POLY(2-OXAZOLINE) BOTTLE-BRUSH BRUSHES FOR THE CONTROL OF PROTEIN ADSORPTION AND CELL ADHESION

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Introduction

The modification of surfaces to control protein adsorption and cell adhesion is a key technology for the development of biomaterials as implants or in regenerative therapy. No general recipe for the design of protein-resistant or "non-fouling" surfaces is available, as the responds of an organism towards foreign materials is highly developed and complex. However, for specific applications various coating designs have been development and research focuses on defined systems that allow the identification of a structure-property relationship. Among those, self-assembled monolayers (SAMs) [1,2] and polymer brushes are the most intensely studied systems.[3]

In detailed studies the groups of Whitesides [4] and Grunze [5,6] reported on the non-fouling properties of oligo(ethylene glycol)-terminated SAMs (OEG-SAMs) on metals and since then, OEG and longer poly(ethylene glycol) (PEG) brush surface coatings became the gold standard for "bioinert" model surfaces. Later on, Chilkoti *et al.*[7,8,9] introduced highly crowded bottle-brush brushes of acrylates featuring OEG as defined side chains as protein resistant surfaces. In general it is believed that "non-fouling" surface coatings should be (i) hydrophilic, (ii) present hydrogen-bond acceptors but (iii) no hydrogen-bond donors and (iv) be of neutral net charge.[2] Moreover, for hydrophilic polymer brush coatings, the polymer has to be highly hydrophilic, flexible and amorphous to exhibit protein repellency based on the "steric repulsion" effect.[10] While PEG is now the most widely used polymer for "biocompatibilization" of solids, poly(2-oxazoline)s (POx) are currently entering the field of biomaterials,[11] especially the very hydrophilic poly(2-methyl-2-oxazoline) (PMeOx) and poly(2-ethyl-2-oxazoline) (PEtOx) which is still good water soluble but shows a slight amphiphilicity, similar to PEG.[12,13] Already in early studies, Rühe *et al.*[14] reported preliminary studies on PEtOx brushes on gold that significantly reduce the adsorption of fibronectin and recently, Textor *et al.*[15] presented adsorbed layers of PLL-PMeOx bottle-brushes as protein repellent surfaces. While the latter system showed protein-repellent properties equal to PEG systems, a more stable coating via covalent bonding and a bottle-brush brush morphology similar to the Chilkoti system may be desirable for the long term use in organisms.

Here we present the first account on the preparation of POx-based bottle-brush brushes (BBBs) by means of surface-initiated polymerization (SIP) on silanized silicondioxide substrates and the control of protein adsorption and subsequent control in cell adhesion as a function of POx side chain composition, length and end function.

Experimental

Materials. Chemicals were purchased from Aldrich (Steinheim, Germany) or Acros (Geel, Belgium). Chemicals for the LCROP were dried by refluxing over CaH_2 under a dry argon atmosphere and were freshly distilled prior to use. **2-Isopropenyl-2-oxazoline (IPOx) and 2-*n*-propyl-2-oxazoline (*n*PrOx)** were synthesized according to published procedures.[16,17]

SAMs of α,ω-aminopropyltrimethoxysilane (APTMS). Silanization was performed according to a modified RCA-procedure for substrate activation [18] and silanization using ultrasonication.[13]

Synthesis of POx-bottle brush brushes by surface-initiated photografting and photopolymerization (SIPGP) and consecutive surface-initiated living cationic ring-opening polymerization (SI-LCROP). The synthesis of POx BBBs was adopted to silane SAMs on silicon dioxide performed from recently published procedures on glassy carbon,[19] diamond [20] and carbon templates.[21] Variation of POx composition (used monomers: MeOx, EtOx and *n*PrOx) and side chain length and end group was performed using one larger sample and subsequent division into comparable samples during synthesis to ensure maximum comparability. Variation of side chain length was realized by different SI-LCROP times from 1 to 4 h.

Protein adsorption (fibronectin) and cell culture experiments (human endothelial cells, HUVECs) were performed similarly to procedures described recently.[22] The protein amounts remaining on the substrate surface were quantified in terms of fluorescence intensities using Openlab software. Reference intensities were collected from fibronectin-coated poly(octadecen-*alt*-maleic anhydride) (POMA) surfaces and uncoated surfaces.

Fluorescence microscopy. The microscopy analysis was performed on an inverse epi-fluorescence microscope (DMIRE2, Leica Microsystems, Germany) with a 40x oil immersion objective using Openlab software (Perkin Elmer). Carboxytetramethylrhodamine FluoReporter (Invitrogen, Karlsruhe, Germany) was used to label fibronectin prior to the experiments. After 1 h of cell culture samples were fixed with 4% paraformaldehyde for 10 min and stained with DAPI (Sigma) and phalloidin-Alexa488 (Invitrogen) to visualize nuclei and actin cytoskeleton, respectively.

Surface analysis. AFM was performed on a Nanoscope IIIa scanning probe microscope from Veeco Instruments (Mannheim, Germany), contact angles were measured with a Krüss DSA 10 Mk2 goniometer. FT-IR was performed on a IFS 55 Bruker instrument equipped with a diffuse reflectance Fourier transform infrared (DRIFT) setup from SpectraTech.

Results and Discussion

The preparation of POx-based bottle-brush brushes by means of (a) SIPGP [23] of 2-*iso*propenyl-2-oxazoline to poly(2-*iso*propenyl-2-oxazoline) (PIPOx) brushes on APTMS SAMs and (b) SI-LCROP of MeOx, EtOx and *n*PrOx from surface bond macroinitiator brushes is outlined in **Figure 1**.

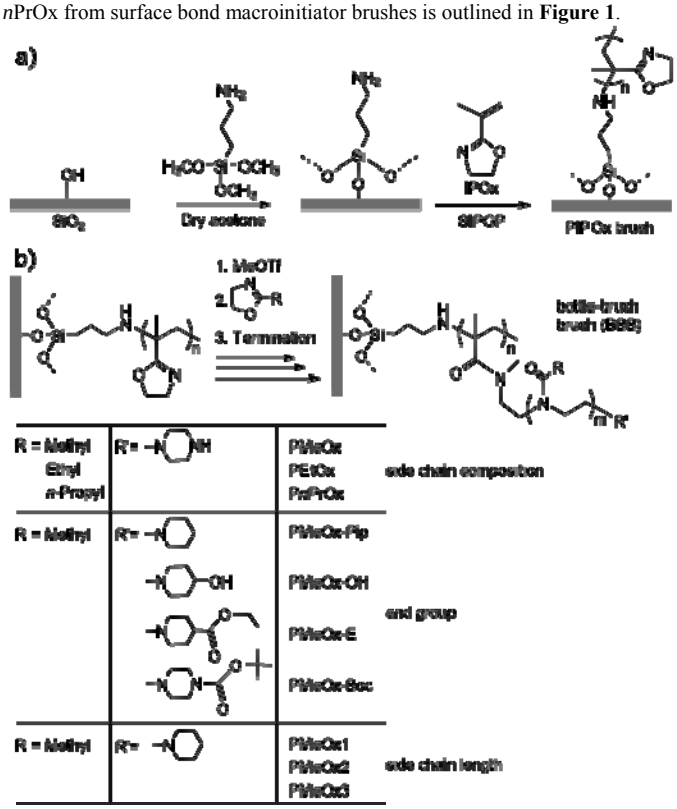

Figure 1. Preparation of POx BBBs on silicon dioxide substrates. a) Formation of APTMS SAMs and subsequent growth of PIPOx brushes by SIPGP. b) Conversion of the PIPOx brush backbone to the macroinitiator salt and SI-LCROP of 2-oxazolines. The LCROP was terminated by different reagents to give POx bottle-brush brushes of systematical variation of side chain composition, side chain length and end functions.

The two-step polymerization as well as the introduction of the various end groups were followed by means of AFM measurements, contact angle measurements and surface-sensitive FT-IR spectroscopy. As an example, the FT-IR spectra confirmed the formation of PIPOx brushes and the subsequent conversion to PMeOx-Pip by SI-LCROP is shown in **Figure 2**.

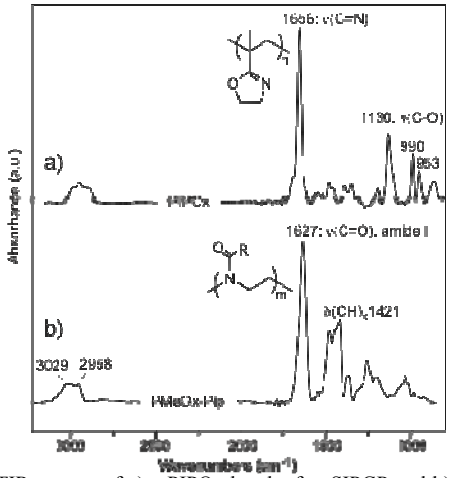

Figure 2. FTIR spectra of a) a PIPOx brush after SIPGP and b) PMeOx-Pip bottle-brush brush after SI-LCROP and termination using piperidine.

After SI-LCROP, the (C=N) and (C-O) stretching bands as well as the two ring skeletal vibration bands from the pendant 2-oxazoline ring in PIPOx brushes are no longer observed and a new intensive band appeared around 1627 cm^{-1} which is characteristic for the carbonyl stretching mode of the amide function (amide I band). Moreover, the characteristic CH_x deformation modes for PMeOx-Pip are observed around 1421 cm^{-1}. Furthermore, AFM measurements showed a significant thickness increase of the polymer layer from e.g. 56±3 to 140±10 nm after the SI-LCROP due to the stretching of the BBB backbone by the side chain crowding. In addition, BBBs with PMeOx side chain of different length were prepared. One substrate with a PIPOx brush was divided and SI-LCROP of MeOx was performed for 1h, 2h and 4h, respectively. The relative increase of layer thickness was found to be a linear function of the SI-LCROP polymerization time, which is in good agreement with early reports.[19] The contact angle of BBBs varied significantly with the side chain length of e.g. from 51° for PMeOx1 to 43° for PMeOx4. Contact angles for BBBs with PMeOx side chain (LCROP=4h) and different end groups showed only minor changes of the contact angles due to surface reconstruction. Strong differences in the wettability could be observed for BBBs featuring different POx-side chains. While BBS with P*n*PrOx show a relatively high contact angle of 53°, PEtOx (43°) and PMeOx (38°) are hydrophilic. However, also here the slight amphiphilicity of PEtOx is noticeable. All surfaces were used for protein adsorption experiments using fibronectin. A POMA surface with a known amount of coupled protein (~600 ng/cm^2) was used as a reference in order to quantify the surface concentration of adsorbed protein on the BBBs.

Figure 3 summarizes the results of all surfaces and gives a conclusive picture of the relevant structural (side chain length) and compositional factors (side chain composition and end group) that influence the fibronectin adsorption. Relatively high protein adsorption (90 ng/cm^2) was observed for P*n*PrOx BBBs and low protein adsorption for PEtOx. The most hydrophilic PMeOx brushes showed almost no protein adsorption (≤6 ng/cm^2) and the values are comparible to the PLL-PMeOx bottle-brush surface reported by Textor *et al*.[15] Interestingly, the PMeOx side chain length had a significant influence on the fibronectin adsorption (from ~45 to ≤11 ng/cm^2) as well as on the static contact angle, while the nature and polarity of the end group had only a slight effect. However, also here, the "rule" for protein adsorption on hydrogen-bonding donors vs. acceptors [2] is nicely obliged. All results on the protein adsorption correlate with the water contact angle values. The lower the contact angle, the lower is the protein adsorption.

Finally, cell adhesion studies using endothelial cells were performed on the various BBB surfaces after the protein adsorption measurements (data not shown). As expected, a very similar trend was found. While, e.g. on a P*n*PrOx BBB surfaces good cell adhesion (highly spread cells) were observable, no cell adhesion could be observed on hydrophilic BBBs with PMeOx and PEtOx of medium or long side chains.

Conclusions

The SIPGP-LCROP approach allows the preparation of bottle-brush brushes of defined architecture and composition. POx-based BBBs can effectively control the protein adsorption behavior and the cell adhesion onto surfaces. The relative and absolute amount of protein adsorption correlates with the water contact angle value.

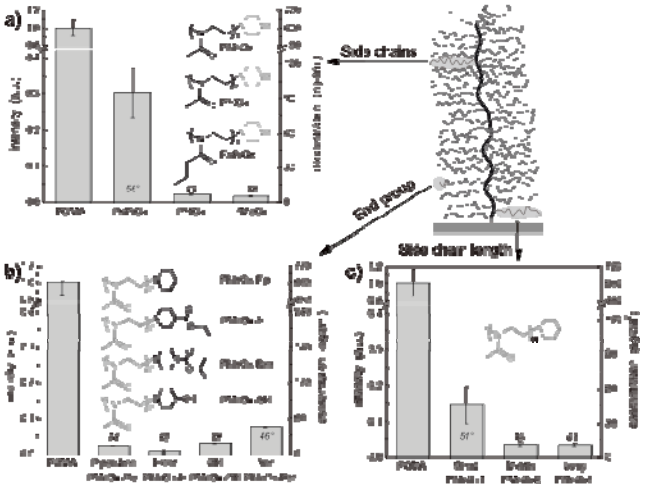

Figure 3. Relative intensity and absolute amount of fibronectin adsorption on POx-BBBs as a function of BBB architecture and composition. a) BBBs with PMeOx, PEtOx and P*n*PrOx side chains. b) BBBs with PMeOx side chain of identical side chain and stem length but variation of the side chain end group. c) BBBs of identical stem length and PMeOx side chain of different length. The numbers in italic are the respective water contact angle values.

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