PROTEIN ADSORPTION, CELL ADHESION AND POLYMER SURFACES: MOLECULAR PROCESSES AND EXPERIMENTAL METHODS

P.G. Rouxhet, J.L. Dewez, Th. Marchal, Ch. Dupont-Gillain, Y. Dufrene

Unité de Chimie des interfaces, Université catholique de Louvain, Louvain-la-Neuve, Belgium

Abstract

Adsorption of extracellular matrix (ECM) proteins in competition with other substances is a key to explain the relationship between substratum surface hydrophobicity and mammalian cell adhesion: when polystyrene substrata were exposed simultaneously to ECM protein and a PEO-PPO-PEO polymer surfactant (Pluronic F68), either by pre-conditioning or through protein cell secretion, a weaker substratum hydrophobicity favoured adsorption of the protein and subsequent cell adhesion. This knowledge was used to achieve a selective adhesion of different types of mammalian cells on tracks (a few tens of μm wide) produced on polystyrene by photolithography and oxygen plasma treatment, conditioning the substratum with a solution of ECM protein and Pluronic F68.

Examination of a broader range of substrata confirmed that inhibition of cell adhesion on hydrophobic substrata is due to adsorption of substances competing with extracellular matrix proteins. However it also showed that substratum surface properties more subtle than overall wettability are important.

In situ observation of the nanoscale organisation of collagen adsorbed in the absence of competitor, using atomic force microscopy (AFM), showed that a smooth substratum surface allows collagen mobility and aggregation of molecular ends in the adsorbed phase. The organisation obtained after drying (smooth film, pattern) was examined by combining AFM, XPS and radiolabelling and found to be influenced by substratum roughness, substratum hydrophobicity and drying rate.

Key words: *protein adsorption, cell adhesion, polymers, surface patterns, atomic force microscopy (AFM), X-ray photoelectron spectroscopy (XPS), radiolabelling, nanotechnology.*

Introduction

Better understanding of the interaction between mammalian cells and material surfaces is essential in biomaterial engineering, whether the aim is to achieve a particular cell behaviour (in terms of proliferation, migration, physiology, architecture, organisation) or to minimise any influence of the surface on the biological system (passive biocompatibility). This contribution is a summary of recent work focused on the influence of polymer surface properties on protein adsorption and on mammalian cell adhesion. Exciting new possibilities offered by atomic force microscopy to probe, in situ, the organisation of adsorbed proteins are described. More extensive literature review and experimental details can be found in cited references.

Competitive adsorption of proteins: key of the relationship between substratum surface properties and adhesion of mammalian cells

The adsorption of type I collagen was investigated [1] using polystyrene substrata characterised by different oxygen surface concentrations and wetting properties: bacteriological grade dishes BGPS-S and BGPS-F, and tissue culture dishes TCPS, with O/C atomic concentration ratios determined by X-ray photoelectron spectroscopy (XPS) < 0.001, about 0.02 and about 0.20, and water contact angles equal to 97°, about 82° and about 56°, respectively. The adsorption was performed in PBS buffer at pH 7.2 and ionic strength 0.165 M and quantified using labelled collagen and radiocounting, on the one hand, and XPS, on the other hand.

The adsorption of collagen solution in PBS is only slightly affected by the surface properties of the supports. The adsorption isotherms show that collagen has a higher affinity with the more hydrophobic substratum and reaches a plateau of 0.7 μgcm^{-2} for a residual concentration of 20 μgml^{-1}. With TCPS, no plateau is observed. However the amount adsorbed for a residual concentration of about 30 μg ml^{-1} is similar for the three substrata.

Strong changes occur when adsorption takes place from a solution of collagen containing Pluronic F68. This is a poly(ethylene oxide) - poly(propylene oxide) - poly(ethylene oxide) triblock copolymer surfactant (PEO_{80} - PPO_{30} - PEO_{80}) which is frequently added in serum-free media. A drastic decrease of collagen adsorption is observed for BGPS while, for TCPS, the amount adsorbed decreases more progressively as a function of Pluronic F68 concentration. This is revealed both by radiocounting and by XPS, as illustrated by FIGURE1. A similar trend is observed when the compounds competing with collagen for adsorption are human serum albumin or constituents of fetal calf serum.

This observation explains the adhesion behaviour of human hepatoblastoma cell line, Hep G2, cultured in chemically defined nutritive medium [2]. The substrata were BGPS-F and TCPS conditioned by collagen and rinsed with PBS. When the substratum was pre-conditioned with a collagen solution in PBS, the cells attached and spread both on TCPS and BGPS. When Pluronic F68 was present in the conditioning solution, either brought by a commercial medium or added in the laboratory, attachment and adhesion took place on TCPS and not on BGPS. It turns out that the effect of substratum hydrophobicity on Hep G2 cell adhesion is mediated by the adsorption competition between an extra-cellular matrix (ECM) protein and another compound, collagen and Pluronic F68 respectively in the present case.

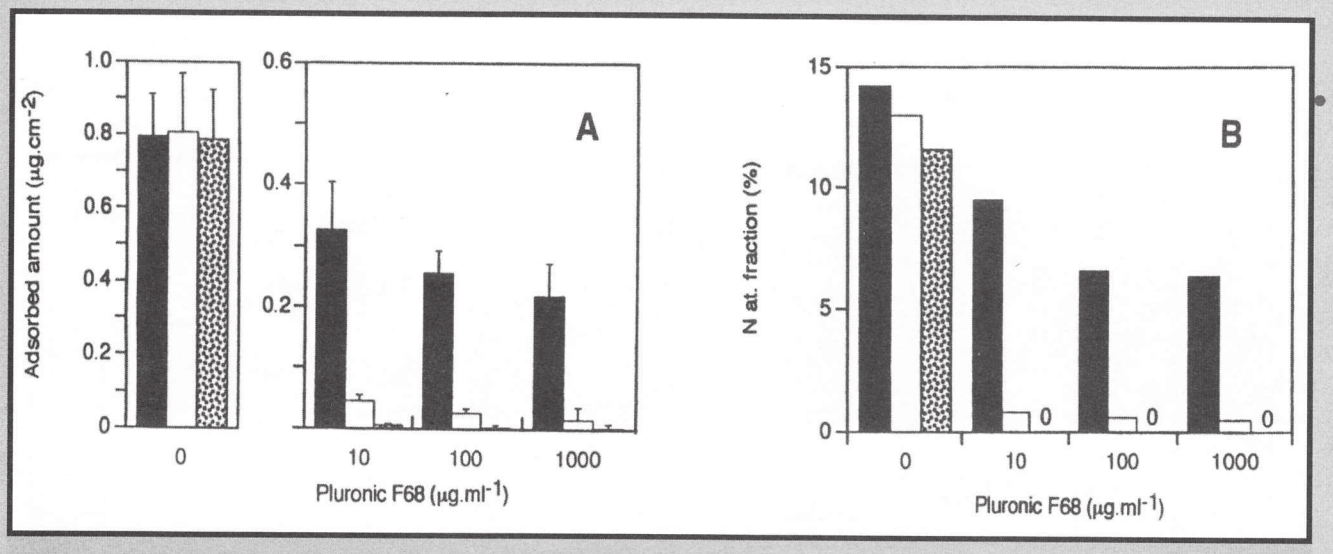

FIG.1. Adsorption of collagen on TCPS (black), BGPS-F (white) and BGPS-S (dotted) substrata in the presence of the indicated initial concentrations of Pluronic F68. A, amount adsorbed determined by radiolabelling; B, mole fraction of nitrogen determined by XPS. Initial collagen concentration = 30 μg·ml^{-1}; adsorption time = 1 h; T = 36°C; pH 7.2; I = 165 mM.

Competitive adsorption also explains the influence of substratum hydrophobicity on Hep G2 cell attachment and spreading in the presence of Pluronic F68 when the substratum is not pre-conditioned, the cell response depending on the production of proteins by the cells themselves [3]. On strongly hydrophobic polystyrene substrata, adhesion was hindered in the presence of the surfactant, which prevented the adsorption of secreted proteins. Cell adhesion on substrata pre-conditioned with a solution of ECM proteins (collagen, laminin, fibronectin) was not hindered by Pluronic F68 present in the culture medium, indicating that pre-adsorbed proteins were not markedly displaced by the surfactant. This was found whatever the substratum surface hydrophobicity, whether protein synthesis was going on or switched off. When the substratum was pre-conditioned with fetal calf serum (1 %), the effect of ECM proteins of the serum dominated with respect to the effect of albumin; the cells attached and spread.

Application to selective cell adhesion on defined patterns

Understanding the influence of substratum surface hydrophobicity on cell adhesion was applied to achieve adhesion following defined patterns [4,5]. The procedure involved the following steps :

- a photosensitive resin is spin-coated on a polystyrene substratum;
- it is submitted to UV irradiation through a mask grooved with tracks (width of the order of tens of mm);
- the irradiated resin is dissolved (development);
- the system is treated by an oxygen radio-frequency plasma discharge;
- the remaining resin is dissolved;
- the substrate is conditioned with a solution of a ECM protein and Pluronic F68;
- cells are inoculated.

FIGURE 2A shows an image of the material processed by photolithography and oxygen plasma treatment. The image was obtained by atomic force microscopy (AFM) in the lateral force mode. The contrast is created by the friction of the AFM tip, which is weaker on the oxidised tracks (PSox) compared to polystyrene (PS). The water contact angle measured on wide zones of PS and PSox is 86° and 52°, respectively. FIGURE 2B shows a ToF-SIMS image of the material conditioned with a solution of fibronectin (33 mg ml^{-1}) and Pluronic F68 (150 μg ml^{-1}). The image was made using CNO^- ions, which are specific to the protein. It shows that fibronectin adsorbs selectively on the oxidised tracks. FIGURE 2C gives an optical micrograph of MSC mouse schwannoma adhering on the pre-conditioned substratum. This demonstrates that cells adhere selectively on the oxidised tracks thanks to the selective preadsorption of fibronectin. Similar results were obtained for PC 12 rat adrenal pheochromocytoma, as well as for rat hepatocytes (primary culture) on a substratum pre-conditioned by a solution of fibronectin and type I collagen, respectively, containing Pluronic F68.

Confining cell adhesion to particular regions of dimension close to the cell size influences cell morphology, migration and metabolism [6] and thus offers new prospects in cell culture and tissue engineering. The method described above has the particularity that patterning is made directly on the polymer surface, without silicon or metal deposition.

Complexity of reality

The above investigations were extended to polymers of different natures [7]: polypropylene (PP), poly(ethylene terephthalate) (PET) and poly(methyl methacrylate) (PMMA), characterised by O/C surface concentration ratios equal to 0.01, 0.43 and 0.40, and water contact angles of 100°, 77° and 74°. In the absence of competitors (Pluronic F68, serum constituents), both adsorption of extracellular matrix proteins (collagen, fibronectin, laminin) and attachment of WI 38 fibroblasts and ECV 304 endothelial cells did not differ significantly according to polymer wettability. In the presence of Pluronic F68, adsorption of proteins was almost completely prevented, except adsorption of fibronectin and laminin on PET, which was only reduced by a factor of 2 or 3. In the presence of Pluronic F68 (0,01 %) or fetal calf serum (1 %) or both, cell attachment on PP and PMMA was appreciably lower, compared to that observed

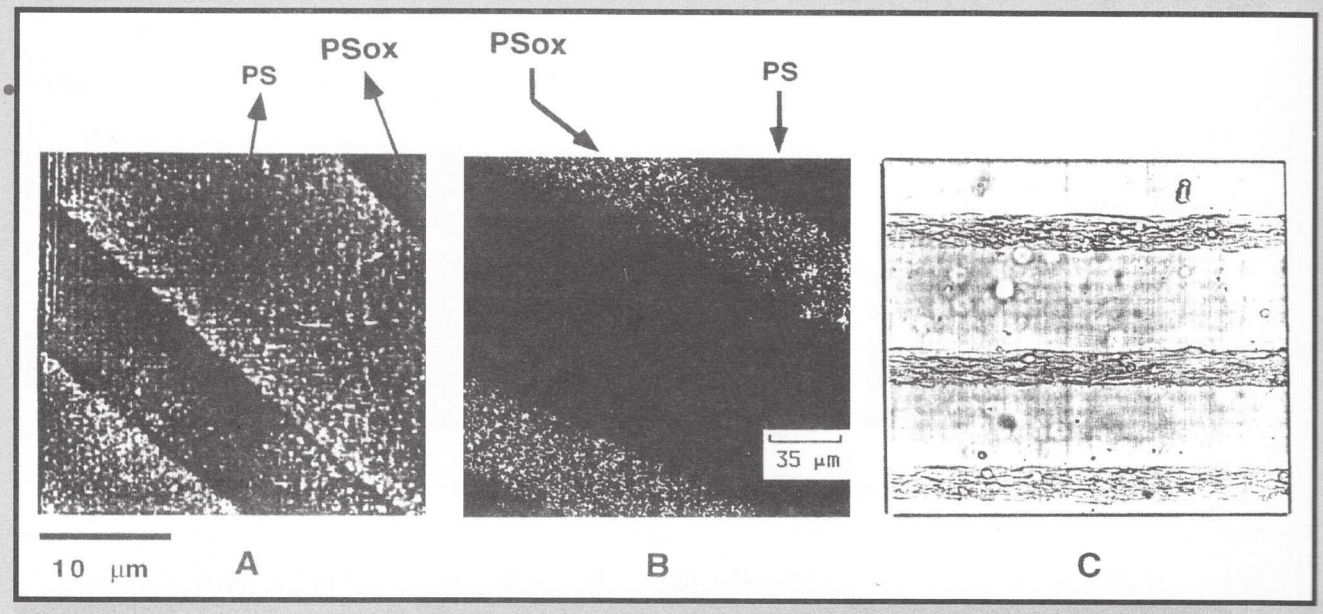

FIG.2. A, lateral force microscopy image of PS processed by photolithography and oxygen plasma treatment. B, ToF-SIMS image recorded with CNO- ions on a patterned substrate conditioned with a solution of fibronectin and Pluronic F68. C, optical micrograph of MSC 80 mouse schwannoma on substratum processed as illustrated in a and b.

on PET. As the water contact angle of PMMA is very close to that of PET, this shows that overall wettability is not the only factor controlling the adsorption of ECM proteins in competition with other compounds. A first approach along that line, in the absence of competitor is presented below.

Nanoscale organisation of adsorbed collagen

The adsorption of type I collagen was further investigated by AFM [8] with polymer substrata covering a wide range of surface roughness and surface hydrophobicity : bisphenol A polycarbonate (PC), PET, and poly(vinylidene difluoride) (PVdF) used as such or treated by an oxygen plasma discharge (ox). The untreated polymers differed markedly by their surface roughness (rms from 0.5 to 11 nm) but had fairly close water contact angles (75 to 89°). Oxidation did not modify significantly the surface roughness but decreased the water contact angle (down to 52°, 31° and 69° for PCox, PETox and PvdFox, respectively).

On substrata exhibiting vertical topographic variations smaller that the collagen molecular diameter (1.5 mm), dot-like (on PC) and elongated structures (on PCox) were observed (FIGURE 3 A) and attributed to aggregated ends of collagen molecules (FIGURE 3 C). Extended rupture lengths were detected in the retraction force-distance curves (FIGURE 3 B), suggesting the progressive tearing-off of the collagen molecules from the adsorbed phase (FIGURE 3 C). In contrast, on substrata showing vertical topographic variations larger than the collagen molecular thickness, the adsorbed collagen formed a film devoid of topographic features and no extended rupture lengths were observed. This indicates that a critical substratum height variation close to the collagen molecule size may affect the mobility of the adsorbed molecules and their tendency to aggregate in the adsorbed phase; this is not markedly influenced by surface hydrophobicity.

After drying (quick drying under nitrogen flow) the substrata with adsorbed collagen were examined by AFM in air. Patterns of aggregated structures were still visible on PC and PCox. With rough substrata, holes were observed

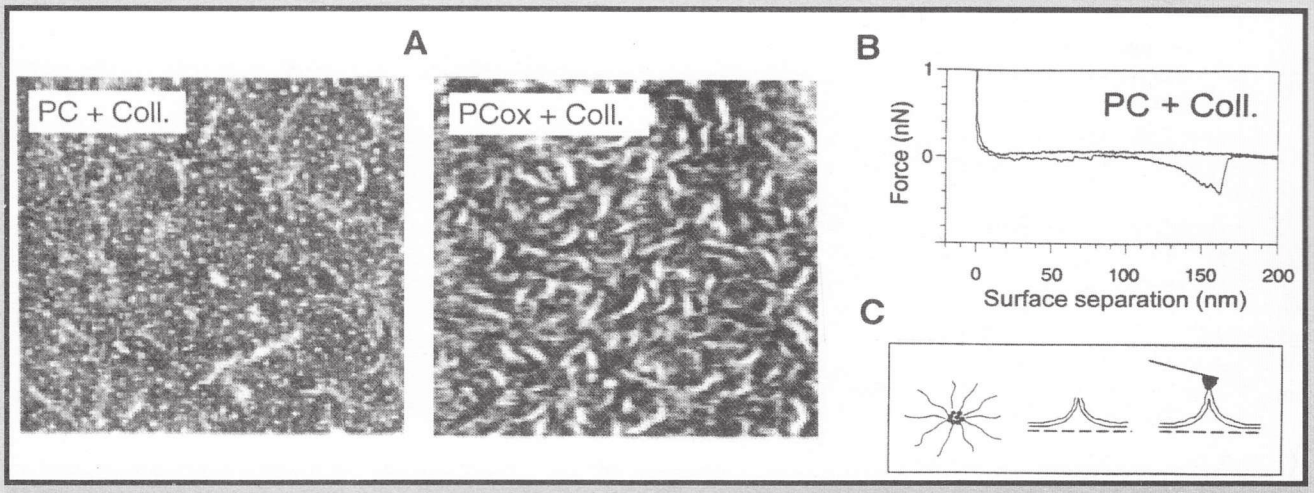

FIG.3. A, AFM topographic images (size 5 µm x 5 µm; z range = 15 nm) in water of PC and PCox after collagen adsorption (lighter = higher level). B, typical force-distance curve recorded (50 % of spots) in water after collagen adsorption. C, schematic representation of the film of collagen adsorbed on PC: top view (left), cross-section (middle) and interaction with the AFM probe upon retraction (right).

on PET and PVdF and not on PETox and PVdFox. They are attributed to the release of stresses created by shrinkage of a stiff adsorbed layer, occurring upon drying on a hydrophobic support.

Differences in the organisation of the adsorbed collagen layer (surface coverage, layer thickness) observed by AFM fitted well those found by models obtained from XPS and quantification of the adsorbed amount by using radiolabelled collagen [9]. This illustrates the complementarity of AFM, XPS and radiolabelling. The importance of the mobility of adsorbed collagen was further demonstrated by the influence of drying rate on the nanoscale organisation of the film adsorbed on PMMA [10]: fast drying by flushing with a nitrogen flow; slow drying under 95 % relative humidity. AFM revealed the formation of a net-like structure at slow drying rates. Comparison between the surface organisation, as observed by AFM, and surface models provided by XPS and contact angle measurements, indicated that chemically heterogeneous surfaces were produced at slow drying rates, PMMA being exposed at the outermost surface in the holes left by the collagen net.

Conclusion

Competitive adsorption of proteins is a key to understand the influence of substratum hydrophobicity on mammalian cell adhesion. This concept offers interesting prospects for biomaterial applications. However hydrophobicity (or surface energy) is not the only factor influencing the adsorption behaviour. AFM demonstrated that the organisation of films of collagen adsorbed in the absence of competitor depends on collagen mobility, the latter being influenced by surface roughness rather than by surface hydrophobicity. The organisation of the film obtained after drying is determined by substratum roughness, substratum hydrophobicity and drying rate.

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