Antithrombotic and hemocompatible properties of nanostructured coatings assembled from block copolymers

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Abstract

We describe the antithrombotic properties of nanopatterned coatings created by self-assembly of poly(styrene-block-2-vinylpyridine) (PS-b-P2VP) with different molecular weights. By changing the assembly conditions, we obtained nanopatterns that differ by their morphology (size and shape of the nanopattern) and chemistry. The surface exposition of P2VP block allowed quaternization, i.e. introduction of positive surface charge and following electrostatic deposition of heparin. Proteins (albumin and fibrinogen) adsorption, platelet adhesion and activation, cytocompatibility, and reendothelization capacity of the coatings were assessed and discussed in a function of the nanopattern morphology and chemistry. We found that quaternization results in excellent antithrombotic and hemocompatible properties comparable to heparinization by hampering the fibrinogen adhesion and platelet activation. In the case of quaternization, this effect depends on the size of the polymer blocks, while all heparinized patterns had similar performance showing that heparin surface coverage of 40% is enough to improve substantially the hemocompatibility.

1. Introduction

The assembly of block copolymers (BCP) is a versatile bottom-up approach that is used for biomaterials fabrication and modification [1-3]. It can be applied to coat large surface areas with nanostructured films, whose topography is finely tuned by the choice of the blocks, their molecular weight, and the processing conditions [2,4,5]. The interplay between these parameters results in distinct microphase separation and formation of discrete nanodomains from the respective blocks, thus, generating nanopatterns with unique protein(s) adsorption and biological response [6-10]. BCP nanopatterns targeting different cells responses, e.g. surfaces that
promote cell adhesion, stem cell differentiation, bactericidal and antifouling coatings, have been developed [5,11-15]. Most of the final applications of these nanopatterns such as coatings of implants, vascular stents, and heart valves require direct contact with blood. In these cases, hemocompatibility is a major issue considering that the management of thrombus formation is still challenging [16]. Indeed, the hemocompatibility of micro- and nanostructured surfaces with chemically homogeneous composition has been already addressed [3,17-20]. As an example, submicron structures generated by phase separation of polyurethane grafted with alkyl chains have shown a reduction of platelet adhesion [21]. In spite of this promising results from the 80’s, the response of the blood components to well-defined BCP nanopatterns, i.e. surfaces made of polymer nanodomains, has not been investigated comprehensive.

The contact between the blood components and the implant’s surface can cause different complications [22]. The most common issue is the blood coagulation and consecutive thrombi formation that obstructs the normal blood flow. This process is triggered by proteins adsorption onto the surface of the implanted device and initiated immediately upon contact with the blood. The formed proteins layer then promotes the adhesion and activation of the platelets and the formation of fibrin (via interaction of the fibrinogen with the thrombin). The generated network traps the red blood cells (RBCs) and the blood clots are formed [23-25]. Therefore, any blood-contact device must have anti-thrombogenic characteristics. The hemocompatible materials must also present low hemolysis - the disruption of RBCs membrane results in the release of naked hemoglobin, for example, that can promote diverse pathological outcomes like inflammation, renal impairment, or thrombosis [26]. Finally, the blood-contact materials must be also cytocompatible and promote the proliferation of endothelial cells needed for the recovery of the endothelium that has been damaged after the intervention (e.g. vascular implantation) [27-30].

Herein, we assembled nanopatterned coatings from the widely used poly(styrene-block-2-vinylpyridine) diblock copolymers, PS-b-P2VP, were purchased from Polymer Source (Quebec, Canada). PS-b-P2VP with three molecular weights were used: PS (127 kDa)-b-P2VP (92 kDa) designated as PS127-b-P2VP92, PS (130 kDa)-b-P2VP (135 kDa) herein named PS130-b-P2VP135, and PS (320 kDa)-b-P2VP (398 kDa) labelled as PS320-b-P2VP398. The polydispersity indexes Mw/Mn (where Mw is the weight-average molecular weight and Mn is the number-averaged molecular weight) are 1.10 for PS127-b-P2VP92, 1.3 for PS130-b-P2VP135, and 1.25 for PS320-b-P2VP398. Toluene and chloroform were purchased from Fisher Chemical. ALOCHROMIX powder for cleaning, 1,4-diiodobutane and heparin sodium salt from porcine intestinal mucosa were purchased from Sigma-Aldrich. Glass coverslips (20 mm2) were purchased from Carl Roth. Human blood samples were obtained from Serviço de Imuno-Hemoterapia - Centro Hospitalar de São João (Portugal) under an approved institutional board protocol (ethical commission of CHSJ/FMUP approved at 18/13/2018). The samples were collected in sterile BD Vacutainer® coagulation tubes (BD-Plymouth, UK), which contained 3.8 % sodium citrate.

2. Materials and methods

2.1. Materials

Poly(styrene-block-2-vinylpyridine) diblock copolymers, PS-b-P2VP, were purchased from Polymer Source (Quebec, Canada). PS-b-P2VP with three molecular weights were used: PS (127 kDa)-b-P2VP (92 kDa) designated as PS127-b-P2VP92, PS (130 kDa)-b-P2VP (135 kDa) herein named PS130-b-P2VP135, and PS (320 kDa)-b-P2VP (398 kDa) labelled as PS320-b-P2VP398. The polydispersity indexes Mw/Mn (where Mw is the weight-average molecular weight and Mn is the number-averaged molecular weight) are 1.10 for PS127-b-P2VP92, 1.3 for PS130-b-P2VP135, and 1.25 for PS320-b-P2VP398. Toluene and chloroform were purchased from Fisher Chemical. ALOCHROMIX powder for cleaning, 1,4-diiodobutane and heparin sodium salt from porcine intestinal mucosa were purchased from Sigma-Aldrich. Glass coverslips (20 mm2) were purchased from Carl Roth. Human blood samples were obtained from Serviço de Imuno-Hemoterapia - Centro Hospitalar de São João (Portugal) under an approved institutional board protocol (ethical commission of CHSJ/FMUP approved at 18/13/2018). The samples were collected in sterile BD Vacutainer® coagulation tubes (BD-Plymouth, UK), which contained 3.8 % sodium citrate.

2.2. Coatings preparation and characterization

PS-b-P2VP was dissolved in toluene (1 or 2 wt%, stirring overnight at room temperature, RT). Squared glasses were washed by...
immersion in a sulfuric acid solution of ALNOCRiMIX™ overnight, rinsed with osmotized water, and dried with a nitrogen stream. Subsequently, they were spin-coated (3000 rpm, 40 s) with the PS-b-P2VP in a clean room environment and subjected to a solvent vapor annealing (SVA) as described elsewhere [5]. Briefly, the coated glasses were placed in a closed container with a saturated atmosphere of chloroform or toluene at controlled humidity (under 50 %) for 3 h at RT. After this treatment, the samples were removed and stored at RT until further use. The topography of the coatings was analyzed by atomic force microscopy (AFM, Dimension Icon, Bruker) in a PeakForce™ Tapping mode (cantilevers with a spring constant of 0.4 N/m and frequency of 70 Hz) and high-resolution scanning electron microscopy (HR-SEM, Auriga Compact, Zeiss). Roughness measurements were obtained from the AFM images processed by the software NanoScope Analysis 1.5. Surface wettability was evaluated by static contact angle using the sessile drop method and OCA 15+ equipment (DataPhysics). The analysis of the polymer block surface exposition was performed by immersing the nanopatterns in an acidic solution of gold salts (10 mM HAuCl₄ in 0.9 % HCl (aq.) solution, gold has an affinity to P2VP) followed by AFM analysis as previously described [5]. Samples with surface-exposed P2VP blocks were submitted to modification with 1,4-diiodobutane. This modification was performed with a double aim: to crosslink the coating and to introduce charge on the surface enabling following electrostatic deposition (Fig. 1). The PS-b-P2VP coated samples were placed in a glass tube oven (Büchi, B585) together with a petri dish containing 20 μL of 1,4-diiodobutane, heated at 75 °C under vacuum to create an atmosphere of 1,4-diiodobutane, and kept in this atmosphere for 42 h. After this time, the samples were removed from the oven and stored at RT or further modified by immersion in aqueous solutions of heparin (5 mg/mL, 1 h at RT). After rinsing with distilled water and drying under vacuum overnight, the samples were analyzed by X-Ray photoelectron spectroscopy (XPS, Kratos Axis-Supra) or tested for their antithrombotic and hemocompatible properties as described below. For the XPS measurements, a X-ray source (aluminum Kα monochromatized radiation at 1486.6 eV) was used in a Constant Analyser Energy mode (CAE) with a pass energy of 160 eV for survey spectra and of 20 eV for high-resolution spectra [4].

### 2.3. Protein adsorption

Bovine serum albumin (BSA, Sigma-Aldrich, Mw 68,000) and human fibrinogen plasminogen depleted (Fg, Enzyme Research Laboratories, Mw 341,000) were used as models for proteins adsorption tests. The coated substrates were immersed in 2.5 mg/mL BSA (in phosphate buffer saline, PBS, pH 7.4) or in 0.5 mg/mL Fg (PBS, pH 7.4) and incubated at 37 °C for 1 h. These different concentrations were chosen according to the protein molecular weight: Fg is five-fold bigger than BSA. After 1 h, the samples were washed and the adhered proteins quantified. The samples were immersed (1 h, 37 °C, orbital agitation 160 rpm) in 2 % sodium dodecyl sulfate to detach the adsorbed proteins and Pierce Micro BCA Protein Assay Kit (ThermoFisher Scientific) was used for quantification. The reported data are averaged values from three independent experiments. Tissue culture polystyrene (TCPs) was used as a control substrate.

### 2.4. Hemocompatibility

The blood of three different donors was used for these experiments. The whole blood was centrifuged at 200 g for 20 min to separate it into its components (platelet-rich plasma (PRP), red blood cells (RBCs), anduffy coat), which were used in the hemocompatibility assays. For the hemolytic activity, RBCs were washed with PBS (centrifuged three times at 3700 rpm for 5 min with PBS washes between them) and the obtained RBCs concentrate was diluted to a concentration of 5 vol% in PBS. This suspension was seeded on the coated substrates and cultured for 1 h at 37 °C, 5 % of CO₂ with constant orbital agitation. To assess the hemoglobin release, the PBS was removed from RBCs culture and centrifuged at 120 g for 10 min. The supernatant was transferred into a 96-well plate and the absorbance was measured on a microplate reader (Synergy HT, Bio-Tec) using an excitation wavelength of 540 nm. To check the integrity of the cultured RBCs, the substrates were washed with PBS, fixed with 2.5 % glutaraldehyde, dehydrated using ethanol with increasing concentrations (50, 70, 90, 100 %), and observed under high resolution scanning electron microscopy (HR-SEM, Auriga Compact, Zeiss). RBCs cultured on glass coverslips were used as a control for this analysis. PBS (non-hemolytic) and 1 % SDS (hemolytic) were added to the original RBCs suspension and used as negative and positive controls of hemolysis, respectively. The hemolytic ratio was calculated from equation (1) (eq. (1)):  

\[
\text{Hemolytic ratio} = \frac{\text{Sample} - \text{Negative control}}{\text{Positive control} - \text{Negative control}} \times 100 (1)
\]

The hemolytic ratio was interpreted as follow: < 2 non-hemolytic, 2−5 slightly hemolytic, and > 5 hemolytic.

Platelet adhesion was also studied. PRP was obtained after the centrifugation of the whole blood as described above. Two thirds of the PRP were transferred to a new tube to avoid contamination with cells present on the buffy coat. An HEPES based buffer (140 mM NaCl, 2.7 mM KCl, 3.8 mM HEPES, 5 mM EGTA, pH 7.4) supplemented with prostaglandin E1 (to prevent platelet activation) was added to PRP at a ratio of 1:1 (v/v). After mixing gently, the solution was centrifuged at 100 g for 15 min. The supernatant was transferred into a new plastic tube and the platelets were pelleted at 800 g for 15 min. The pellet was rinsed with a washing buffer (10 mM sodium citrate, 150 mM NaCl, 1 mM EDTA, 1 % (w/v) dextrose, pH 7.4) and then resuspended in Tyrode’s buffer (134 mM NaCl, 12 mM NaHCO₃, 2.9 mM KCl, 0.34 mM Na,HPO₄, 1 mM MgCl₂, 10 mM HEPES, pH 7.4) supplemented with glucose (5 mM) and BSA (3 mg/mL). The platelet suspension (300 μL) was added on the coated substrates and cultured for 1 h at 37 °C with 5 % CO₂. The buffer was removed and the samples were washed with PBS to remove the non-adherent cells. The adhesion was determined by quantification of the viability of the platelets on each coating with the CyQUANT LDH Cytotoxicity Assay Kit (TermoFisher Scientific). Samples were incubated in 2 % Triton X-100 at 37 °C for 45 min and the kit was used following the indications of the manufacturer. A bare glass was used as a control substrate. The samples for microscopy analysis were fixed (2.5 % glutaraldehyde), dehydrated using ethanol gradients, and observed under HR-SEM (Auriga Compact, Zeiss).

### 2.5. Cell adhesion and reendothelialization

Human Umbilical Endothelial Cells (HUVECs, Gibco) were cultured in Medium 200 (M–200, Gibco) supplemented with Large Vessel Endothelial Supplement (LVES), at 37 °C with 5 % of CO₂. Upon confluence of 80 %, HUVECs were trypsinized and cultured on the coated substrates at a density of 5000 cells/cm² (for evaluation of cell morphology and adhesion) or 50,000 cells/cm² (for evaluation of reendothelialization) using Low Serum Growth Supplement (LSGS, Gibco). After 24 h of culture, cell viability was quantified with Alamar Blue (BIO-RAD) following the manufacturer’s instructions. The substrates were then washed with PBS and fixed with 10 % formalin for the following immunocytocchemical analysis. The expression of von Willebrand factor (vWF), a key endothelial...
lial marker, was visualized by staining with anti-vWF antibody (0.5 μg/mL, ab201336, abcam) and secondary antibody anti-mouse IgG1 (Alexa Fluor 488, 1:400, ThermoFisher Scientific). Nuclei were stained with DAPI (1:500) and actin filaments with phalloidin (1:50). Images were taken with a confocal laser scanning microscope (TCS SP8, Leica). Cell morphology analysis was performed with Fiji (Image J). TCPS was used as a control substrate.

2.6. Statistical Analysis

Statistical analysis was performed with GraphPad Prism 8 (California, USA). The normality of the data was evaluated using a Sapiro-Wilk test ($p < 0.05$). All the data was followed a normal distribution and thus, one-way ANOVA was applied to analyze the differences between groups, using Tukey test for post hoc assessment. Statistical significance was defined at different levels,

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**Fig. 2.** Atomic force microscopy images of the coatings used in this study. (A) Coatings with micellar morphology and surface-exposed PS were generated by annealing in toluene while (B) nanopatterns with both blocks (PS and P2VP) exposed on the surface and different morphology (fingerprint for PS$_{127}$-b-P2VP$_{92}$, lamellar for PS$_{130}$-b-P2VP$_{135}$, and perforated lamellar for PS$_{320}$-b-P2VP$_{398}$) were achieved by annealing in chloroform. (C) Coatings with P2VP surface exposition were crosslinked with 1,4-diiodobutane. (D) The crosslinking process induces quaternization of P2VP block (Fig. 1) and thus, allows following electrostatic deposition of heparin. Scale bar = 500 nm.
namely $p < 0.1$, $p < 0.01$, $p < 0.001$, $p < 0.0001$. In all cases, the data is presented as mean ± standard deviation from three independent experiments.

3. Results and discussion

3.1. Assembly and modification of the nanostructured coatings

The used herein block copolymers are known for their ability to self-assemble into nanopatterns with different morphology [31]. Indeed, we were able to generate coatings with different nanopatterns and chemical composition (Fig. 2) by deposition of the PS-b-P2VP copolymers dissolved in toluene and following solvent vapor annealing (SVA). The SVA process lowers the glass transition temperature of the blocks, allowing their mobility and promoting microphase separation, thus resulting in coatings with different morphology that depends on the used solvent [32,33]. Coatings with micellar nanodomains were generated when toluene was used for the SVA (Fig. 2A). Toluene is a good solvent for PS but not for P2VP and promotes assembly of micellar structures with

Table 1

Surface characteristics of the developed coatings: pattern size (PS/P2VP), roughness (arithmetic average of the absolute values, Ra), and contact angle (CA). All values are represented as mean ± standard deviation.

<table>
<thead>
<tr>
<th></th>
<th>PS$<em>{127}$-b-P2VP$</em>{92}$</th>
<th>PS$<em>{130}$-b-P2VP$</em>{135}$</th>
<th>PS$<em>{320}$-b-P2VP$</em>{398}$</th>
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<tbody>
<tr>
<td></td>
<td>Pattern size (PS/P2VP)</td>
<td>Ra [nm]</td>
<td>CA [deg]</td>
</tr>
<tr>
<td></td>
<td>[nm]</td>
<td></td>
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<tr>
<td>SVA in toluene*</td>
<td>24.5 ± 3.0/ 19.4 ± 6.0</td>
<td>0.6 ± 0.1</td>
<td>87 ± 1</td>
</tr>
<tr>
<td>SVA in chloroform</td>
<td>24.8 ± 6.0/ 34.8 ± 5.8</td>
<td>1.4 ± 0.2</td>
<td>84 ± 3</td>
</tr>
<tr>
<td>Crosslinked</td>
<td>28.8 ± 5.0/ 36.2 ± 7.4</td>
<td>1.1 ± 0.2</td>
<td>95 ± 2</td>
</tr>
<tr>
<td>Heparinized</td>
<td>45.7 ± 6.9/ 31.6 ± 7.5</td>
<td>3.4 ± 0.9</td>
<td>89 ± 7</td>
</tr>
</tbody>
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* In the case of micellar nanopatterns (PS block is exposed on the surface), the second value (italic) in the PS/P2VP column shows the size of the intermicellar space.

Fig. 3. X-ray photoelectron spectra of the developed coatings. (A) N1s, (B) I3d, and (C) S2p core level spectra of nanopatterns generated by SVA in chloroform (red), and the respective crosslinked (black) and heparinized (green) coatings.
a PS shell (exposed to the solvent) and a P2VP core (Fig. 1) as confirmed by the negative staining with gold (metals have an affinity to the P2VP domain and can form complexes with it, Fig. S1A) [34].

As expected, the size of the micelles increases with the increase of the molecular weight of the copolymers (Fig. 2A, Table 1). This difference was also reflected in the change of the surface roughness (Ra, Table 1): Ra (PS_{127}-b-P2VP_{92}) < Ra (PS_{130}-b-P2VP_{135}) < Ra (PS_{320}-b-P2VP_{398}) that can influence the protein adsorption and cell-material interactions [35].

When chloroform (good solvent for both blocks) was used instead of toluene for the SVA, phase separation was induced and coatings with nanodomains composed of either polymer, i.e. PS and P2VP were assembled (Fig. 2B). The morphology of the generated patterns depended on the molecular weight of the copolymers and the ratio between the blocks: nanodomains generated by SVA in chloroform from PS\textsubscript{127}-b-P2VP\textsubscript{92} (\textasciitilde 40 wt\% of PV2P) are a mix of lamellae and cylinders arranged vertical to the surface, as expected from the close limit to the interface between cylinders in the classical BCP phase diagram [1]; PS\textsubscript{130}-b-P2VP\textsubscript{135} (\textasciitilde 51 wt\% of PV2P) formed lamellas, while the composition of PS\textsubscript{320}-b-P2VP\textsubscript{398} (55 wt\% of PV2P) is at the border between a cylindrical and a lamellar organization in the phase diagram, i.e. this copolymer was assembled in structures that correspond either to vertical cylinders or perforated lamellae (most probable given the thin film thickness [5]). The use of the chloroform altered not only the morphology of the coatings but also their surface chemistry: positive gold staining was observed for the patterns generated by SVA in chloroform (Fig. S1B) evidencing the exposition of both blocks of the copolymers on the pattern surface. Of note, the gold staining revealed that in the patterns assembled from PS\textsubscript{127}-b-P2VP\textsubscript{92} and PS\textsubscript{130}-b-P2VP\textsubscript{135} (appear lighter in the AFM images) P2VP is the main component of the ridges and the valleys are composed by PS, while in the case of PS\textsubscript{320}-b-P2VP\textsubscript{398} patterns the gold staining revealed that the valleys were assembled from P2VP.

The presence of P2VP block on the surface allows charge introduction via the quaternization of the pyridine’s nitrogen (Fig. 1). We submitted the coatings with the surface-exposed P2VP blocks to a treatment with 1,4-diiodobutane that has a double role - quaternization of the P2VP domains exposed on the coating surface and their crosslinking (Fig. 1).[4] This treatment did not affect the morphology of the coatings (Fig. 2B vs. 2C, Table 1) but the water contact angle increased (Table 1) indicating the incorporation of the

![Fig. 4](image-url)

Protein adsorption on the developed coatings. Quantification of (A) albumin and (B) fibrinogen adsorbed on nanopatterns generated by SVA in toluene (blue) or in chloroform (red), and the respective crosslinked (black) and heparinized (green) nanostructured coatings. Statistical differences: *p < 0.1, **p < 0.01, ***p < 0.001, ****p < 0.0001; differences between the protein adsorption on the control (CTRL) and each coating are presented with grey symbols: †(p < 0.1), ‡(p < 0.01), ‡‡(p < 0.001), ‡‡‡(p < 0.0001).
alkyl chains/crosslinking occurrence. X-ray photoelectron spectroscopy (XPS) analysis of the coatings corroborated the contact angle data (Fig. 3, Table S1). The crosslinking/quanternization was confirmed by the emerging of a new nitrogen peak at 402 eV that corresponds to the quanternized alkylated nitrogen[36] (Fig. 3A, black signal), the appearance of the typical iodine peaks at 620.4 and 631.9 eV, and the signals at 618.4 and 629.9 eV that correspond to iodine linked to the alkylated pyridine (Fig. 3B, black signal; Table S1) [4]. Previous studies have shown that the induced quanternization enables following electrostatic deposition of negatively charged polyelectrolytes [4]. Herein, we used heparin because of its known anticoagulant activity. The heparinization was confirmed by XPS - the emergence of the sulfur peak was observed in the spectra of the coatings modified by heparin (Fig. 3C, green signal; Table S1) [37]. Considering the weight percentage of PV2P to PS in the block copolymers, we can estimate about 40 to 55 % surface coverage with heparin.

3.2. Protein adsorption

Proteins are the main components of the plasma serum and have a central role in body response to a foreign device/biomaterial. We studied the adsorption of BSA and Fg on the developed coatings (Fig. 4).

These two proteins were selected because: (i) they are abundant in plasma; (ii) their size is different - BSA is a small globular protein (68 kDa, hydrodynamic diameter of ca. 6 nm) [35], while Fg is bigger and has a tri-nodular elongated shape (341 kDa, length of ca. 45 nm)[38]; and (iii) albumin can exert either anti- or procoagulation effects [39], while Fg mediates platelet adhesion and acts as a structural component of the blood clots [24]. The isoelectric points of Fg and BSA at physiological conditions (pH = 7.4) are 5.8 and 5.1 respectively, i.e. both proteins have a net negative charge, although they contain positively charged domains [40,41].

Surface properties such as morphology, roughness, and chemistry determine protein adsorption [42-44]. Data on protein adsorption on BCP nanopatterns have been mainly obtained for copolymers in which one of the blocks is PS that promotes the protein adsorption, while the other, usually PEG, has antifouling properties [6,8,11,14]. For such copolymers, the relative surface exposure of each block determines the protein adsorption, e.g. the predominant presence of the antifouling block results in passivated surfaces [12,15]. The used herein copolymers are different as they do not contain an antifouling block - comparable fibrinogen adsorption has been reported on the PS and P4VP - a homopolymer that is similar to the P2VP [45]. We observed higher BSA and Fg adsorption on unmodified coatings, especially in the case of micellar nanopatterns generated from PS320-b-P2VP398, i.e. the copolymer with the longest PS block (Table 1). Of note, the micellar nanopatterns differ by their morphology (biggest Ra was determined for PS127-b-P2VP398) but not surface chemistry and thus, these results demonstrate the possibility to tune the protein adsorption by changing the morphology.

Quanternization of the patterns reduced proteins adsorption especially in the case of Fg on PS127-b-P2VP92 and PS130-b-P2VP135. At first sight, this result seems paradoxical: the introduction of positive charge on the surface is expected to increase the electrostatic deposition of the negatively charged proteins. Similar Fg behavior has been previously reported for positively charged latex particles and explained by a side-on deposition and stretching of Fg molecules resulting in the adsorption of lower amounts of Fg [46]. Such explanation is supported by the fact that different Fg conformations are observed upon its deposition on PS, P4VP, and PS-b-PMMA BCP nanopatterns [8,45]. This is an important result given the known influence of Fg conformation on platelet adhesion [47]. In the case of BSA, this difference is not well pronounced because of the globular shape of this protein [48]. Of note, the protein adsorption on quanternized and unmodified patterns assembled from PS320-b-P2VP398 by SVA in chloroform was similar and can be explained by the spacing and the relative ratio between the surface-exposed blocks. The large Fg (ca. 45 nm length and 7 nm width) cannot accommodate in the PS nanodomains as summarized from PS127-b-P2VP92 and PS130-b-P2VP135 without exposition to the quanternized P2VP domains. On the other hand, the PS domains assembled from PS127-b-P2VP398 are enough larger to custom Fg and no significant differences are observed before and after crosslinking. These results are consistent with the above-mentioned importance of the surface morphology for the development of biocompatible coatings.

As expected, the heparinized coatings were the least adhesive ones for the studied proteins. Heparin is a well-known anticoagulant and its surface immobilization can be advantageous over the intravenous supplementation that requires high dosage and can cause side effects [37]. Previous reports describe heparin immobilization via a covalent bond, i.e. polymer grafts or by electrostatic deposition in a layer-by-layer fashion [49-57]. The decreased BSA and Fg adsorption can be explained by the electrostatic repulsion between the sulfate groups of heparin and the negative charge of these proteins [40,41]. It must be noted, that the protein adsorption was similar for all heparinized patterns. This result indicates

![Fig. 5. Hemolytic potential of the developed coatings. The hemolysis ratio for all coatings is below 2 and comparable to the non-hemolytic control (CTRL) phosphate buffer saline (PBS). Data for coatings with micellar nanopatterns are shown in blue, SVA in chloroform in red, crosslinked in black, and heparinized ones in green.](image-url)
that a relatively low heparinization (ca. 40%) is enough to decrease significantly the protein adsorption and that higher heparanization (up to 60% for PS$_{320}$-b-P2VP$_{398}$) does not result in further improvement of the antifouling properties.

3.3. Hemocompatibility

Preservation of the erythrocytes integrity and morphology is another requirement for blood-contact devices [26,58]. We therefore evaluated the hemolytic potential of the developed coatings (Fig. 5). A non-hemolytic surface should have a ratio < 2 and all developed coatings meet this criterion. Moreover, most of them performed better than the control (PBS). This results were confirmed by SEM characterization of the RBC in contact with the coatings (Fig. S2): the images show cells with normal morphology - a biconcave shape with an indented center [59].

We also studied platelets behavior in contact with the developed coatings (Figs. 6 and S3) because of the involvement of these blood components in the thrombus formation: surfaces that favor platelets adhesion promote thrombogenesis. We observed a significant number of adhered platelets on coatings with micellar nanopattern (Fig. 6A, blue). Nanotopography can affect the platelet behavior [3,17,19,20] and indeed, fewer platelets were found on the nanopatterns generated by SVA in chloroform and similar adhesion was observed for their crosslinked analogs. The subsequent functionalization of these nanopatterns with heparin, known for its antithrombotic activity [29,56,60], reduced remarkably the adhesion (Fig. 6A, green). These results agree with the data obtained for the proteins adsorption that showed least amount of
proteins on heparinized coatings and most adsorbed proteins on
the micellar coatings. (Fig. 4).

Besides the number of the adhered platelets, it is important to
assess their activation state. The adhesion can activate signal tran-
sduction, which leads to platelets activation expressed by cytoskele-
tal/shape change and release of bioactive components that trigger
the platelet-mediated thrombosis. In their non-activated state pla-
telets have a discoid shape, which upon activation is continuously
changed passing by a dendritic to a fully spreading morphology
[61,62]. The developed herein coatings had a different effect on
platelet activation (Fig. 6B and S3) and this effect depended greatly
on the surface chemistry. Mostly activated platelets were found on
unmodified coatings assembled by SVA either in toluene or in chlo-
roform (Fig. 6B1 blue and red bars) that have PS and PS/PV2P
exposed on the surface, respectively. Crosslinking/quaternization
of the nanopatterns with P2VP exposed blocks decreased the acti-
vated platelets and this effect depends on the size of PV2P block
(Fig. 6B1 black bars), i.e. on the amount of quaternized species

![Image](image_url)

**Fig. 7.** Ability of the developed coatings to induce reendothelization. (A) Confocal microscopy images of Human Umbilical Vein Endothelial Cells (HUVEC) cultured on the
coatings (vWF immunostained in green, cytoskeleton stained with phallolidin in red, and nucleus with DAPI in blue) and (B) viability of the HUVECs 24 h after seeding on the
coatings. Data for micellar coatings assembled by SVA in toluene are shown in blue, the nanopatterns generated by phase separation in chloroform are presented in red,
crosslinked in black, and heparinized ones in green. Statistical differences: *p < 0.1. Scale bar = 1 μm.
available on the surface: PS_{127-b-P2VP98} < PS_{130-b-P2VP135} < PS_{120-b-P2VP398}. This result is related to the amount of Fg deposited on these patterns (Fig. 4): after the initial activation of platelets on the pattern, they release Fg, vWF, and coagulation factors and promote a feedback loop towards activation of additional platelets [22]. Thus, high adhesion of the released factors on the patterns can promote platelet activation and low protein adhesion might decelerate and reduce the activation process.

Further functionalization with heparin had a positive effect on the PS_{127-b-P2VP98} and PS_{130-b-P2VP135} coatings but did not improve additionally the PS_{320-b-P2VP398} ones – a similar number of activated platelets was determined for crosslinked and heparinized PS_{320-b-P2VP398} coatings. However, we observed significantly different adhesion (Fig. 6A) for these two coatings and thus, we can conclude that the heparinized coatings are most appropriate in terms of antithrombotic effect associated with platelets response.

3.4. Cytocompatibility and reendothelialization

Rapid reendothelialization is crucial for blood-contact materials [30]. The recovery of the endothelium that is usually damaged during the intervention is needed to maintain the vascular system homeostasis and avoid thrombus formation [28,63,64]. The vascular endothelium, for example, releases continuously nitrogen oxide that prevents platelet activation and aggregation [65].

We assessed the cytocompatibility of the coatings and their capacity to induce reendothelialization by evaluating the viability, morphology, and expression of von Willebrand factor (vWF, specific endothelial glycoprotein) by HUVECs cultured in contact with the nanopatterns. The cells adhered to all coatings and presented high viability (Fig. 7B) and similar vWF expression (Fig. 7A) and morphology (Fig. 54). These results evidenced the good cytocompatibility of the developed coatings in agreement with previous data about BCP nanopatterns [5-9,11-15]. The results also indicated the promising potential of the coatings towards reendothelialization that has not been previously assessed. A comparison between the tested copolymers showed higher HUVECs viability with the increasing size of the blocks. This tendency was most obvious for the nanopatterns with micellar morphology (Fig. 7B, blue bars) and agrees with the results obtained for protein adsorption (Fig. 4).

4. Conclusions

We have demonstrated that the self-assembly of block copolymers is a feasible strategy for the development of nanostructured cytocompatible and hemocompatible coatings. While previous investigations have only addressed the role of morphology on the hemocompatibility of chemically homogeneous or partially phase-separated surfaces [3,17-20], we provide data about the effect of other nanopatterns features, such as chemistry and size, on their antithrombotic and hemocompatible properties. The specific assays showed the following performance order: heparinized > crosslinked/quaternized > coatings generated by SVA in chloroform > micellar coatings. Remarkably, quaternization hinders Fg adsorption and platelet activation and its effect is comparable to heparinization for copolymers with shorter blocks, i.e. PS_{130-b-P2VP135}, PS_{127-b-P2VP98}.

All surfaces presented good cytocompatibility and reendothelialization capacity. The copolymers of different molecular weight showed similar tendencies although the differences between the four types of coatings - generated by SVA in toluene, by SVA in chloroform, and the respective crosslinked and heparinized analogs - were more pronounced for longer blocks, i.e. PS_{120-b-P2VP398} > PS_{130-b-P2VP135} > PS_{127-b-P2VP98}, and are a consequence of the effect of nanospacing on the protein adsorption.

Considering the reported herein results and our previous data about the antibacterial properties of these nanopatterns [5], we envision their applications as efficient coatings for implants and implantable devices. The next steps should be devoted to validation of these coatings in complex and dynamic bioenvironments using such devices.

Credit authorship contribution statement

R. Fontelo: Conceptualization, Methodology, Formal analysis, Investigation, Writing original draft. D. Soares Costa: Methodology, Investigation. R.L. Reis: Resources, Funding acquisition. R. Novoa-Carballal: Conceptualization, Methodology, Writing the manuscript, Supervision, Project administration, Funding acquisition. I. Pashkuleva: Conceptualization, Methodology, Writing the manuscript, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments


Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jcis.2021.10.076.

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