Nanoscale tailoring of the surface properties of biomedical devices by layer-by-layer technique

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ABSTRACT

In this work, the layer-by-layer (LbL) technique was applied for the coating of 316L stainless steel model substrates for stents, after surface priming by functionalisation with 3-aminopropyl) triethoxysilane (APTES). APTES coatings were prepared through the deposition of an APTES acqueous-ethanol solution on the metal substrates. The morphological and physico-chemical characteristics of APTES coatings were investigated by fluorescence and atomic force microscopy (AFM), scanning electron microscopy (SEM) and X-ray photoelectron spectroscopy (XPS) analysis. APTES films consisted of globular submicrometer domains, coating the stainless steel substrates. XPS analysis demonstrated the presence of amino groups on the APTES-functionalised surface, although the preferential surface exposure of APTES alkyl chains and/or the roughness of the coatings strongly decreased the static water contact angle. Fluorescence microscopy also showed the multilayered structure of APTES coatings on stainless steel substrates. Heparin/ poly(diallyl dimethylammonium) chloride (HE/PDDA) LbL coatings were prepared on the APTES-modified surface. SEM analysis showed that surface morphological features of LbL coated samples were not homogeneous probably due to the intrinsic roughness of metal substrates. AFM phase images revealed that LbL coatings were also not homogeneous, as submicrometer domains with different features were observed. In addition, the LbL coated metal surfaces were hydrophilic with an intermediate contact angle between the ones of pure HE and PDDA: this finding again confirmed the non-homogenity of the LbL coating. Preliminary *in vitro* platelet adhesion tests showed that LbL coated substrates exposing HE as the last layer strongly inhibited platelet adhesion. Future efforts are in progress to increase the density of amino groups on the APTES modified coatings with the aim to promote the deposition of more homogeneous LbL coatings on model stent surfaces.

Keywords: endothelization; layer-by-layer; stent; tissue engineering

1 INTRODUCTION

The layer-by-layer (LbL) technique is a versatile solvent-free process allowing the coating of surfaces with uniform ultrathin multilayered films to tailor surface properties and structure at the nanoscale. LbL is based on the alternating exposure of a charged substrate to solutions of positively and negatively charged polyelectrolytes. A rinsing step is included between each the two previously described adsorption processes to remove excess material as well as to prevent cross-contamination of the polyelectrolyte solutions [1]. LbL approach is a promising tool to engineer interfaces with biomimetic properties. The simplicity of coating with LbL films on geometrically complex substrates provides a method for the control of cell adhesion on biomaterial substrates.

Currently, there is great demand for a new generation of coronary stents allowing rapid re-endothelialization on the stent surface, which could provide protection against thrombus as well as minimize restenosis. Different approaches have been attempted to accelerate surface endothelialization of stents: for instance stents have been pre-seeded with endothelial progenitor cells (EPCs) [2] or coated with anti-CD34 antibodies for the capture of patient's EPCs [3]. However, the use of biologically active substances make the production and sterilization processes difficult. Another limitation may lay in the relatively low content of the EPCs in blood. LbL is an alternative and simple approach for the preparation of functional surfaces with anti-thrombogenic properties and supporting endothelization for vascular tissue engineering and stent

coating. However, stable application of LbL coatings on implantable devices requires an initial surface priming treatment. 3-Aminopropyl) triethoxysilane (APTES) is commonly used to functionalize inorganic substrates because it can form an amine-reactive film that is tightly attached to the surface. Silanization of solid surfaces with APTES has been widely used to prepare substrates which may be functionalised by immobilized proteins and to prepare selective absorbents or organic/inorganic hybrid materials. Chemically adsorbed silane on an inorganic surface provides a platform for further functionalisation [4]. Silane film morphology can affect the accessibility of reactive groups for subsequent coupling with reactive molecules. The understanding of the relationship between morphology and amino group accessibility may lead to a more effective APTES application. In this study, the morphology and physico-chemical features of APTES films prepared on stainless steel substrates were investigated as a tool for stent priming before LbL coating. APTES coatings were prepared using an acqueous-ethanol solution deposition method. Subsequently, LbL coatings were prepared on the APTES-modified stainless steel surface, based on heparin/ poly(diallyl dimethylammonium) chloride (HE/PDDA) with the aim to confer antithrombogenic properties to the metal surface. In addition, the multilayered structure of the coating could allow the incorporation of drugs/growth factors into the different layers to favor re-endothelisation although avoiding thrombus formation.

2 EXPERIMENTAL PART

2.1 Materials

316L stainless steel plates (2.5 x 1.0 cm^2) with 100 μm thickness were as model substrates for implantable devices. Aminopropyltriethoxysilane (APTES), heparin (HE) and poly(diallyl dimethylammonium) chloride (PDDA) were supplied from Sigma-Aldrich.

2.2 Methods

Metal plates were aminolysed using APTES according to the method described by Meng et al. [5]. Substrates were washed into an ethanol:water (1:1 v:v) solution for 4 h to remove impurities. Then, metal samples were dipped in bi-distilled water and dried in an oven at 37°C for 24h. Metal substrates were then incubated in an APTES solution in ethanol with 0.1 % (w/v) concentration for 4 h at 37°C. Then, they were dipped again in bi-distilled water and dried in an oven at 50°C for 24h. After that, samples were treated in a 0.01M HCl solution for 3 hours at room temperature; then, they were dipped in bidistilled water and dried in an oven at 50°C for 24h.

APTES modified metal substrates were coated by LbL techniques with alternate HE/PDDA layers using a KSV DC Dip-Coater allowing for three simultaneous strokes. In detail, APTES-functionalised metal samples were dipped into alternate aqueous solutions (0.1 % w/v) of HE and PDDA for 15 min each, with intermediate washing steps in bi-distilled water for 5 min. After the last layer deposition, samples were dipped in bi-distilled water for 10 min. Dipping speed was set at 100 mm/min. Coatings with 1-11 layers were prepared.

2.3 Characterisation techniques

The elemental chemical surface composition of APTES functionalised metal substrates was determined by XPS analysis in a PHI 5000 Versaprobe spectrometer with an AlKa X-ray source. For each sample, O 1s, N 1s, C 1s, and Si 2p core levels were collected. All core-level peak energies were referenced to the saturated hydrocarbon peak at 285.0 eV.

APTES-coated samples were also analysed under a fluorescence microscope (Axiovert Zeiss Oberkochen). Photomicrographs were taken using a 100 W mercury arc light source with a standard fluorescein (488 nm excitation/510 nm emission) filter set.

AFM analysis was performed in true non-contact (NC) operational mode to avoid sample damage due to the softness of the investigated layers. μ-Masch silicon cantilevers, with a resonance frequency of 350 kHz and nominal radius smaller than 10 nm were used.

Surfaces were sputter coated with Au and then evaluated for surface morphology by scanning electron microscopy (SEM Philips 525M).

Static contact angles were measured at room temperature in a CAM 200 KSV Instrument equipped with an Attention Theta software for data acquisition. Sessile drop method was applied, using a 5 μl double distilled water droplet. Results were expressed as average values ± standard deviation (n=6).

In vitro tests were performed on coated and uncoated stainless steel plates, using human platelets. The blood (25 ml) was collected from healthy human donors in Vacutainer tubes (Becton-Dickinson, USA) containing an acid-citrated dextrose anticoagulant. Platelet rich plasma (PRP) was generated by centrifuging the blood at 225 rpm for 15 minutes at 25°C. The platelet concentration was adjusted to 10^8 platelets/ml with phosphate buffered saline (PBS). The platelet suspension was adhered to the different materials for 1h at 37°C. After the adhesion, the adhered platelets were quantified using a lactate dehydrogenase (LDH) assay.

3 RESULTS AND DISCUSSION

3.1 APTES-functionalised stainless steel substrates

Figure 1 shows images of the intrinsic fluorescence of uncoated (Figure 1a) and APTES-coated (Figure 1b) stainless steel substrates. Clean metal substrates were dark when observed under a fluorescence microscope. A monolayer APTES film has been reported not to be fluorescent: the fluorescence was a result of a three-dimensional silane structure on the substrate [4]. Therefore, the fluorescent intensity was found to increase with increasing the APTES film thickness suggesting the formation of a multilayered film [4]. In addition, fluorescent signal of the APTES-coated substrates was uniform, suggesting a continuous coating of the metal surface at the micrometer scale.

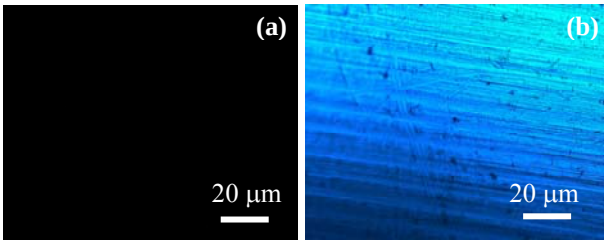

Figure 1: Photomicrographs of the intrinsic fluorescence of unmodified (a) and APTES-modified (b) metal surfaces.

However, fluorescent images only provide two-dimensional microscale information. Therefore, the surface topography was also analysed by AFM. The AFM technique is particularly suitable for detecting either topographical inhomogeneities (causing changes in the oscillation amplitude of the cantilever) or material inhomogeneities (causing changes in the oscillation phase of the cantilever) at the sample surface. Topographic images of APTES-modified stainless steel substrates showed the presence of particle agglomerates with submicrometer diameter and variable heights (Figure 2a).

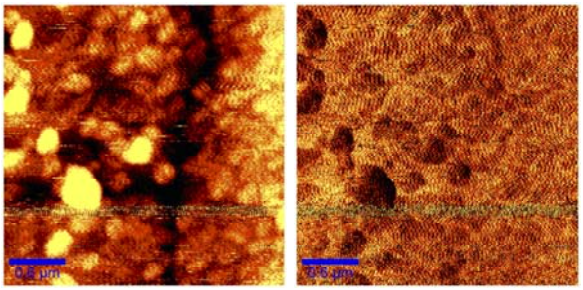

Figure 2: NC-AFM topographic (a) and phase (b) images APTES-modified stainless steel substrates.

On the other hand, stainless steel substrates were flat (data not shown). AFM phase images confirmed the continuous coating of substrates with APTES (Figure 2b).

Static contact angle analysis evidenced that surface wettability of stainless steel substrates increased from 59.1°± 2.9° for unmodified samples to 89.5°± 5.1 ° after APTES functionalisation. The increase of water contact angle with respect to freshly cleaned substrates was due to the presence of alkyl chains of APTES molecules, which imparted a more hydrophobic character to the surface. Moreover, the presence of water during the APTES treatment probably induced hydrolysis of ethoxy groups of silane molecules with their conversion to Si–O–Si bonds by a catalytic process involving the aminopropyl groups of APTES molecule [6]. In addition, the increase in hydrophobicity could be tentatively ascribed to the presence of areas with increased surface roughness due to the presence of silane clusters: they could cause the inward orientation of amine moieties, leading to a more pronounced exposure of the hydrophobic methylene groups of the alkyl chains [7].

XPS analysis was carried out on uncoated and APTES functionalised stainless steel substrates, to get an insight into the chemical composition of the coating. Figure 3 shows the XPS characterization, through the N 1s core line for APTES functionalised samples. XPS spectrum showed a peak centered at around 400.0 eV due to nitrogen atoms of the NH_2 groups. Through peak deconvolution, a shoulder at 400.4 eV was evidenced, ascribed to positively charged nitrogen in the NH^+_3 form. The presence of positively charged molecules was ascribed to the proton transfer from the relatively acidic –OH groups to the amino species of the silane moieties, especially in the presence of adsorbed water, which can promote the transferring of protons from silanols to amino groups of a silane molecule [8].

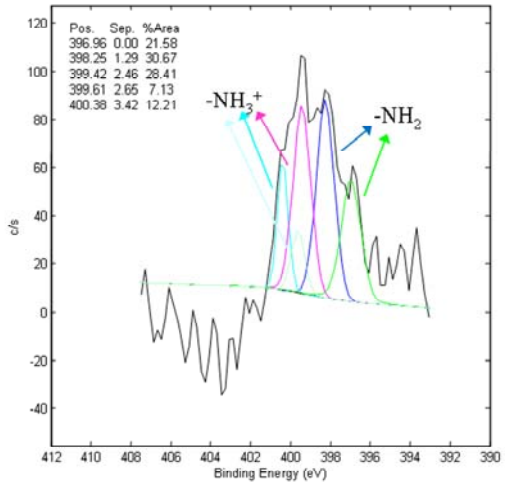

Figure 3: High resolution N1s spectrum of an APTES-modified stainless steel substrate.

The relative amounts of NH_+^3 and NH_2 groups has been found to depend on the amount of water present at the substrate surfaces. Silane chemisorption does not occur in the absence of water, but the presence of multilayered water represents the main cause of self-polymerization of the silane molecules and thus the lack of reproducibility. Furthermore, the sensitivity to water is enhanced for aminosilane species because the amino functionality in the

aminopropyl is known to self-catalyze the hydrolysis of the ethoxy groups.

3.2 HE/PDDA LbL coating on APTES-modified stainless steel substrates

LbL HE/PDDA coatings on APTES-modified stainless steel samples were characterised by static contact angle analysis. The contact angle of pure HE and PDDA cast films were found to be 59.0°±6.8° and 68.5°±0.2°. For LbL coated APTES-modified stainless steel substrates, contact angles varied between around 55° (for odd layers) and 62° (for even layers). Typically, contact angle of LbL-coated samples have been reported to vary between the values of pure layer components. The deviation from this typical behavior was probably due to a not-homogeneous coating of the samples, as suggested by AFM analysis.

Topographic images of LbL-modified stainless steel substrates revealed the presence of particle agglomerates with submicrometer diameter and variable heights (Figure 4a). AFM phase images suggested a not homogenous coating with HE/PDDA layers of the stainless steel substrates (Figure 4b).

SEM analysis of the surface of each deposited layer showed that surface morphology was not homogeneous probably due to the initial roughness of metal substrates (data not shown).

Preliminary *in vitro* studies revealed that platelet adhesion on the stainless steel substrates functionalised by LbL technique was lower in the case of coatings having HE as the last layer as compared to the ones having PDDA as the last layer (Figure 5).

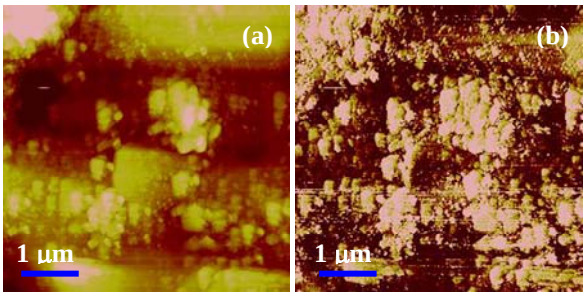

Figure 4: NC-AFM topographic (a) and phase (b) images of LbL coated APTES-modified stainless steel substrates: 11 layers (HE was the last layer).

4 CONCLUSION

A method was proposed for the surface modification of stainless steel substrates as models for implantable devices. Surface "priming" of stainless steel substrates for further LbL coating was performed under mild conditions through the deposition of APTES water/ethanol solutions on the samples. The proposed APTES deposition method allowed the preparation of continuous coatings with increased hydrophobicity as compared to bare metal substrates and accessible amino groups. The poorly hydrophilic behavior of APTES-modified samples was probably a consequence of surface roughness and/or the prevalence of APTES alkyl chains on the surface. HE/PDDA LbL coatings on APTES-modified metal surfaces were not homogeneous at submicron scale as suggested both by contact angle and AFM analyses. Preliminary *in vitro* tests suggested that the exposure of HE layers strongly decreased platelet attachment. Further trials are in progress to increase the density of surface amino groups on the APTES modified samples to get HE/PDDA homogeneous coatings.

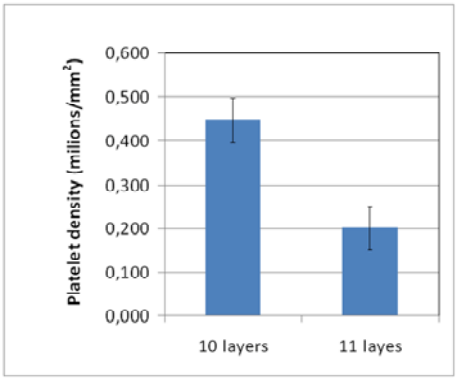

Figure 5: Platelet density on LbL coated APTES-modified stainless steel substrates after 1h incubation.

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