stability within cell-culture environments would provide more robust platforms for cell manipulations and more efficient, functionalizable coatings for implants and tissue engineering scaffolds¹³.

Incubating POEGMA homopolymer brushes in DMEM cell culture medium at 37°C (see experimental section), resulted in film wrinkling and concomitant detachment following 1 day of immersion (Figure 9). Similar behaviour was previously attributed to a combination of osmotic stress and hydrolysis of the Si-O-Si bonds which anchor the brush to the substrate^{18,19}. In addition, the detachment of the polymer coating as a freestanding film suggests that crosslinking is taking place within the brush structure. It was hypothesized that this phenomenon could be due either to transesterification between hydroxyl side functions and methacrylate groups of

neighbouring grafts or the formation of complexes between the monomer units and the metal ions present in the culture medium^{18,65}.

Figure 9. Optical micrographs of POEGMA films a) before and b) after a day of incubation in DMEM cell-culture medium at 37°C. c) AFM tapping mode height micrographs of (b).

Overall, the structural stability of the films was significantly improved by introducing PAMA substrate-bound layers (Figure 10). These improvements could be attributed to the shielding of the substrate by the underlying hydrophobic layers coupled to the reduced osmotic force exerted by the interfacial POEGMA, which in all the block-copolymer films presented a lower grafting density compared to the homopolymer analogue^{18,19,66}. Remarkably, PMMA-b-POEGMA demonstrated the most stable bilayered film among the different studied, not showing any sign of detachment or wrinkling after 7 days of incubation in a cell-culture environment (Figure 10b and 10c). It is notable that this is the minimum incubation time required for studying cell differentiation on several substrates or scaffolds⁶⁷.

At first glance, our results seem to contradict those of Quintana et al.³¹. They concluded that higher hydrophobicity of the substrate-bound layer will result a more stable system, thus in our case one would expect PLMA-b-POEGMA to be the most stable. However, they did not probe or take in to account the difference in mechanical properties of their substrate-bound layers (PS and PMMA). Here, we attribute the substantially improved stability of PMMA-b-POEGMA, compared with the other samples, to the mechanical properties of the PMMA block. As we have

already shown, the PMMA brush is in its glassy state at the culture temperature of 37°C, whereas both PBMA and PLMA brushes are above their T_g . Hence, the rigid PMMA block both protected the silane anchors from hydrolysis and counteracted the mechanical stress that is inherent in a polymer brush. This is evident from the lack of wrinkles on the PMMA-b-POEGMA in comparison to PBMA- and PLMA-b-POEGMA.

Comparison of the stabilities of PBMA-b-POEGMA and PLMA-b-POEGMA shows the effect of hydrophobicity of the substrate-bound layer on the stability of the films. The onsets of wrinkling for PBMA-b-POEGMA and PLMA-b-POEGMA were 4 and 6 days, respectively. Since at experimental temperature, PBMA and PLMA are above their T_g and thus have similar mechanical properties, this different performance could be due to the higher hydrophobicity of the PLMA block, which allowed more efficient shielding of the hydrolysable anchoring groups against the cell-culture medium. One could also argue that the improved stability of PLMA-b-POEGMA compared to PBMA-b-POEGMA was partially due to the higher grafting density and/or chain length of the POEGMA layer in the latter block-copolymer film. As was already demonstrated in several reports, a higher osmotic force, originating from the crowding of the grafted chains, can promote a kinetically faster detachment of brushes from the grafting substrate^{18,66,68}. In order to address this issue a PBMA-b-POEGMA film presenting a lower POEGMA thickness of 24 ± 3 nm was incubated in DMEM cell-culture medium at 37 °C (Table S2). Interestingly, this sample also showed initial signs of failure (wrinkles) on the 4th day of incubation. This result indicated that within the POEGMA thickness range tested, the stability of PAMA-b-POEGMA films was basically independent from the thickness of the POEGMA block (Figure S5).”

Figure 10. Optical and AFM tapping mode height micrographs of PAMA-b-POEGMA brush films before and after incubation in DMEM cell culture medium at 37°C. a) Optical micrographs of PMMA-b-POEGMA before and b) after incubation for 7 days. c) AFM tapping mode height micrograph of (b). d) Optical micrographs of PBMA-b-POEGMA before and e) after incubation for 4 days. f) AFM tapping mode height micrograph of (e). g) Optical micrographs of PLMA-b-POEGMA before and h) after incubation for 6 days. i) AFM tapping mode height micrograph of (h). The optical and AFM micrographs of the same samples prior and after the failure are reported in the Supporting Information.

CONCLUSION

This paper focused on the synthesis and characterization of well-defined block-copolymer brushes, to yield bilayered films comprising a hydrophobic substrate-bound layer and a

hydrophilic, biopassive interfacial brush. The application of selective solvents during sequential SI-ATRP allowed the first block to inhibit the growth of the second polymer from the remaining, unreacted initiator molecules at the substrate, thus ensuring a distinct layered structure. The designed bilayer architecture was demonstrated to determine the physico-chemical properties of the coatings, their biopassivity and their stability in cell culture. In particular, the structure of the hydrophilic layer exposed to the medium governed all the interfacial properties of the films. Evidently, the composition and mechanics of the hydrophobic underlayers determined film stability. Notably, all bilayered block-copolymer brushes showed enhanced stability under cell-culture conditions in comparison to a corresponding hydrophilic homopolymer brush. Furthermore, the stability depended both on the mechanical properties and the hydrophobicity of the underlayer, with the former being the overriding factor. The results originating from this study thus highlight how the structural and chemical parameters of brush films can be tuned in order to successfully fabricate biopassive films that also display long-term endurance in biological media.

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TOC FIGURE

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