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Hemocompatibility of pyrolytic carbon in comparison with other biomaterials

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ABSTRACT

The present generation of mechanical heart-valves prostheses offers significantly improved blood compatibility, combined with appropriate physical and mechanical properties and durability; however, thromboembolism persists as a crucial clinical complication because of the key role played by adsorbed plasma proteins and adherent platelets in determining hemocompatibility. In the present work human plasma protein adsorption and platelet adhesion and activation were evaluated on four materials, selected according to their different physico-chemical and morphological properties: pyrolytic carbon (PyC), titanium alloy Ti–6Al–4V (Ti), tissue culture polystyrene (TCPS) and polystyrene sterilized by γ-irradiation (StPS). Morphological and chemical properties of surfaces were assessed by Time of Flight Secondary Ion Mass Spectrometry (Tof-SIMS), X-ray Photoemission Spectroscopy (XPS), Atomic Force Microscopy (AFM) and Contact Angle (CA) analysis, while protein adsorption and platelet adhesion and activation were evaluated by colorimetric and immunofluorescence tests and Scanning Electron Microscopy (SEM), respectively. Among the analyzed materials, PyC induced the lowest level of protein adsorption and platelet adhesion and activation. In particular, immunofluorescence analysis of fibronectin (FN), fibrinogen (FG), von Willebrand factor (vWF), Hageman factor (FXII) and albumin (ALB) showed that PyC surface was characterized by high levels of FG adsorption, low levels of FN, ALB and vWF and the absence of FXII. Finally, when analyzing the biological response as a function of surface properties, it was found that protein adhesiveness increased with increasing contact angle values. To the contrary, platelet activation related to the total amount of adherent proteins was poor while cell activation was possibly dependent on the detailed composition of the underlying protein layer. Furthermore, differences in surface roughness of the examined materials did not seem to influence the biological response in terms of platelet activation and protein adsorption.

1. Introduction

Among biomaterials available for blood-contacting medical devices, pyrolytic carbon (PyC) offers a fairly good combination of durability, physical and mechanical properties and biocompatibility [1–3]. However, patients with cardiovascular implants made of PyC still require chronic anticoagulant therapy to minimize biomaterial-associated thrombosis [4,5]. In fact, when a device comes into contact with blood, its surface acquires a complex layer of plasma proteins, which can trigger a series of interlinked events, including the recruitment and activation of platelets and leukocytes, as well as the activation of the coagulation and complement cascade [6]. The composition and the structure of the initially adsorbed protein layer are the key factors that determine further biological reactions and, vice versa, they are strongly dependent on the surface properties of the material [7]. In fact, PyC coating seems not significantly reduce major adverse events compared with bare devices of the same design and in particular, PyC allows platelet adhesion and activation [5,8]. Recent studies analyzed carbon-based materials in terms of their surface properties on blood compatibility [9,10], however a detailed study on the composition and structure of the protein layer adsorbed on PyC is still lacking.

In this study, we characterized the hemocompatibility properties of PyC in comparison with three different materials: titanium alloy Ti–6Al–4 V (Ti), tissue culture polystyrene (TCPS) and γ-irradiation sterilized polystyrene (StPS). To better simulate the complexity of the *in vivo* physiological environment, whole plasma was used instead of simplified protein mixtures or isolated cellular components. The aim of this study is to elucidate the mechanisms involved in the initial interactions between the material and the blood components at the molecular level and to investigate the role played by surface morphological and physico-chemical properties in determining biological response. A better understanding of these mechanisms is a prerequisite for developing innovative surface modification

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strategies for improving blood compatibility and increasing the performance of cardiovascular devices.

2. Materials and methods

2.1. Materials and surface analysis

Four different biomaterials were analyzed in this study: PyC supplied by Sorin Biomedica Cardio (Saluggia, IT) as plain circular samples (18 mm diameter) and manufactured for use in clinical devices; three commercial biomaterials: Ti supplied as plain circular discs (11 mm diameter); polystyrene from bacteriological Petri dish (StPS) and tissue culture treated polystyrene (TCPS), both from BD Biosciences, USA (15×10 mm rectangles). All materials were cleaned before analysis: PyC was ultrasonically cleaned in chloroform and rinsed once in ethanol; Ti was ultrasonically cleaned in isopropanol, then rinsed in distilled water three times; polystyrene surfaces were cleaned in 1% sodium dodecyl sulfate (SDS) solution and ultrasonically washed three times in distilled water. All surfaces were air dried.

ToF-SIMS spectra were acquired using a CAMECA TOF IV reflectron instrument using Ga^+ 15KeV in the bunched mode (800 ps pulse width) of operation. All the spectra were acquired for 200 s and a 0.04 mm^2 analysis area was sampled. Operating conditions were maintained within the static regime. Charge neutralization was achieved with a pulsed low-energy (0–20 eV) electron flooding. The mass resolution of the TOF-SIMS analyses was sufficient to identify most of the fragment ions observed in the spectra ($M/\Delta M > 6000$).

XPS measurements were performed using a Scienta ESCA 200 instrument, equipped with a hemispherical analyzer, operated in transmission mode, and a monochromatized Al Kα (1486.6 eV) X-ray source. After cleaning, the samples were immediately inserted, through a load-lock chamber, into the analysis chamber whose base pressure was about 5×10^{-9} mbar. The emission angle between the sample surface and the analyzer axis was 90, corresponding to a sampling depth of approximately 10 nm. For each sample, a survey was acquired in a wide energy range (0–1300 eV), to detect the elements present on the surface. Then the core level material characteristics were recorded at the pass energy of 150 eV with an energy resolution of 0.4 eV. To compensate for sample charging, low electron energy flood gun was used for insulating samples. The quantification is made as relative elemental percentage using the integrated area of the core lines after linear background subtraction and applying atomic sensitivity factors. This procedure gives semiquantitative analysis allowing the surface chemistry comparison among the samples.

AFM measurements were taken in semi-contact mode using a NT-MDT (Russia) Unisolver instrument, equipped with a $120\times120\ \mu m^2$ scanner. NSG-11 (NT-MDT) cantilevers with resonance frequency of approximately 150 and 255 kHz (nominal force constants of 5.5 and 11.5 N/m, respectively) were utilized. For every material, several images of different areas were acquired, ranging from $1\times1\ \mu m^2$ to $40\times40\ \mu m^2$ to determine on different scale sizes the average roughness parameter (S_a), that is the arithmetic mean of the absolute distances of the surface points from the mean plane. Acquired images were plane fitted and leveled line-by-line using an image processing software (SPIP v 4.1.6, Image Metrology A/S, Denmark). The same software was used to compute the roughness of the acquired areas.

CA measurements were performed using the sessile method as modified by Della Volpe and others [11] determining the so called Vibration Induced Equilibrium Contact Angle (VIECA). Each experimental result is the mean of at least 5 measurements; the measurements were carried out at room temperature using a self-developed goniometer. Briefly, VIECA allows to obtain the equilibrium contact angle on rough and/or heterogeneous surfaces using acoustical vibrations generated by a loudspeaker in a controlled way (at a definite frequency and amplitude able to optimally transfer mechanical energy to the liquid meniscus).

2.2. Adherent protein quantification and immunofluorescence analysis

Platelet-rich plasma (PRP) from healthy donors ACD (acid-citrate, dextrose)-anticoagulated human blood was kindly provided by the Immunohematology Department, S. Chiara Hospital (Trento, IT) and maintained at 22 °C with gentle shaking until use. Platelet poor plasma (PPP) was obtained by centrifugation at 3000 *g* for 20 min. Material samples were incubated in static conditions with 3 ml of PPP for 2 h at 37 °C and 5% CO_2. After incubation, the plasma was washed off by rinsing twice in Dulbecco's phosphate buffered saline (DPBS) and adsorbed proteins were eluted by exposing the surfaces to a 2% SDS solution for 2 h at 37 °C and 5% CO_2. After collecting the desorbed proteins, surfaces were further exposed to the same solution for an overnight (o.n.). The eluates were then concentrated by evaporation and stored at −20 °C until use. The quantity of protein eluted from the surfaces was determined using a Micro-BCA Protein Assay Reagent Kit (Pierce, Rockford, IL) according to the manufacturer's instructions. Solutions of purified bovine serum albumin (BSA) ranging in concentration from 1.5 μg/ml to 250 μg/ml were used to generate a standard curve. The albumin standard, as well as the concentrated protein samples, was assayed in a microtiter plate format in duplicate. Absorbance was measured at 575 nm using a microtiter plate reader (Molecular Devices, Sunnyvale, CA).

For immunofluorescence analysis PyC and Ti samples were incubated with 3 ml of PPP for 2 h at 37 °C and 5% CO_2, in static conditions. Plasma was washed off with DPBS and specimens were placed on a cooling plate at 0 °C. Samples of both biomaterials were incubated for 20 min at 0 °C with the following specific primary antibodies: mouse anti-ALB, mouse anti-FN, mouse anti-FG, rabbit anti-vWF (Sigma) and goat anti-FXII (US Biological) antibodies. The incubation was followed by rinsing in DPBS and by a second incubation for 20 min at 0 °C with fluorescein-conjugated goat anti-mouse, goat anti-rabbit, rabbit anti-goat antibodies (Sigma) in the dark. Control experiments were performed by excluding primary antibody incubation. The second incubation was followed by rinsing in DPBS and then the samples were examined and photographed with a fluorescence microscope (Olympus IX 50), utilizing a cooled CCD detector with resolution depth of 10 bits per channel (Olympus) and an Olympus filter set (U-MWIBA2) with excitation band of 460–495 nm and emission band of 510–550 nm. Magnification (100×) and exposure time were standardized and kept constant in all experiments. Several images, each with an acquired area of $172\times130\ \mu m^2$, were taken for every sample. The mean fluorescence brightness of the pictures above background level, represented by the control experiment, was used as a measure of the amount of bound antibodies and was expressed in arbitrary units.

2.3. Platelet adhesion and activation on material surfaces

Substrates were incubated with 3 ml of PRP (5×10^5 platelets/μl) for 2 h at 37 °C and 5% CO_2 in static conditions. They were then washed twice with DPBS and adherent platelets were prepared for electron scanning microscopy (SEM) analysis by fixation in 2% glutaraldehyde (Sigma) in DPBS for 1 h at room temperature. Samples were then dehydrated in an ethanol-graded series (50, 70, 85, 95 and 100%) twice for 15 min each, followed by a post fixation in hexamethyldisilazane (Sigma) for 1 h.

After sputter-coating with gold/palladium (80:20), the samples were then investigated with a Jeol JSM6100 scanning electron microscope, tilting the samples at 40 (tungsten filament, about 10^{-3} Pa specimen chamber pressure, accelerating voltage between 5 and 20 kV). For morphological and quantitative analysis of platelet–material interaction, scanning electron microscopy (SEM) images were obtained from randomly selected areas at 400× magnification. The total number of platelets per unit area was determined. Platelet spreading was then examined by categorizing platelet shapes into five morphological forms