Honeycomb structured porous interfaces as templates for protein adhesion

Juan Rodríguez-Hernández,1,2 Alexandra Muñoz-Bonilla,1 Emmanuel Ibarboure,2 Vanesa Bordegé,1 Marta Fernández-García1

1 Instituto de Ciencia y Tecnología de Polímeros (ICTP-CSIC), C/ Juan de la Cierva nº3 28006 Madrid Spain.
2 Laboratoire de Chimie des Polymères Organiques, LCPO-ENSCPB-CNRS, 16, Av. Pey Berland 33600 Pessac (France)
E-mail: jrodriguez@ictp.csic.es

Abstract. We prepared breath figure patterns decorated with a statistical glycopolymer, (styrene-co-2-[[D-glucosamin-2-N-yl]carbonyl]oxy)ethyl methacrylate, S-HEMAGl). The preparation of the glycopolymer occurs in one single step by using styrene and S-HEMAGl. Blends of this copolymer and high molecular weight polystyrene were spin coated from THF solutions leading to the formation of surfaces with both controlled functionality and topography. AFM studies revealed that both the composition of the blend and the relative humidity play a key role on the size and distribution of the pores at the interface. The porous films shows the hydrophilic glycomonomer units are oriented towards the pore interface since upon soft annealing in water, the holes are partially swollen. The self-organization of the glycopolymer within the pores was additionally confirmed both by reaction of carbohydrate hydroxyl groups with rhodamine-isocyanate and by means of the lectin binding test using Concanavalin A (Con A).

1. Introduction

Surface engineering of a biomaterial surface has to take into account several aspects including the modification and fine tuning of the surface chemical composition, interfacial morphology (roughness, topography) and optionally the coexistence and distribution of micro/macrodonts.[1] A large variety of recent examples have evidenced the key role of these parameters in the surface properties including adhesion/friction, wettability, or surface conductivity. For instance, the wettability can be varied between a hydrophilic and a superhydrophobic behavior by choosing the appropriate chemical function at the interface.[2] In particular, patterned surfaces serve as templates to create a pattern of proteins that can, in turn, be employed to study the interactions between proteins or antibodies with other biomolecules.[3]

Breath figures are formed by water condensation during solvent evaporation thus leading to a physical micropatterning (‘top-down’ approach) in which the topography of the polymer surface can be modified by creating holes of controlled dimensions. The affinity between the hydrophilic segments of the copolymer and the condensed water droplets, offers unique opportunities to modify the pore surface by with the glycomonomer units. The surfaces prepared following this approach could be of potential interest as 3D cell culture platforms. It is worth to mention that topographically controlled surfaces have been until now obtained by using tedious multistep procedures using lithographic techniques. In particular, glycopolymers, i.e. synthetic polymers containing carbohydrate units along
or at the end of the main chain, because their “glyco-cluster effect”, are interesting candidates to modify polymer surfaces creating bioactive materials. Carbohydrates are involved in multiple biological processes in living systems by participating in a variety of mutual recognition processes including immunological protection, virus infection, or recognition in the nervous system, amongst others.

We prepare regularly ordered 3D patterned polymer surfaces from blends of polystyrene and a statistical glycopolymer (styrene-co-2-(D-glucopyranosyl) aminocarbonyloxyethyl acrylate, S-HEAGl) using the breath figures methodology as dynamic templating method. In contrast to previous reported approaches in which the pores formed by using this methodology have been decorated with different functional groups or nanoparticles the preparation of breath figures with a glycopolymer is rather unusual. Nishikawa et al. in 1999 [4] described the preparation of poly-ion complexes at the surface with anionic polysaccharides. Very recently, Ting et al. [5] have reported the patterning of proteins onto galactosylated porous films. This article presents the formation of a regular film formation by using 6-arm star polystyrene which by itself present honeycomb structure with an ordered hexagonal array. Moreover, the preparation of the copolymer was carried out in multiple polymerization and protection/deprotection steps. The methodology employed throughout this manuscript leads to glycopolymers in one single step without protective chemistry. In addition, the parameters (humidity, temperature, polymer concentration, etc.) that significantly modify the surface topography in the breath figures mechanism have been widely investigated. Finally, we demonstrate the capability of the surfaces to selectively interact with proteins within the cavities.

2. Materials and methods

2.1. Materials
Styrene (St), (Aldrich, 99%) was distilled under reduced pressure. 2,2'-azoisobutyronitrile (AIBN) (Aldrich, 98%) was purified by successive crystallizations from methanol. The synthesis of 2-{{(D-glucosamin-2-N-yl) carbonyl]oxy}ethyl methacrylate (HEMAGl) will be described elsewhere. All the solvents used in the course of experiments and characterization (dimethyl formamide (DMF) and dimethyl sulfoxide-d) were employed without further purification. Rhodamine B isothiocyanate (Aldrich) and lectin-fluorescein isothiocyanate conjugate from Canavalia ensiformis (ConA-FTIC) (Sigma) were used as received.

2.2. Copolymerization reaction
Free-radical copolymerization of styrene and HEMAGl with a molar feed composition in styrene of 0.80 was carried out in pyrex ampoule sealed in argon atmosphere at 70°C in a global concentration of 1 mol/L DMF solution with 3•10^2 mol/L of AIBN as the initiator concentration. The reaction was conducted up to total conversion. The resulting copolymer, S8Gl2, was purified by dialysis and further lyophilization.

2.3. Characterization
The statistical glycopolymer was characterized by \textsuperscript{1}H-NMR with a Bruker Advanced 400 MHz spectrometer at room temperature. A probe of deuterated DMSO was introduced in a copolymer solution of dimethylformamide (DMF). Average molecular weights and dispersities were determined by size exclusion chromatography (SEC) using a Jasco system equipped with two PL gel 5 \(\mu\)m (300 x 7.5 mm) mixed-C columns and a PL gel 5 \(\mu\)m (50 x 7.5 mm) guard column, a Jasco 1530 differential refractive index detector and a Jasco 875 UV detector. N,N-Dimethylformamide (HPLC grade) was used as eluent containing 0.1 M LiBr with a flow rate of 0.8 ml/min at 60 °C. Calibration was obtained from narrowly distributed polystyrene standards.

2.4. Film preparation
Mixtures having 10-50 wt% of copolymer S8Gl2 and 90-50% high molecular weight polystyrene (\(M_n= 250000 \text{ g/mol}\)) were dissolved in THF (solution concentration 30 mg/mL). The polymer solutions were filtered with a 0.1 \(\mu\)m Millipore membrane and spin-coated (4000 rpm for 60 s) onto silicon wafers purchased from SC (Siebert Consulting e.K., Germany). The silicon wafers were cleaned prior to use in piranha solution (3:1 v/v of \(H_2SO_4\) in \(H_2O_2\)) and rinsed several times with ethanol. For the preparation of samples under controlled relative humidity, beakers containing water and saturated aqueous solutions of sodium bromide were placed inside the spin coating chamber in order to obtain values of relative humidity between ~40 and ~57% respectively. The humidity degree and the temperature were measured by means of a hygrothermograph.

2.5. Annealing
The sample annealing was carried out to further study the variations of the surface chemical composition as a function of the environment. Upon analyzing (by AFM imaging) the films obtained after spin-coating, the samples were exposed either to air at 90 °C for 3 days or placed in a tightly closed stainless steel vessel saturated in water vapor. The annealing in a humid environment was carried out during 36 h at 90 °C. After each treatment, the samples were dried under vacuum at room temperature.

2.6. Rhodamine-isocyanate immobilization
The films previously annealed to water vapor were immersed in an aqueous Rhodamine-isocyanate solution (0.25 mg/mL) for 2 h at room temperature. After washing with deionized water, the film was dried at room temperature.

2.7. Lectin interaction
The film was immersed into a phosphate-buffered saline solution (PBS, 7.4) at room temperature containing 0.2 mg/mL of fluorescein-conjugated Concanavalin A (Con A-FITC). After 6 hours, the film was sequentially washed with PBS buffer and distilled water.

2.8. Methods
Atomic Force Microscopy, AFM, images were recorded in air at room temperature with a Nanoscope IIIa microscope operating in tapping-mode. The probes were commercially available silicon wafer with a spring constant of 42 N/m, a resonance frequency of 285 KHz and a typical radius curvature in the 10-12 nm range. Both the topography and the phase signal images were recorded with a resolution of 512x512 data points.

Fluorescence Microscopy and Image Processing. Images were performed on a Zeiss Axiovert 40 CFL inverted microscope equipped with a 12-V, 35-W halogen lamp (for the phase-contrast images) and an HBO 50 W/AC mercury lamp (for the fluorescence images). The objectives used were a 5×/0.12 A-Plan, a 10×/0.25 A-Plan, a 20×/0.50 EC Plan-NEOFLUAR, and a 40×/0.50 LD A-Plan (Zeiss). Images were acquired by using a CANON A640 CCD camera.

3. Results and discussion
The preparation of structured interfaces was carried out by mixing a statistical copolymer (\(M_n= 42900 \text{ g/mol; PD}=1.73\)) prepared by conventional free radical polymerization with linear high molecular weight polystyrene (\(M_n=250000 \text{ g/mol}\)). The copolymer consists of styrene and 2-\{[(D-glucosamin-2-N-yl) carbonyl]oxy\}ethyl methacrylate (HEMAGl) units in 80 to 20 molar ratio. The composition of the copolymer can be varied by modifying the initial feed. Herein, we employed a copolymer with a relative high amount of styrene (80% of styrene and 20% of HEMAGl). This proportion was chosen due to the following reasons: first, by increasing the amount of styrene content, the compatibility between the glycopolymer and the homopolymer matrix is enhanced. Second, since in a subsequent step the prepared films will be annealed in water. The amount of hydrophilic glycomonomer has to be
low in order to form stable films that do not dissolve in aqueous media and could resist the annealing conditions.

Films of the blends varying the relative composition of glycopolymer to linear polystyrene matrix were prepared by spin coating from THF solutions (30 mg/mL) in an atmosphere under controlled humidity (44% RH). As it has been reported, during solvent evaporation the temperature of the solvent-air interface decreases and water vapor starts to condense (Figure 1). As the solvent evaporation continues the condensed water droplets grow until, finally, upon complete evaporation of both solvent and water the resulting surface contains holes randomly distributed reflecting the positions where the water droplets condensed. The conditions in which the films were prepared influence the size and distribution of the holes obtained.[6] Hence, solvent, blend composition, concentration of solution and humidity are important external parameters that have to be controlled.

In Figure 2 are illustrated the AFM images of films obtained for a blend composed of 20% in weight of statistical glycopolymer and 80% of polystyrene. Amphiphilic copolymers are hygroscopic and may interact with water during the spin coating process. Thus, increasing the amount of hydrophilic polymer in the mixture enhances water uptake and as a consequence the diameter of the holes formed. Blends with amounts of S8G12 above 50% lead to rather disordered surface structures as a consequence of the coalescence of the water droplets. Equally, films prepared in highly moist atmospheres above 50% RH lead to a similar effect. Hence, within this range of blend compositions and humidity the average size of the holes can be controlled.

Figure 1. Breath figures approach to obtain porous polymeric interfaces by water condensation.
Figure 2. AFM height (left) and phase images (right) (10 µm x 10 µm) of films obtained by spin coating in a 44% humid atmosphere with a blend composition of 20/80 (w/w, S8Gl2/PS).

The interaction between the polar glycomonomer units within the copolymer and water droplets condensed during the spin coating modifies the distribution in terms of chemical composition at the interface. As a consequence, the glycopolymer in contact with water rearranges around the water droplet and after drying is principally localized in this area. Hence, the surface composition is different between the holes and the rest of the interface. The hygroscopic nature of the carbohydrate moieties reveals upon annealing in humid air a preferential swelling this area. This behavior is evidenced by AFM in films obtained directly after spin coating and annealed in humid air. Annealing in humid water vapor provokes reversible changes on the micropatterned surface. Hence, the holes are transformed in island-like structures after 3 days of annealing. Similarly, drying the surface under mild conditions (60 °C, overnight) removes the water and recovers the original topography.

Figure 3. Tapping mode height AFM images (10 µm x 10 µm and 5 µm x 5 µm) of: film obtained by spin coating 50/50 S8Gl2/PS (w/w) after annealing in water vapor at 80 °C for 3 days.
The employment of the surfaces as templates needs the availability of recognition/reactive sites to which the biomolecules could be attached. Whereas the presence of glycopolymer and therefore of saccharide units within the pore surface is proven by AFM upon imaging the surfaces obtained after annealing in water vapor, the availability of the hydroxyl groups of the saccharide units is evidenced by reaction with rhodamine isothiocyanate (Rho-FITC). The images obtained with a fluorescence microscope for the blends with 50% of S8Gl2 are depicted in Figure 4. The ring-shaped fluorescent pattern indicated the attachment of the rhodamine to the pore surface by covalent reaction between the isothiocyanate and the hydroxyl moieties. These images clearly indicate the capability of the hydroxyl groups to accomplish chemical reactions with other functional groups.

The ability to these glycopolymer-modified surfaces to specifically recognize lectins is evidenced by using Concanavalin A which is a protein which specifically interacts with glucopyranosyl residues. The films are immersed into PBS solution at room temperature containing 0.2 mg/mL of fluorescein-conjugated Concanavalin A (Con A-FITC). After 6 hours, the film is sequentially washed with PBS buffer and distilled water. Figure 4 (right) shows the fluorescence microscopy of the interaction between the S8Gl2 glycopolymer with Con A-FITC. As can be observed, such interaction is produced in the external part of the pore confirming that the hydrophilic part of glycopolymer is mainly exposed to the surface.

Figure 4. Top view images of the films obtained by using a fluorescence microscope. Left: the fluorescence detected in the inner part of the pore evidenced the functionalization of the inside of the pore with a rhodamine-isocyanate by reaction with the hydroxyl groups of the glycomonomer units. Right: images of the surface obtained upon chemical recognition between the glycopolymer and fluorescein-conjugated Concanavalin A within the pores.
4. Conclusions

We described the elaboration of micropatterned surfaces by using the breath figures methodology. By controlling the parameters involved in the film preparation such as, composition of the blend, humidity and temperature we are able to prepare surfaces with defined pore size and distributions. By swelling the polar carbohydrate moieties we evidenced the selective enrichment of glycopolymer in the inner part of the pore. Depending on the size of the pores created, the pores are either transformed in hills or only partially swollen. Finally, the potential of these structures to serve as templates for the attachment of bioactive molecules is clearly evidenced. Firstly, the hydroxyl groups of the saccharide are able to react inferring specific functionality as demonstrated by the reaction with the isothiocyanate functional group of the rhodamine. And secondly, the specific interaction with Con A lectin clearly verified the accessibility of the carbohydrate groups into the surface, indicating again the transformation from hydrophobic to hydrophilic surface.

5. Acknowledgments

The authors gratefully acknowledge financial support from the Spanish National Research Council (CSIC) through the PI 200860I037, MAT2009-12251, MAT2007-60983 and CCG08-CSIC/MAT-3643 programs.

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