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Letter to the Editor

Bio-inspired surface modification of PET for cardiovascular applications: Case study of gelatin

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ABSTRACT

An aqueous-based bio-inspired approach was applied to chemically bind a bio compatible and cell-interactive gelatin layer on poly(ethylene terephthalate) (PET) for cardiovascular applications. The protein layer was immobilized after an initial surface activation *via* a dopamine coating. The individual and synergetic effect of the dopamine deposition procedure and the substrate nature (pristine *versus* plasma-treated) was investigated *via* XPS, AFM, SEM and contact angle measurements. Dependent on the applied parameters, the post dopamine coating presented various surface roughnesses ranging between 96 nm and 210 nm. Subsequent gelatin immobilization mostly induced a smoothening effect, but the synergetic influence of the deposition protocol and plasma treatment resulted in different gelatin conformations. In addition, a comprehensive comparative study between chemically-modified (*via* dopamine) and physically-modified (physisorption) PET with gelatin was developed within the present study. All investigated samples were submitted to preliminary haemocompatibility tests, which clearly indicated the direct link between blood platelet behaviour and final protein arrangement.

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1. Introduction

In the 1900s constructs manufactured from a variety of materials including aluminium, silver, glass or fabrics were tested *in vivo* and emerged as the first generation of artificial veins or synthetic grafts [1]. Upon their use, the importance of anti-thrombogenic properties (prevention of thrombus formation) and material durability (maintenance of mechanical and structural integrity) was highlighted.

Poly(ethylene terephthalate), PET, has been widely used for synthetic graft manufacturing due to its excellent bulk properties, such as good mechanical properties and chemical inertness [1,2]. However, its surface properties represent an impediment, as its hydrophobic nature fails to provide the much needed haemo- and bio compatibility. When in contact with blood, hydrophobic surfaces induce a specific and/or prolonged protein adhesion [1,3] that can lead to thrombus formation and ultimately graft occlusion or blockage. This is one of the main reasons of graft failure and an unwanted consequence of inadequate graft surfaces. Hence, the appropriate tailoring of the surface properties of PET grafts is of prime importance when targeting biomedical applications.

Up to now, a wide variety of surface modification methods have been pursued, all having the aim to 'wrap' the substrate, PET, into a body-friendly, body-recognizable compound without compromising its bulk properties. Accomplishment of this task is particularly difficult because the surface of PET lacks any functional groups. Nevertheless, biomolecules derived from or part of the extracellular matrix (*e.g.* gelatin, collagen, fibronectin), anti-inflammatory agents or anti-coagulant agents (*e.g.* heparin) were immobilized through physical or chemical methods [4–6]. The main drawbacks currently encountered are either the use of toxic substances, such as formaldehyde [7], or the compromise of the mechanical integrity of the substrate, by applying harsh reaction condition like in the case of alkalization [8] and aminolysis [9] methods, or time-consuming multistep protocols performed in organic solvents such as graft polymerization reactions [3,10]; latter can also present toxicity issues due to incomplete removal of solvents. Consequently, novel concepts are still required to properly surface modify PET.

In the present study, we report on a straightforward surface modification approach in aqueous conditions to immobilize gelatin on PET using dopamine coatings as intermediate layer. Dopamine is the synthetic equivalent of DOPA motifs present within mussel adhesive proteins, which are responsible for the adhesion properties of mussels. Pioneered by Messersmith et al. [11], dopamine coatings mimic the properties of their alike biological siblings and are capable to adhere on a variety of substrates including metals, ceramics and polymers upon incubation in aqueous alkaline media. The precise mechanism of adhesion is not completely unravelled, but non-toxicity and non-inflammatory responses of dopamine-coated materials were reported in literature, rendering them suitable for a vast range of biomedical applications [11–14]. The aim herein is to exploit the beneficial bio compatibility features of the dopamine coating and take advantage of its ability to enable (covalent) anchoring of gelatin – a protein known to be non-toxic, biodegradable and biocompatible, and that is easily recognized and accepted by the human body [15]. The main advantage of the applied procedure is given by a combination of factors: (1) the exclusion of toxicity issues due to the lack of organic solvents needed for the surface modification procedures – all reactions are performed in aqueous media; (2) the proven bio

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compatibility of the selected compounds – dopamine and gelatin; (3) the ease of the surface modification procedures – simple incubation procedures; and (4) the maintenance of the structural integrity of PET – mild alkaline conditions, pH 8.5, are used to induce dopamine polymerization. In addition, we have taken the opportunity to investigate possible effects of plasma-pretreatments and dopamine coating procedures on the final gelatin coating.

To the best of our knowledge, a study in which the application of a plasma-pretreatment, a dopamine coating procedure and the covalent immobilization of a protein onto PET intended for cardiovascular applications has not been reported to date.

2. Materials and methods

Materials. Biaxially oriented poly(ethylene terephthalate), PET, 0.1 mm thickness, was purchased from Goodfellow, UK. Gelatin type B, GelB, isolated from bovine skin by an alkaline process and with an isoelectric point of ~5 was kindly supplied by Rousselot, Belgium. Hydroxytyramine hydrochloride (otherwise called dopamine hydrochloride), 2-amino-2-(hydroxymethyl)-1,3-propanediol (Trizma base), 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), Triton X-100, Trypan blue (0.4% solution), hydrochloric acid (HCl, 0.1 N) and glutaraldehyde were purchased from Sigma Aldrich. Disinfectol was purchased from ChemLab, Belgium. All chemicals were used as received without further purification. Human whole blood with citrate-phosphate-dextrose-anticoagulants was obtained from "Rode Kruis", Belgium, to be used for research purposes only.

Substrate and buffer solution preparation. PET samples (0.5 cm × 2 cm) were ultrasonically cleaned with disinfectol for 15 min prior to surface modification. Tris–HCl buffer solution was prepared from 10 mM Trizma salt, pH 8.5. HEPES buffered saline solution (HBS pH 7.4, containing 20 mM HEPES and 150 mM NaCl) and a phosphate buffered saline solution (10 mM PBS, pH 7.4, containing 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 2 mM KH_2PO_4) were prepared for whole blood processing.

Dopamine coating procedure. Cleaned PET was incubated in a dopamine solution (2 g/L in Tris–HCl buffer) for 24 h, at ambient temperature on a shaking bed. Two grafting protocols were elaborated and compared. Conventional dopamine coating procedure was used for both procedures [11], but the solution's ageing time was varied. In protocol 1, a dopamine stock solution was prepared and stirred for 5 min and afterwards PET was incubated in it. In protocol 2, PET samples were initially incubated in Tris–HCl buffer and immediately dopamine hydrochloride was added up to a concentration of 2 g/L [16]. The surface-modified samples were washed twice with distilled water and stored in a desiccator in the absence of light until further use.

Post-plasma dopamine coating procedure. The influence of the substrate nature on coating properties was investigated through a plasma treatment prior to dopamine coating. Argon plasma treatment (0.6 mbar, 100 W, 60 s irradiation) was applied to pristine PET within the device described in [17]. The plasma-(pre)treated samples were subsequently immersed in dopamine solutions, following the aforementioned protocols.

Immobilization of gelatin on dopamine-modified PET films. Dopamine-modified PET was subsequently incubated in GelB solution (5% (w/v) in Tris–HCl buffer) for 24 h at 37 °C on a shaking bed. Next, the samples were rinsed twice and incubated overnight in distilled water at 37 °C in order to remove any physically adsorbed gelatin. Finally, samples were dried in air and stored in the fridge until further use. Two blank samples physically-modified with gelatin were also prepared accordingly to the aforementioned strategies, but in the absence of dopamine. Only the presence of the pretreatment was taken into account, as the applied protocol for dopamine coating has no relevance in this case.

Immobilization of radiolabelled gelatin on dopamine-modified PET films. Radiolabelled GelB was produced by first synthesizing a stock solution of 5% (w/v) Bolton-Hunter gelatin (BHG) subsequently radiolabelled with ^{125}I (GE/Amersham Health, Einhoven, Holland) by means of the Iodogen-method, as described in [18,19]. The concentration of BHG in the final GelB solution was adjusted to 5% (w/v) in Tris buffer. Dopamine-modified PET films were immobilized in thus-obtained radiolabelled GelB solution overnight and then washed in distilled water for 24 h at 37 °C in order to remove any physisorbed gelatin. At this point, the amount of immobilized radiolabeled GelB was determined by measuring the sample associated activity using a dose calibrator (CAPINTEC CRC-15R, Capintec Instruments, USA). All experiments were repeated four times.

Surface characterization methods. X-ray photoelectron spectroscopy (XPS) measurements were recorded as mentioned in [17]. Surface wettability was investigated on the device reported in [17]. Water contact angles (WCA, 1 μL) were determined in triplicate. The sample roughness was investigated through atomic force microscopy (AFM) measurements on the device reported in [15]. Different scan sizes were recorded (5 μm × 5 μm and 25 μm × 25 μm) in "tapping" mode. Both root-mean-square roughness (R_{RMS}) and average roughness (R_a) values were recorded (see more details in Appendix). Surface topography was investigated by scanning electron microscopy (SEM) on the device reported in [16]. The samples for platelet adhesion investigations were dehydrated in different volumetric ethanol/water mixtures (30, 75, 90, 95 and 100%) before gold sputtering and consecutive SEM analysis.

Preliminary in vitro blood compatibility assays. The whole blood was manipulated into platelet-rich plasma (PRP) or erythrocyte-rich plasma (*i.e.* red blood cell, RBC). All experiments were performed within 24 h of unsealing the whole blood bag.

Platelet adhesion. PRP with a cell density of 464000 cells/mL was obtained by whole blood centrifugation (1500 rpm, 10 min). Cell density was calculated with a Burker counting chamber and Trypan blue staining solution (1:1 v/v in PRP). Samples were placed in direct contact with freshly obtained PRP (100 μL/sample) and incubated at 37 °C for 60 min. Next, multiple preparation steps including rinsing with PBS, fixation, dehydration and drying were performed. Platelets were counted from at least two images showing a 1000× magnification per sample type.

Haemolysis. Healthy RBCs were obtained after the total removal of the anticoagulants from the whole blood through centrifugation (5 min, 3700 rpm). After supernatant removal, the resulting RBC suspension was gently washed three times with HBS buffer at room temperature and centrifuged. The procedure was repeated three times. After each centrifugation step, the supernatant and the top layers were removed, while the remaining dark-red liquid was considered the new concentrated RBC suspension. The final RBC concentrate was diluted in HBS buffer down to a 2% suspension and used for sample incubation (2 mL RBC suspension, 37 °C under continuous shaking). After 24 h, a final centrifugation was applied and the extent of the haemolysis was quantified using an UV–Vis spectrometer (Uvikon XL) at the wavelength of 545 nm. Triton X-100 (1% (v/v) in 2% RBC solution) was used to realize 100% haemolysis (*i.e.* the positive control), while the HBS buffer was applied as negative control (crf. 0% haemolysis). All measurements were performed in triplicate.

Statistical analysis. Student's *t*-test was performed in order to evaluate the reproducibility and the significant differences between samples with a confidence level of 95% and $p > 0.05$.

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