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**Coating of cobalt chrome substrates with thin films of polar/hydrophobic/ionic
polyurethanes: characterization and interaction with human immunoglobulin G and
fibronectin**

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Abstract

Biomaterial implants often lead to specific tissue reactions that could compromise their bio-integration and/or optimal cellular interactions. Polyurethanes (PU) are of particular interest as coatings to mask CoCr's bioactivity, since they are generally more biocompatible than metal substrates, present a broad range of chemistry, and have highly tunable-mechanical properties. In the current work, complex polyvinyl-urethanes (referred to as D-PHI materials) are studied for their surface phase structures: specifically, an original D-PHI polymer (O-D-PHI) and a differential formulation with relatively higher hydrophobic and ionic content (HHHI) are of interest. The PUs are diluted in tetrahydrofuran (THF) to generate thin films which differentially influence the physical and chemical properties of the D-PHI coatings. AFM images over time show the gradual appearance of domains exhibiting crystalline organisation, and whose shape and size were dependent on D-PHI thickness (thin films vs non-solvent cast resin materials). After three weeks, a complete stabilization of the crystal state is observed. The thin coatings are stable in an aqueous and 37°C environment. The adsorption of two human plasmatic proteins Immunoglobulin G (IgG) and Fibronectin (Fn), involved in inflammation and coagulation, was studied. The exposure of specific protein sequences (IgG-Fab, Fn-Cell Binding Domain and Fn-N-terminal domain) were dramatically reduced on both D-PHI materials when compared to bare metal CoCr. The implications of these findings would be relevant to defining coating strategies used to improve the blood clotting and immune cell reactivity to CoCr implant materials.

Keywords

Thin film surfaces, Polyurethane, Cobalt Chrome, Immunoglobulin G, Fibronectin.

Introduction

Medical devices are clinically used in a wide range of applications and their performance is highly dependent on the nature of their surfaces and the latter's interaction with defined proteins and cells. Post implantation of any biomaterial, a tissue reaction occurs which results in an inflammatory response driven, in large part, by immune related proteins and cell contact activation. An appropriately modulated innate immune response would improve the acceptance of a given biomaterial by the host and its bio-integration with tissues. Polyurethanes (PU) are attractive polymers for the biomedical industry^{1,2}, as they present a broad range of chemistry, and their inherent chemical characteristics yields tailored mechanical properties. This in turn may enable enhanced biocompatibility, when the polymers are synthesized with defined compositions and microdomain features.

Recent work has focused on the synthesis of advanced-urethane vinyl based systems and their properties for reducing inflammatory responses by human blood monocytes^{3,4}. These polyfunctional polyurethanes (referred to as D-PHI, or degradable polar/hydrophobic/ionic) contain in their original formula, a divinyl polycarbonate-lysine urethane oligomer –DVO-, methacrylic acid –MAA- and methyl methacrylate –MMA- (**Supplementary Figure S1**). The original D-PHI (O-D-PHI) had a molar ratio of 1:5:15, respectively, with DVO representing a polar non-ionic unit, MAA yielding anionic character, and MMA providing hydrophobic character. O-D-PHI has been shown to be biocompatible and non-toxic to cells when used in the engineering of vascular tissues with vascular smooth muscle cells (VSMCs)⁵, endothelial cells⁶ and fibroblasts⁷. Furthermore, O-D-PHI has been shown to reduce the pro-inflammatory activation of monocytes when compared to tissue culture polystyrene (TCPS) and polylactic glycolic acid (PLGA)^{4,8}, two prominent biomaterials reported on in the literature.

The block structure chemistry of D-PHI can be tuned by varying the hydrophobic and anionic monomer content relative to the polar divinyl oligomer content in order to obtain formulations that interact with human monocytes differentially. For example, an alternate DVO/MAA/MMA ratio of 1:20:39 (referred to as HHHI, high hydrophobic and high ionic) appears to promote a further reduced inflammatory response by human blood monocytes relative to that of O-D-PHI with a 1:5:15 ratio^{3,8}.

The combination of polar, hydrophobic and ionic character presents an opportunity to display diverse chemical group presentation, within different micro-domains, at material surfaces when exposed to unique environments, such as protein solutions. Such chemical group diversity is not unlike that which is also exhibited by different peptide residues found in proteins. Previous research showed that O-D-PHI's unique chemical structure enables one to direct specific binding sites and their respective exposure states for adsorbed immunoglobulin G (IgG)⁸ and human plasma-derived fibronectin (Fn)³. The latter are important modulators of cellular responses, and specifically to those of monocytes. Immediately after implantation, when blood comes into contact with any biomaterial, there is a spontaneous protein adsorption on the surface⁹, before the interaction of the biomaterial with inflammatory cells. Therefore, the type, concentration, and conformational arrangement of the adsorbed proteins influence the recruitment and activation of immune cells^{9,10}. The monocyte / macrophage interaction with a surface has been shown to depend on a defined number of plasma proteins, including IgG¹¹ and Fn¹².

IgG is the most widespread immunoglobulin in extracellular fluids. Fragment antigen binding (Fab) enhances long-term macrophage adhesion^{8,13} and their exposure at **the biomaterial/protein interface** influences the activation of immune cells, more particularly monocytes and macrophages.

Fn is an extracellular matrix glycoprotein and exists as a compact soluble form within plasma. It is composed of three types of repetitive modules (I, II and III), which contain binding domains to a wide range of extracellular and cellular surface molecules such as the Cell Binding Domain (CBD) and N Terminal domain¹⁴ previously shown to play a role in inflammation^{15,16} and coagulation^{17,18} respectively.

Cobalt chrome (CoCr) has been used as an implant biomaterial because of its physico/chemical properties and is common in cardiovascular medical devices such as, stents, heart valves and pacemakers. It is non-magnetic, resistant to wear, corrosion and heat and is considered by many literature studies to be biocompatible^{19,20}, although there is some controversy related to this material. Previous studies have shown that bare CoCr is pro-thrombotic and pro-inflammatory due to its ability to activate platelets and coagulation proteins; and to induce leukocyte adhesion and activation^{21,22}. Therefore, it would be of interest to improve the surface properties of CoCr.

The goal of this study was to investigate the potential use of thin film D-PHI materials for coating CoCr surfaces which come in contact with the innate immune cells. This is of significant importance as D-PHI has never been characterized in a thin film form. Specifically, there would be concern if D-PHI's critical anti-inflammatory nature was lost. The absence of this knowledge is vital to the premise that coating reactive CoCr materials will yield low inflammatory character^{5,8}. Moreover, there are no reports in the polyurethane literature which describe the multiphase structure characterization of a dual phase polymer with vinyl chemistry combined with step growth chemistry. Despite the dominant effect of cobalt chrome chemistry on proteins, it has never been conceived to use complex polyurethanes in thin film form to alter the reactivity of cobalt chrome.

Hence, it was essential to characterize these thin films and their interaction with CoCr in order to better understand the structure and molecular organization of D-PHI in contact with CoCr. We thus focus on the physico-chemical surface characterization of the D-PHI coatings on CoCr and the subsequent IgG and Fn, behavior on these surfaces.

Materials and Methods

Cobalt Chrome surface

CoCr surfaces (L605, Goodfellow) were electropolished and the specimens designed to fit into the wells of 96-well plates (6 mm diameter, 0.25 mm thickness). CoCr surfaces were cleaned with pure water for 1 h, and then immersed in TFD 9-10 (detergent) for 2 h. Finally, the surfaces were washed in 70% ethanol for 2 h, sonicated in water (3 times during 10 min) and autoclaved.

Polyurethanes resins

D-PHI synthesis was carried out using a previously established method⁵. Divinyl oligomer (DVO) were mixed with a methacrylic acid (MAA) and methyl methacrylate (MMA) mixture and added to the initiator benzoyl peroxide (BPO, 0.032 mol/mol vinyl group) to generate the polyurethane precursor solutions (Supplementary Figure S1). Respectively for DVO/MAA/MMA, the resin formulation referred to as original D-PHI (O-D-PHI) had a 1: 5: 15 stoichiometry, whereas HHHI (High Hydrophobic, High Ionic relative to O-D-PHI) had a stoichiometric ratio of 1: 20: 39⁸. The viscosity of the as prepared resin mixtures for O-D-PHI and HHHI precursors, prior to the addition of solvent, was measured using a MCR 301 rheometer (Anton Paar), equipped with a plane-plane geometry in

aluminum (measuring system was 25 mm in diameter). The values of 2.404 ± 0.693 Pa.s and 0.04 ± 0.011 Pa.s were determined for O-D-PHI and HHHI respectively.

Polymerized vinyl-urethane materials

The two DVO/MAA/MMA precursor resin mixtures were used to generate i) the bulk cast material or ii) the thin films materials from solutions containing THF.

For the bulk polyurethane material, briefly, the samples were generated by direct deposition of a drop (50 μ L) solution consisting of the resin mixtures in 96-well polypropylene plates as previously reported⁸.

Thin films (in the low μ m-range of thickness) were prepared by first diluting the PUs resin mixtures in THF in order to decrease their viscosity. Different ratios of the resin mixtures and THF were first investigated and it was established that a respective resin: THF ratio of 1:32 and 1:16 for the O-D-PHI and HHHI resin mixtures would enable the generation of thin films of similar thicknesses for the two compositions. The viscosity of the resin: THF solutions was measured to be 0.0003 Pa.s for both solutions. A drop of resin: THF solution (10 μ L) was deposited on the 6 mm diameter CoCr discs before polymerization.

In both cases (thick bulk or thin film), the polymerization was initiated at 65°C during 1h (to allow solvent evaporation), followed by 24h under nitrogen at 110°C^{4,8}. [Note: all experiments were carried out in triplicate, 3 times with 3 different syntheses].

Characterization of polyurethane based-materials

Profilometry measurement

A profilometer (Dektak 150 from Veeco) was used to obtain thickness measurements of D-PHI coatings. The reported thickness values correspond to the measurement of 3 independent scratches.

Scanning electron microscopy (SEM) and Energy Dispersive X-ray spectrometry (EDS)

Samples were subjected to nickel metallization (2 x 30 seconds) and then underwent SEM using a LEICA S430i microscope operating in secondary electronics mode. The same field of view was then scanned using an energy-dispersive X-ray spectrometer (using Silicon Drift detector) to acquire a set of X-ray maps for C, O and N using the PhiRhoz method (Bruker AXS Microanalysis GmbH) and to characterize the atomic composition of the polymer surfaces.

Atomic Force Microscopy (AFM)

AFM experiments were performed in Peak Force QNM mode with a Dimension ICON microscope from Bruker. All measurements were carried out in air at room temperature with a ScanAsyst tip (Constant of stiffness $k = 0.4 \text{ N/m}$, Bruker). The sample measurements were carried out using both dry and wet states (water 18M-Ohm). All images were obtained using the same probe with the same peak force. Images were analyzed using NanoScope Analysis software version 1.5 from Bruker Nanoscope.

Differential Scanning Calorimetry (DSC)

DSC was carried out with a DSC Q100 model (TA Instrument). Polymer samples were placed in an aluminium cell (approximately 5 mg) and cooled at -80°C . Upon initial scans it was observed that the crystalline phases were below 50°C , and hence the heating thermograms were recorded from -60°C to $+50^{\circ}\text{C}$ at $5^{\circ}\text{C}/\text{min}$ under argon flow. Melting temperature values were taken as the onset point from the first heating cycle.

Water Contact Angle (WCA)

The samples were dried at room temperature for 72h in a desiccator and the contact angle measurements were performed on the different surfaces using a 10 μ L drop of pure water (18 M-Ohm resistivity) deposited with a micro syringe³. Values were measured for three drops on each of three separate samples per test group. The contact angle measurements were performed with a Drop Shape Analysis System KRUSS DSA 10MK2 goniometer. The results were analyzed using Drop Shape Analysis DSA 1 software.

Protein analysis

Fibronectin preparation

Fn was purified from pooled plasma isolated from different human donors following a three-step affinity chromatography process, as previously described by Poulouin et *al.*²³. This purification process has been shown to yield protein in solution with a purity of approximately 98% (w/w). Fn concentration was determined by optical absorbance measurement $A^{1\%}_{280\text{nm}} = 12.8$.

Protein adsorption

Protein adsorption was performed using human IgG (0.2 mg/mL, *sigma I4506*) or human plasma-derived Fn (0.1 mg/mL). Solutions were exposed to the substrates and protein adsorbed for 2h at 37°C. The substrates were then gently rinsed three times in PBS to remove loosely retained protein. Protein adsorbed substrates were incubated in 120 μ L of 2% Sodium dodecyl sulfate for 24h at room temperature. The bicinchoninic acid (BCA) assay was used to quantify the SDS eluates and was performed according to the manufacturer's protocol (Thermo Scientific). After elution by SDS, the surface was further assessed for residual protein by BCA assay, and none could be detected above the background of the substrates themselves, confirming the effectiveness of the SDS treatment for protein removal.

Quantification of specific protein sequences using monoclonal antibodies

The presentation of Fn epitopes in the adsorbed protein state was measured by ELISA (Enzyme Linked ImmunoSorbent Assay) as described in previous work³. Samples were incubated with monoclonal antibodies against specific Fn sequences: anti Cell-Binding Domain (dilution 1/30000) (abcam, ab26245) (III-9/10 domain), and anti N Terminal (dilution 1/43000000) (Millipore, MAB1936) (II-1/2 I-7/8/9 domain). Then, samples were incubated with a secondary antibody, anti-mouse IgG (dilution 1/1000) (Thermo scientific, prod#31430). Bovine serum albumin (BSA) was used as a control to assess for non-specific interactions. The absorbance was normalized to the total Fn, as measured by the BCA assay described above.

Following the substrate coating with IgG, Fab exposure was quantified using an HRP-conjugated Fab-specific IgG (Sigma, A0293) and a tetramethylbenzidine (TMB) substrate solution (eBioscience), with absorbance read at a wavelength of 450 nm. The absorbance was normalized to total IgG as measure by BCA assay described above.

Statistical analysis

Statistical analysis was performed using the SPSS Statistics 17.0 software (SPSS Inc., Chicago, IL) for an analysis of variance (ANOVA). Statistical significance was reported for $p < 0.05$. Unless otherwise stated all experiments were repeated in triplicate with three technical replicates for each experimental value.

Results and Discussion

Surface characterization for thin film of polyurethanes

To mask the inherent bio-reactivity of metallic medical devices, some research has focused on the use of material coatings such as polyurethanes²⁴, complex co-polymers (*e.g.*

poly(hydroxystyrene-*b*-isobutylene-*b*-hydroxystyrene))²⁵. However, it has been reported that polymer coatings are not entirely inert, and display hypersensitivity reactions to the polymer²⁶ with long-term adverse effects of increased inflammation²⁷.

In the current study we have investigated CoCr substrates coated with a novel lysine-derived vinyl polymerized urethane platform material, by generating two formulations, O-D-PHI and HHHI. To work with thin films of D-PHI materials, it was essential to dilute the O-D-PHI and HHHI polymer resins in THF prior to their polymerization, in order to generate the desired range of film thickness (note: the original resins were too viscous to generate low micron-thin films). The use of THF with D-PHI rather than its application in a neat form was anticipated to possibly influence the physical state of the material surfaces, as a previous study showed that the use of different solvents had an impact on the nature and crystallization of polypyrrole films²⁸. The thickness was effectively reduced with the solvent cast materials, from the order of mm for bulk materials (*i.e.* O-D-PHI = 1.4 ± 0.1 mm and HHHI = 1.3 ± 0.2 mm) to μm for thin films when THF was used (*i.e.* O-D-PHI = 4.9 ± 1.3 μm and HHHI = 5.2 ± 1.8 μm). Analysis using SEM/EDS images show that the CoCr surfaces coated with thin films of D-PHI were homogenously covered (**Supplementary Figure S2**). The atomic composition analysis of the polymer coated surfaces (**Table 1**) shows a slight but significant decrease in carbon content between the bulk materials and thin films coated on CoCr. There was also a significant increase in oxygen and nitrogen on the thin films. Both of the latter atomic elements would be associated with the polar groups of the polymer which are interacting with the evaporating THF from the surface, and would draw the polar groups to the surface.

Surfaces Atoms (%)	O-D-PHI bulk	O-D-PHI thin film	HHHI bulk	HHHI thin film
Carbon	66.9 ± 2.1	64.6 ± 3.0 ^Φ	67.7 ± 0.8	65.6 ± 2.0 [#]
Oxygen	29.8 ± 1.3	31.7 ± 2.6 ^Φ	29.3 ± 0.6	31.1 ± 1.8 [#]
Nitrogen	3.3 ± 0.8	3.7 ± 0.5 ^Φ	3.0 ± 0.2	3.5 ± 0.4 [#]

Table 1: Atomic composition of surfaces with different polyurethane formulations obtained using EDS. Data are expressed in % as mean ± SD, n=3 repeated 3 times, 5 different spots.

^Φp<0.001 between O-D-PHI bulk and thin film; [#] p<0.001 between HHHI bulk and thin film.

The inherent differences in surface chemical changes resulting from the solvent process subsequently led to the need to characterize the surface physical structure of the different materials as well as their chemical differences. **Figure 1** shows the presence of crystals (relatively hard domains by AFM) and amorphous zone (relatively soft domains by AFM) on the surface of the materials. Given that the oligomeric carbonate is the only component of the polymer system known to crystallize, it was hypothesized that the crystalline domains implicated the oligomeric urethane carbonate monomer within these crystal domains²⁹. These crystal structures were different in appearance depending on film thickness (solvent cast *vs* non-solvent). On thin films, crystals were smaller but distributed over the entire surface whereas on bulk materials, crystals were much larger and not present across the whole surface.

Figure 1 shows the results of a **time course** study that reveals a slow transformation of crystals over time, with the crystalline domains eventually forming homogeneously over the entire surface at some time between 11 and 20 days, although the density of crystals was greater for the THF processed thin films. The crystallization evolved more slowly for the non-solvent cast films *vs* the THF processed thin films of D-PHI. The process stabilized for all

surfaces by approximately 20 days post polymerization. This is in accordance with other studies where the use of solvent in the generation of thin films based on polylactic acid reveals different re-organization of the chains by AFM³⁰. The findings of the current work imply that a minimum of 20 days are required after the polymerization of the coated materials to enable a stabilized surface to be established for bio-characterization. All subsequent surface characterization and protein experiments were carried out at 20 days post synthesis. This included the data reported on **Table 1**.

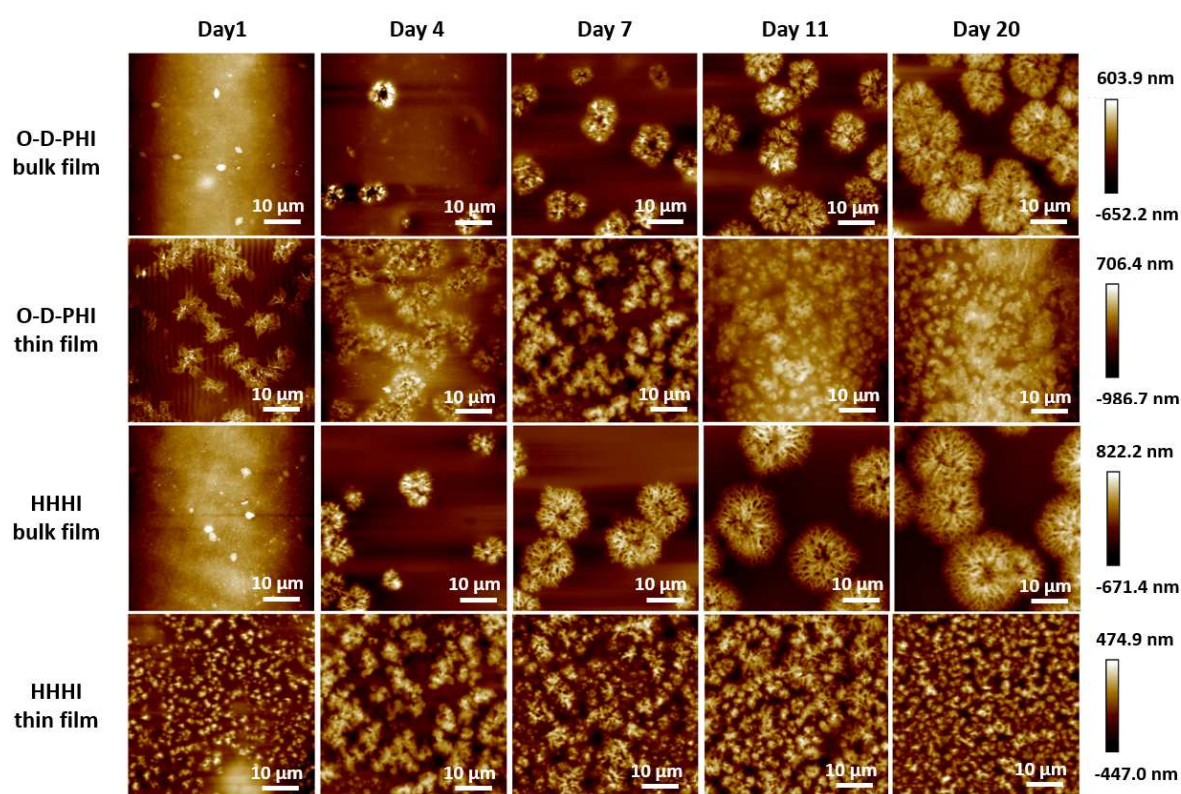


Figure 1: Topography of CoCr surfaces with different D-PHI formulations: Crystal formation as a function of time **in a dry environment with light**. Images were obtained by Atomic Force Microscopy at 1, 4, 7, 11 and 20 days post synthesis. n=3, repeated 3 times. Scale Bar: 10 μm.

Since the crystallization of the D-PHI PUs was suspected to be driven by the presence of the polycarbonate (*e.g.* DVO) moiety and not the lower molecular weight vinyl monomer

segments, we performed additional AFM experiments (**Figure 2**), to define the features of each relevant component.

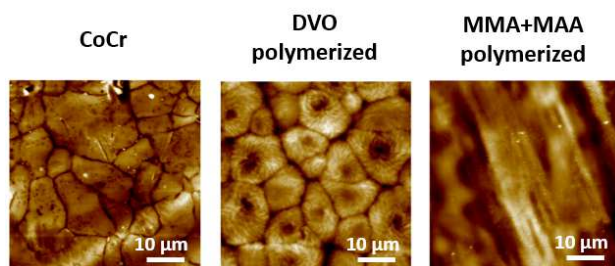


Figure 2 : Topography of CoCr surfaces and verification of the presence or not of crystals for polymerized DVO or MAA/MMA alone on the CoCr substrates. Images were obtained by Atomic Force Microscopy. n=3, repeated 3 times. Scale Bar:10 μm .

Figure 2 shows that only the DVO component and not the MMA/MAA component, when isolated alone and reacted, crystallizes from 1-day post polymerization. These results were further verified using DSC (**Table 2**), where a T_m° was observed for the polymerized DVO, but no crystalline melt phase was found for the polymerized MMA/MAA material. Note that the T_m° was the same for the four cast surfaces of D-PHI (thin film or bulk film) as well as for the polymerized DVO (*i.e.* approximately $34^\circ\text{C} \pm 1$). These T_m° values were identified during the first heat cycle. However, they were no longer detected in a second heat cycle, which suggests that it takes a long time for them to reform, as indicated by the kinetics of crystallization (**Figure 1**). The reported T_g for MAA is 220°C^{31} , and was also confirmed by DSC in our experiments, while the reported T_g value for MMA is 105°C^{32} , and shown to be 120°C by DSC in our lab. Neither of the materials show a crystal melt value at higher values.

Surfaces	Melting Temperature (°C)	Crystallization Temperature (°C)
O-D-PHI bulk	35°C	ND
O-D-PHI thin film	33°C	ND
HHHI bulk	34°C	ND
HHHI thin film	34°C	ND
Polymerized MAA/MMA	No detected	ND
Polymerized DVO	33°C	ND

Table 2: Thermal properties of different polyurethane surfaces and of different components for these polyurethanes. This table represents the melting temperature on the first heating cycle obtained by DSC. n=1, repeated 2 times. *ND* = No Detected

The **formation** of the crystals for all D-PHI materials (thin film and bulk material for both O-D-PHI and HHHI) was investigated **under different environmental conditions**. Whether **that crystallization was allowed to proceed** in a dry, or wet (*e.g.* water) **environment**, **and in the presence of** light or protected from light at room temperature, the formation of crystals proceeded for all materials **over 20 days in a similar manner (hence we only report data for one condition (dry/light) in Figure 1)**. The final nature of the crystals was more defined by the combination of the polyurethane material and solvent than the combination of environments studied. Further, we examined the stability of the crystalized samples after they were formed. The behavior of the polymerized samples was investigated over three days in different conditions, exposed to a wet environment *vs* dry air, and in the light *vs* dark. Regardless of the combinations of the environmental condition's the crystals were preserved. From this work it was evident that crystals that have been formed in polymerized samples over 11 days at room temperature show relative stability at 37°C *vs* being completely melted if the sample was treated at 60°C for 24h (*Data not shown*). The melting value of pure polycarbonate oligomer as measured by DSC was in the range of 50-60°C which is in

agreement with previous work²⁹. *Tang et al.* showed that the polyurethane oligomer's crystal melt temperature dropped to 45°C when copolymerized with other monomers. In the current study, the presence of other monomers co-polymerized with the polycarbonate oligomer reduced the oligomer's melt temperature to approximately 35°C.

The above findings provide **for the first time** the conditions needed to generate a stable and reproducible D-PHI substrate with respect to the relative distribution of carbonate, urethane, MMA and MAA moieties in the material, as well as their crystalline state. This was required for subsequent studies focused on determining the effect of relative chemical group distribution and concentration (*e.g.* O-D-PHI *vs* HHHI) on protein interactions with thin films formed on CoCr substrates.

Study of protein behavior

Given that previous studies have described the importance of relative surface hydrophobicity to the nature of protein adsorption by entropic effects³³ and that there were shifts in relative polar chemistry associated with thin *vs* bulk cast films (**Table 1**), it was therefore of interest to examine the relative change in hydrophobicity on the thin film *vs* bulk film substrates using contact angle methods, **with and without protein binding** (**Table 3**).

Coating Surfaces	Bare surface	Surface coated with Fn	Surface coated with IgG
O-D-PHI bulk	83° ± 2.8	74° ± 3.6	71° ± 4.3
O-D-PHI thin film	76° ± 2.0	59° ± 5.3	57° ± 5.9
HHHI bulk	80° ± 3.8	72° ± 6.1	71° ± 5.1
HHHI thin film	77° ± 2.0	59° ± 2.0	62° ± 4.0
CoCr	56° ± 5.6	63° ± 5.0	69° ± 8.0

Table 3: Water contact angle measurements of different surfaces with different coatings.

Results are expressed in degrees as means \pm S.D. n=3, repeated 3 times.

Since the mean square roughness of all surfaces was less than 221 nm (**Supplementary Table S1**), physical roughness can be ruled out as a major contributor to the differences in contact angle values, and the question of surface chemistry and crystal domain distributions become more relevant. The results show that the contact angle (**Table 3**) decreases between bulk material (*i.e.* O-D-PHI = $83^\circ \pm 2.8$ and HHHI = $80^\circ \pm 3.8$) and thin films of PUs (*i.e.* O-D-PHI = $76^\circ \pm 2.0$ and HHHI = $77^\circ \pm 2.0$). This is suspected to be a direct reflection of the slightly more polar character of the thin films (**Table 1**), and possibly due to the increased number of microdomains relative to more heterogeneous large domains. The surface of CoCr is much more hydrophilic than that of the coated polymer surfaces, with a value of $56^\circ \pm 5.6$ which is in agreement with the literature (*i.e.* 59°)²². **Table 3** also reveals a decrease of hydrophobicity with the addition of proteins -Fn or IgG- on the four D-PHI surfaces. That change was irrespective of the D-PHI chemistry and was typically twice as great for the thin films (-17 and -18 for O-D-PHI and HHHI respectively) vs the bulk material (-9 and -8 for O-D-PHI and HHHI respectively). In contrast to the polymers with Fn or IgG adsorption, the contact angle increases on CoCr in the presence of protein. The differences observed between the shifted contact angle values on the protein-coated PUs and protein-coated CoCr suggest that the manner by which Fn and IgG adsorbs onto the D-PHI surfaces and the CoCr was very different. It must be pointed out that contact angle analysis used to probe surface energetics alone provides an incomplete interpretation and understanding of the physical interactions with proteins³⁴.

Therefore, we undertook studies at the molecular level using ELISAs. Given the availability of monoclonal antibodies, it was possible to study specific sequences of Fn and IgG that were exposed and therefore may be potentially be available for the cells to interact with. Gradually, as the proteins adsorb to the surface, it was hypothesized that they may undergo conformational changes in order to minimize the total free energy of the system³⁵.

The results presented in **Figure 3** show that exposure of the Fab fragment related to IgG was significantly greater on CoCr than on the four D-PHI surfaces. Note that this exposure was significantly lower on thin films of PUs when compared to bulk material. When the surface contact angle was examined for all the materials (*e.g.* with *vs* without IgG), it was observed that there was a strong correlation ($R^2 = 0.82$) between the change in contact angle and the level of Fab presentation for given surfaces (**Supplementary Figure S3**). Exposure of IgG Fab was previously reported to be a critical factor required to increase monocyte adhesion and propagation^{8,13}. The ability of D-PHI to limit Fab exposure by binding IgG from the solution thus would be anticipated to minimize IgG activation of pro-inflammatory activity on adherent monocytes⁸. The significant decrease in IgG Fab segment exposure on D-PHI materials (**Figure 3**) appears to show that the chemistry of the PUs *vs* bare CoCr allows for a more passive adsorption of IgG, considered to be a marker of low inflammatory nature and hence the results suggest the importance of these biomaterials as immunomodulators. The observed differences in the adsorption and behavior of the IgG protein, between thin film *vs* bulk material in novel, and suggest that small changes in surface atomic composition, as well as shape, size and distribution of the crystals (**Figure 1**) over the surface have a strong impact on presentation of the protein (also supported by contact angle data for the comparison of thin film *vs* bulk (**Table 3**)).

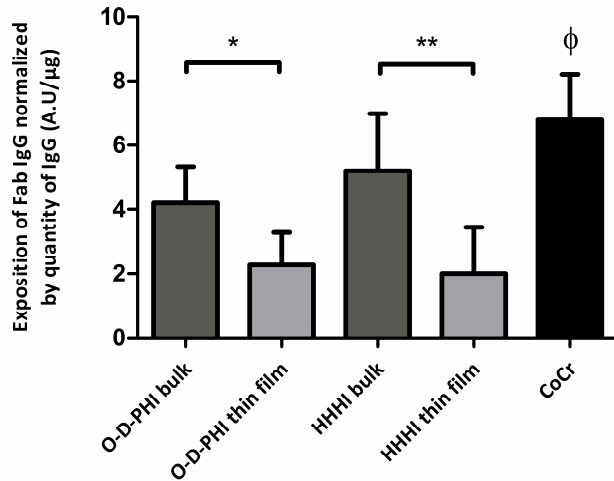


Figure 3: Quantification of Fab exposure measured by ELISA. Absorbance is normalized to IgG adsorbed quantity from the BCA assay. Results are expressed as means \pm S.D. n=3, repeated 3 times., * $p < 0.05$, ** $p < 0.01$, $\phi p < 0.001$ between CoCr vs PUs

Fn is known to have a role in inflammation *via* its Cell Binding Domain (CBD) and in coagulation through its N terminal segment. In order to better show the statistical differences between the four polymer coated surfaces, the values for the CoCr are only reported on in the text here and not compared in Figure 4 so that statistical differences between the four polymers can be clearly seen. The availability of the CBD sequence was found to be 4 times greater on CoCr when compared to the different D-PHI coatings, with the value for CoCr = 85 A.U/μg (Data not shown), while D-PHI thin film or bulk materials being approximately between 10 to 20 A.U/μg respectively (Figure 4 A). Looking more closely at the four polyurethane materials (Figure 4 A), there is a significant difference between O-D-PHI and HHHI bulk ($p < 0.001$) and between O-D-PHI and HHHI thin films ($p < 0.01$), showing that the different chemistries of PU lead to different behavior on adsorption of Fn and presentation of the CBD. The CBD contains the amino acid sequences RGD and PHSRN. The synergy between these two sequences favors the fusion of macrophages to form foreign Body Giant Cells (FBGC)¹⁵ and activates monocytes through the VLA-5 receptor, thereby inducing the

production of proinflammatory cytokines such as IL-1, IL-6 and tumor necrosis factor- α (TNF- α) by these activated cultured human monocytes¹⁶.

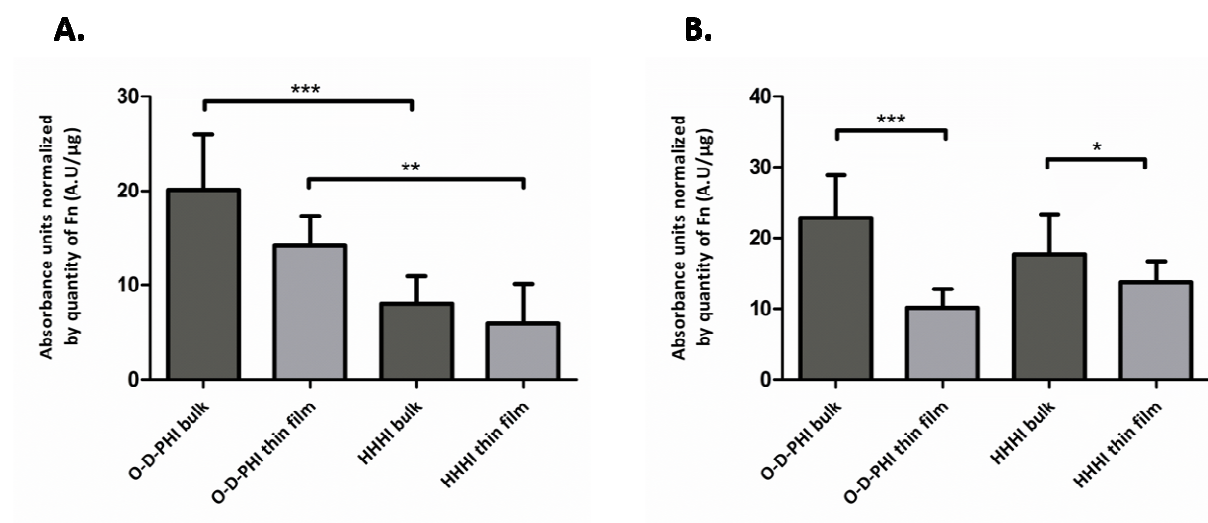


Figure 4: Availability of Fn peptide sequences measured by ELISA on polymer coated CoCr surfaces. Two monoclonal antibodies are used: **A.** Cell binding Domain and **B.** N terminal Domain. Absorbance is normalized to Fn adsorbed quantity from the BCA assay. Results are expressed as means \pm S.D. n=3, repeated 3 times. ***p<0.001, **p<0.01 and *p<0.05

The availability of the N terminal domain on Fn (**Figure 4 B**) was significantly higher (e.g. 4-times) on bare CoCr as compared to bulk and thin D-PHI materials, with a value of 100 A.U./μg for CoCr vs values between 5 to 20 A.U./μg for PUs thin film or bulk polymer (**Figure 4B**). In a specific comparison of the four polyurethane coated materials, a significant difference was observed between O-D-PHI bulk and thin film (p<0.001), as well as HHHI bulk and thin film (p<0.05), suggesting that changes in surface topography related to shape, size and distribution of the crystals influences the availability of N terminal sequence of Fn. Again, the changes in surface contact angles (**Table 3**) also support that there would likely be a change in protein presentation between these surface features. The N terminal domain and

more particularly the 70 kDa fragments of Fn play an important role in coagulation. This latter domain contributes to cross-linking activity within fibrin clots by formation of ϵ -(γ -glutamyl)-lysyl bonds, and catalyzed by the thrombin-activated coagulation factor XIII (FXIIIa, plasma transglutaminase)³⁶. This cross-linking increases both thrombus size and platelet adhesion¹⁸.

The accessibility of Fn sequences (CBD and N-terminal) is therefore influenced differentially by two parameters, *e.g.* the chemical composition and the surface topography with the presence of crystals. These novel results further support fundamental work previously reported on for other materials³ which showed an influence of the intrinsic chemistry of material on the availability of the Fn sequences. In other work, it has also been shown that material chemistry (polystyrene vs hydroxyapatite) influenced the adhesion of osteoblastic cells suggesting a different accessibility of Fn sequences³⁷. Topography has also been shown to have a role in the availability of Fn sequences. Specially, in co-polymer systems, the chemistry of the polymers was kept the same but the distribution of the block components of the co-polymer was different, thereby causing a different topography of surfaces. These results yielded changes in the adsorption as well as availability of Fn peptide sequences³⁸. Hence, our findings suggest that the chemistry of the D-PHI PUs as well as their processing in the form of thin films may allow for a more passive adsorption of proteins, which in turn could modulate the response of cells in a favorable manner with respect to both inflammatory and coagulation processes. Importantly, and certainly not contemplated in earlier studies, this effect is more pronounced with the thin film of PUs than with the bulk cast PUs.

Conclusion

The chemical and physical characterization of biomaterial surfaces with thin films of D-PHI on metallic substrates were studied here and yield novel discoveries with respect to protein/material interactions. The results show that the introduction of copolymeric D-PHI surfaces in combination with thin film structures appears to enhance the homogeneous nature of the surfaces. Exposure of IgG-Fab, and Fn derived CDB and N terminal domains was substantially reduced on all the D-PHI materials when compared to bare metal CoCr, and there was an overlaid differentiation of the D-PHI coatings, although substantially less than the difference observed for the CoCr vs D-PHI. The chemical diversity and the topography present in the structure of the D-PHI PUs may be a critical factor in supporting favorable biomaterial-protein interactions. The results also suggest that the use of polar solvents like THF slightly modified the D-PHI surfaces. Importantly these modifications appear to be favorable regarding the subsequent exposure of specific plasma protein sequences. The results are encouraging and support the need for further investigations with human blood cellular components, implicated in inflammatory and coagulation processes with thin films as these newly processed materials are behaving significantly different from their bulk counterparts. The latter would provide a more extensive assessment of the beneficial phenomena demonstrated here for the D-PHI PU surfaces.

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Materials



Properties

D-PH
Thickness

Structure
(after 3 weeks of
stabilization)

± 2 mm



± 3 µm



Human plasma proteins adsorption

Immunoglobulin G

Fibrinogen



Intermediate
IgG adsorption



Lower C3b
and
iC terminal
exposure



Low IgG adsorption



High C3b and
iC terminal
exposure



High IgG adsorption

