# Controlling the supramolecular organisation of adsorbed collagen layers

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The supramolecular organisation of collagen adsorbed on polymer substrates was investigated as a function of properties of the substrates (chemical nature, roughness) and of characteristics of the collagen solution (concentration, state of aggregation) as well as details of the preparation procedure (adsorption time, drying rate). Elongated structures are formed at the interface by assembly of collagen molecular segments protruding into the solution. This is favoured by using a hydrophobic and smooth substrate, by increasing the adsorbed amount and by increasing the adsorption time, even beyond stages at which the adsorbed amount does no longer vary. Collagen adsorbed at low amount on hydrophobic substrates strongly reorganises into a net-like pattern if drying is performed at low rate. This is due to dewetting and collagen displacement by the water meniscus. Applications derived from the control of collagen organisation are presented. Nanostructured polymer surfaces were created starting from a collagen template. The attachment and the cytoskeletal organisation of mammalian cells (MCF-7/6) were also shown to depend on collagen organisation.


## 1. Introduction

The control of cell–material interactions, which is a key issue in biomaterials science and biotechnology, relies on a better understanding of protein adsorption at the material surface. The latter is indeed immediately submitted to protein adsorption once it is placed in contact with a biological fluid. Mammalian cells then interact with the material through recognition events between cell-surface receptors and adsorbed proteins [1]. The nature of the adsorbed proteins as well as their conformation affect the recognition process. Moreover, the organisation within the adsorbed layer may promote cooperative interactions between several recognition sites, thereby modifying the cell behaviour [2].

Collagen, an extracellular matrix protein, is the most abundant structural protein in the animal kingdom. The type I collagen molecule (length $\sim 300$ nm; diameter $\sim 1.5$ nm) is a helix formed by three polypeptides. Non-helical portions, named telopeptides, are found at each end of the molecule. Collagen may aggregate and form fibrils, *in vivo* as well as *in vitro* [3]. It contains amino-acid sequences which may be recognised by specific cell receptors [4]. Due to its shape and dimensions, and to its self-assembling properties, collagen offers promising perspectives in view of creating adsorbed layers with controlled supramolecular architectures. This would enable to study cell behaviour as a function of the adsorbed protein layer organisation. On the other hand, collagen can be considered as a building block to design nanostructured objects, using the so-called bottom-up approach of nanotechnologies.

This paper gives an overview of the research dedicated to the study of the supramolecular organisation of collagen adsorbed on polymer substrates, which was carried on in our laboratory in the last years [5–15]. In Section 2, the effects of the properties of the substrate and of the collagen solution, and of the sample preparation procedure on adsorbed collagen organisation are discussed. In Section 3, adsorbed layers presenting characteristic supramolecular organisations are used to further produce nanostructured polymer surfaces and to examine cell behaviour.

## 2. Factors affecting the supramolecular organisation of adsorbed collagen

Adsorbed collagen layers were investigated as a function of factors expected to affect their organisation, including the properties of the substrates and of the collagen solution as well as details of the preparation procedure. Therefore, type I collagen solutions were prepared by dilution in phosphate buffer saline (pH $\sim 7.2$; ionic strength $\sim 160$ mM) down to the desired concentration. Adsorption was then performed at 37 °C in static

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conditions, using substrate pieces of about 1 $cm^2$, for a given period of time. Samples were rinsed with water and either examined in water using atomic force microscopy (AFM), or dried and analysed with AFM and X-ray photoelectron spectroscopy (XPS). In some cases, adsorbed amounts were determined using collagen labelled by reductive methylation with $^{14}$C-formaldehyde [13].

## 2.1. Properties of the substrate

### *2.1.1. Chemical nature of the substrate*

Fig. 1(c) and (d) presents AFM images, acquired in air, of collagen adsorbed on polystyrene (PS) and plasma-oxidised PS (PSox), respectively. The collagen concentration was 40 µg/ml, the adsorption duration was 2 h and sample drying was performed under a $N_2$ flow (fast drying, FD). Compared to the PS surface, the PSox surface is characterised by the presence of oxygen-containing functions, as determined by XPS, and by an increased hydrophilicity, as demonstrated using the Wilhelmy plate method. Moreover, it was shown to be covered by a polyelectrolyte layer which can be swelled by aqueous solutions [16]. There is a striking effect of the surface modification of PS on the organisation of adsorbed collagen: while the layer obtained on PS presents a high density of elongated features of the size of a few collagen molecules, the layer formed on PSox is smoother. This difference is accompanied by only small differences of the adsorbed amount, the latter being slightly lower on PSox compared to PS after 2 h, as shown by radioassays and XPS measurements [14].

Other pairs of materials were examined, allowing the effect of the nature of the substrate to be further

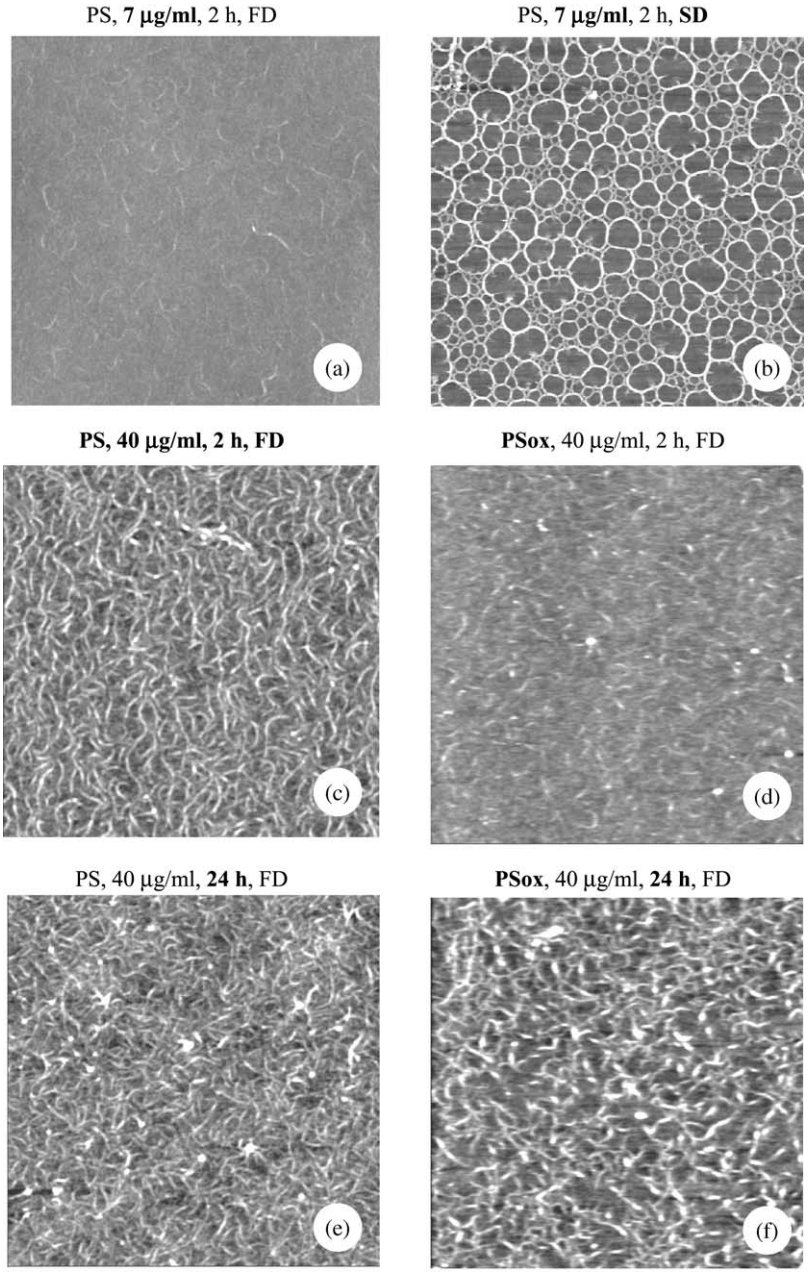


*Figure 1* AFM images ($5 \times 5\ \mu m^2$) acquired in air of collagen layers obtained by adsorption on a PS or a PSox substrate: adsorption from a 7 µg/ml solution for 2 h on PS followed by fast drying (a, $z = 10$ nm) or slow drying (b, $z = 10$ nm); adsorption from a 40 µg/ml solution for 2 h on PS (c, $z = 5$ nm) or on PSox (d, $z = 5$ nm) followed by fast drying; adsorption from a 40 µg/ml solution for 24 h on PS (e, $z = 5$ nm) or on PSox (f, $z = 5$ nm). The indications in bold characters outline the differences of experimental conditions with respect to image c.

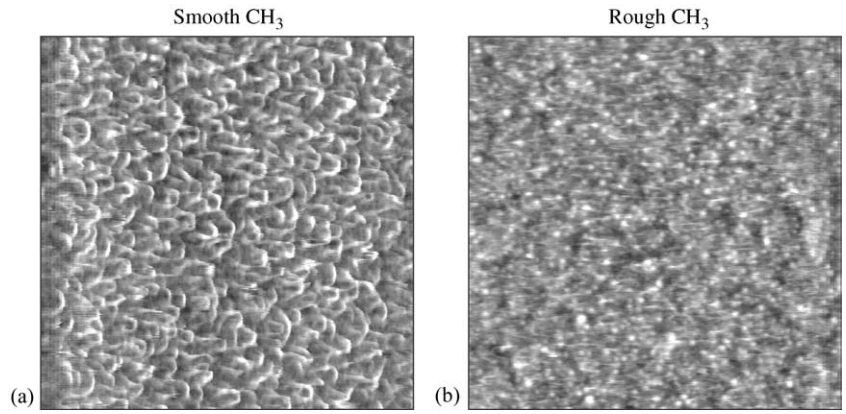


*Figure 2* AFM images ($5 \times 5\,\mu m^2$, $z = 20\,nm$) acquired in water of collagen layers obtained by adsorption from a 30 μg/ml solution for 2 h on a smooth (a) or a rough (b) $CH_3$-terminated self-assembled monolayer. Reprinted in part with permission from Langmuir **18** (2002) 819. Copyright 2002 American Chemical Society.

investigated. Adsorbed collagen layers formed (adsorption for 2 h from a 30 μg/ml solution) on self-assembled monolayers of alkanethiols on gold, terminated with $CH_3$ or OH groups, showed an organisation similar to that observed on PS and PSox: elongated structures on the very hydrophobic $CH_3$ surface, smooth layer on the hydrophilic OH surface. In that case, combination of AFM and XPS results showed that the adsorbed amount was higher on the $CH_3$ compared to the OH surface [11]. Poly(ethylene terephthalate) (PET) was also compared to plasma-oxidised PET (PETox). The collagen adsorbed amount, as estimated by XPS, was equivalent on both substrates. The collagen layer formed after 24 h from a 7 μg/ml solution on the hydrophobic PET substrate clearly showed elongated features, similar to those observed on the PS and the $CH_3$ surfaces. On the more hydrophilic PETox surface, the collagen layer was smoother [10].

The chemical nature of the substrates thus clearly affects the organisation of adsorbed collagen. As a general trend, in the investigated range of concentration, smooth layers are found on the more hydrophilic substrates, while elongated structures, attributed to the association of a few collagen molecules, are formed on the more hydrophobic substrates. This may to some extent be due to the fact that the adsorbed amount tends to increase with the substrate hydrophobicity. However, it must also be related to particularities of collagen–substrate interactions, which are modulated by the physicochemical properties of the substrate. Observations made using AFM in other modes (force–distance curves; nanomechanical probing of the adsorbed layer) indicate that collagen forms a felt of lying molecules on hydrophilic substrates, but leaves free molecular segments in solution on hydrophobic substrates [8, 11, 13, 14]. In the latter case, further aggregation of molecular segments may lead to the observed structures.

### *2.1.2. Roughness of the substrate*

Self-assembled monolayers of $CH_3$-terminated alkanethiols were prepared on gold deposited on silicon wafers (smooth $CH_3$ surface) and on gold presenting nanoscale protrusions (height $\sim 15$ nm; surface coverage $\sim 15\%$) created by colloidal lithography (rough $CH_3$ surface). Fig. 2 presents AFM images of collagen adsorbed on the smooth and the rough $CH_3$ surfaces (adsorption from a 30 μg/ml solution for 2 h). As discussed above, elongated features arising from the association of collagen molecules were observed on the smooth $CH_3$ surface. These structures were not observed on the rough $CH_3$ surface [11]. A similar trend was found for collagen adsorbed on polymer substrates covering a range of surface roughness and hydrophobicity. Structures attributed to associated collagen molecules were found when topographic variations of the substrate surface were smaller than the diameter of the collagen molecule [6].

The effect of surface roughness on the organisation of adsorbed collagen may be related to the mobility of adsorbed molecules. Topographic variations of the substrate which are large compared to the diameter of the collagen molecule may hinder collagen mobility along the surface plane, thereby preventing intermolecular associations.

## 2.2. Properties of the collagen solution

### *2.2.1. Collagen concentration*

Fig. 1(a) and (c) presents AFM images of collagen layers obtained by adsorption from a 7 and 40 μg/ml solution, respectively, during 2 h on PS. At 7 μg/ml, the adsorbed layer is quite smooth, with some thin isolated filamentous structures. At 40 μg/ml, the surface is covered by a dense layer of elongated structures. The amount of adsorbed collagen, measured using radioassays, is equal to 0.6 and 1.0 $\mu g/cm^2$ at 7 and 40 μg/ml, respectively [14, 15].

A similar trend was also found using PET as a substrate [13]. Adsorption from a 10 μg/ml solution during 1 h led to the formation of a layer with a slight granular structure, while adsorption from a 100 μl/ml solution again produced a dense layer of elongated structures. This difference was observed in water as well as on dried samples. The adsorbed amounts were 0.33 and 0.65 $\mu g/cm^2$ at collagen concentrations of 10 and 100 μg/ml, respectively.

Increasing the concentration of the collagen solution

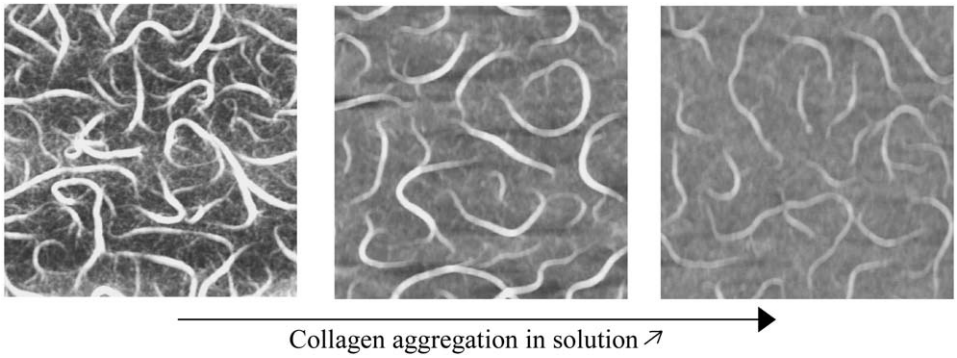


*Figure 3* AFM images ($2 \times 2\,\mu m^2$; $z = 10\,nm$) acquired in air of collagen layers obtained by adsorption from a 40 µg/ml solution for 30 min on PS. Prior to adsorption, the collagen solution was aged at 37 °C for 15 min, two and seven days, from left to right, respectively.

used for adsorption thus leads to the formation of elongated structures. This behaviour may be related to an increased adsorbed amount. When the collagen concentration is low, the low adsorbed amount allows the formation of a layer of molecules in lying position. When the concentration is raised, molecular segments are protruding in solution and may be available for intermolecular association.

### 2.2.2. State of aggregation of collagen in solution

Collagen is mainly found in soluble monomer form in cold acid or neutral solutions [17]. Increasing the pH, the temperature and/or the ionic strength is known to provoke collagen aggregation [18]. Given the presence of supramolecular assemblies in adsorbed collagen layers formed in certain conditions (smooth hydrophobic substrate, high collagen concentration, long adsorption duration), it is important to clarify the role played by aggregates formed in solution on the organisation of adsorbed layers.

Collagen solutions were prepared by diluting a cold (4 °C) acidic (pH 3.0) 3 mg/ml collagen stock solution in cold phosphate buffer (pH $\sim$ 5.8; ionic strength $\sim$ 160 mM) down to a concentration of 150 µg/ml. The obtained solutions were then warmed at 37 °C for 15 min, two or seven days. The absorbance at 400 nm was then measured using a 10-cm long quartz cell; it was of the order of 0.09, 0.75 and 0.95 after 15 min, two and seven days, respectively. The aggregation of collagen thus increased with the ageing time at 37 °C. The solutions were then further diluted, using the same phosphate buffer, to a concentration of 40 µg/ml. Adsorption was performed, with these solutions, for 30 min on PS samples.

Fig. 3 presents AFM images of collagen adsorbed on PS from solutions at pH 5.8 aged for 15 min, two and seven days, that is, solutions presenting an increasing level of aggregation. Elongated structures are observed at the interface. The density and the thickness of those structures decrease with the aggregation of collagen in solution. XPS analysis of the same samples also revealed that the adsorbed amount decreased with collagen aggregation in solution.

This demonstrates that the presence of supramolecular assemblies of collagen at the interface is not directly linked to the presence of aggregates in the collagen solution used for adsorption. These observations, that is, a reduced adsorbed amount and less elongated structures when the solution is more aggregated, indicate that the adsorption process is governed by free collagen molecules in solution, either because they diffuse faster to the interface or have a stronger affinity for the substrate compared to aggregates formed in the solution. Adsorbing from a more aggregated solution thus produces a similar effect as adsorbing from a less concentrated solution (see above).

## 2.3. Procedure of sample preparation

### 2.3.1. Adsorption time

Fig. 1(e) and (f) presents AFM images of collagen layers obtained by adsorption during 24 h from a 40 µg/ml on PS and PSox, respectively. These images can be compared with those recorded after 2 h of adsorption (Fig. 1(c) and (d)). On PS, the surface is already fully covered by elongated structures after 2 h; a similar morphology is found after 24 h. On PSox, the adsorbed layer, which was rather smooth after 2 h, shows elongated structures after 24 h, similar to those found on PS [14].

The adsorption duration may affect the organisation of adsorbed collagen layers through the evolution of the adsorbed amount. The latter increases sharply up to 2 h on PS and PSox, after which a plateau is reached, at about 1.1 and 0.9 µg/cm$^2$, respectively. However, the organisation of adsorbed collagen still changes after 2 h of adsorption, as seen on PSox. These changes, which are not linked to an evolution of the net adsorbed amount, may be explained either by exchanges between adsorbed collagen and collagen molecules in solution, or by reorganisation of adsorbed collagen at the interface.

A similar trend was observed on PET [10]: elongated features were absent after 3 h of adsorption from a 7 µg/ml solution, but appeared after 24 h. In this case again, evaluation of the adsorbed amount using XPS indicated that it was not much higher after 24 h compared to 3 h.

### 2.3.2. Drying of the samples

The rate of drying has a striking effect on the organisation of the adsorbed layer obtained at low concentration and short adsorption times. Fig. 1(a) and (b) presents AFM images of collagen layers obtained by adsorption from a 7 µg/ml solution for 2 h on PS and

respectively fast (i.e. under a nitrogen flow; FD) or slow (i.e. in 95% relative humidity; SD) drying. A smooth layer is formed after fast drying while slow drying produces a net-like structure (holes of a diameter of 50–500 nm; thickness $\sim$ 6–8 nm). It was shown using AFM in the adhesion mapping mode that the surface obtained by slow drying presents a chemical contrast, with PS domains present at the outermost surface in the holes of a collagen net [12]. The formation of such a net-like structure is attributed to the rupture of the liquid film by dewetting, collagen being displaced by the water meniscus.

A similar effect was observed with poly(methyl methacrylate) (PMMA) used as a substrate [5]. However, the formation of a net-like structure was not observed on PSox [8]. This can be due to the hydrophilicity of PSox, which may prevent dewetting, and/or to the organisation of the collagen layer on PSox (see above), which confers more mechanical resistance to the layer and thereby may oppose collagen displacement by the water meniscus.

On PS, the formation of the net-like structure upon slow drying did not occur upon increasing the collagen concentration in the solution used for adsorption. In these conditions, increased intermolecular collagen associations are thought to prevent the displacement of collagen along the surface plane [8]. When the adsorption time is varied at 7 μg/ml, a range of collagen patterns is found, from a dendritic structure to a net and, finally, to a dense layer of elongated features [15]. At adsorption times not longer than 2 h, the patterns recall those observed during a dewetting process. At longer adsorption times, patterns are not formed anymore; again this is attributed to the formation of a collagen mat preventing dewetting and displacement of collagen along the surface plane.

## 3. Applications

Controlling the supramolecular organisation of collagen may lead to applications in various fields, including biomaterials science and nanotechnologies. This is illustrated by the use of a collagen adsorbed layer as a template to create nanostructured polymer surfaces, and by the study of mammalian cells behaviour (human breast cancer cells MCF-7/6) as a function of collagen supramolecular organisation.

### 3.1. Nanostructured polymer surfaces

Fig. 4(a) depicts the method developed to design nanostructured polymer surfaces. A collagen template is first created on a smooth polymer surface, by adsorption followed by slow drying. As discussed above, this leads to the formation of a collagen net, with the polymer present at the outermost surface in the holes of the net. A solvent of the polymer is then spin-coated on top of the template. This provokes the

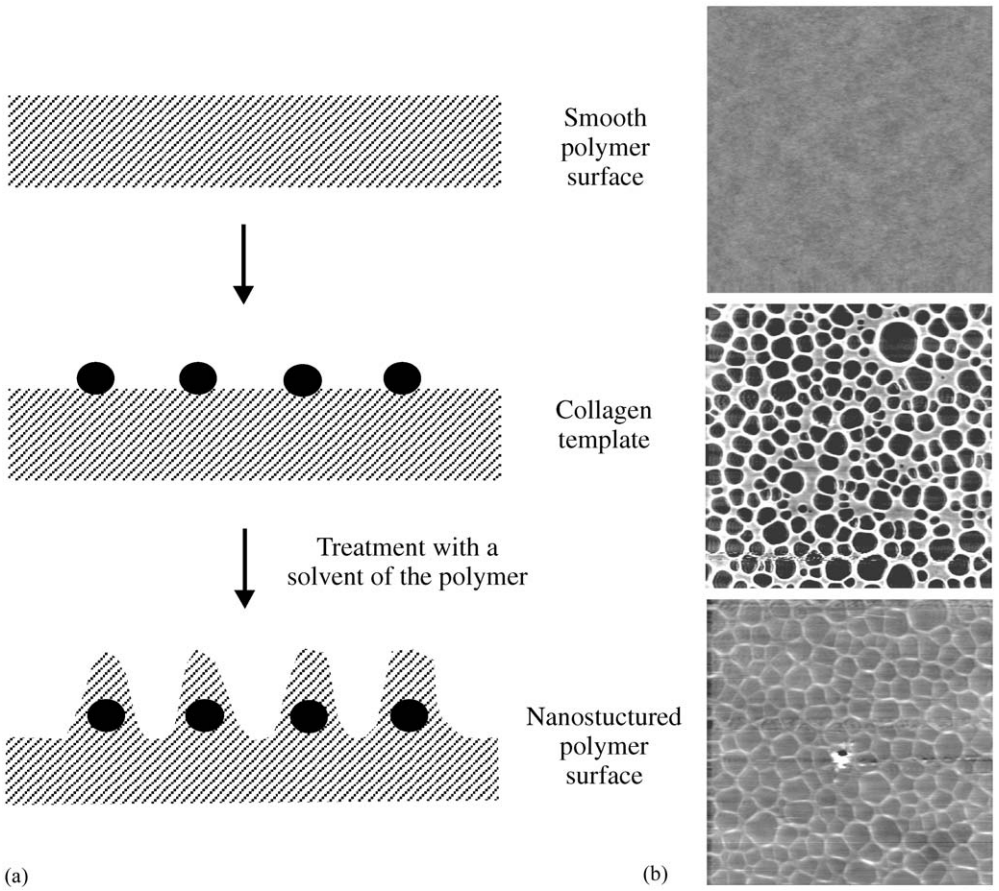


*Figure 4* (a) Principle of the method used to design nanostructured polymer surfaces, starting from a smooth polymer surface on which a collagen template is first created and then treated with a solvent of the polymer. (b) Application of the method to a smooth PS substrate. (AFM images acquired in air, $5 \times 5\ \mu m^2$, $z = 10$ nm): a collagen template was created by adsorption from a 7 μg/ml solution for 2 h followed by slow drying; spin-coating with toluene was then performed.

dissolution of the polymer and its redeposition on the collagen template.

The method was applied to PS, on which the collagen template was first created, and spin-coating was then performed with toluene, as illustrated in Fig. 4(b). XPS analysis showed that a pure PS surface is obtained after spin-coating; collagen from the template is not detected anymore. With this polymer–solvent combination, the initial relief of the template is attenuated: the height of the net threads decreases from $\sim 8$ to $\sim 3$ nm.

The same method was successfully applied to PMMA, using chlorobenzene for spin-coating [9]. In that case, the relief of the collagen template was strongly enhanced (from 3–12 to 50–250 nm) but, again, the obtained surface had a chemical composition typical of PMMA and showed a lateral organisation similar to that of the collagen template.

### 3.2. Cell behaviour as a function of collagen organisation

Collagen was adsorbed from a 7 µg/ml solution on PMMA for 2 h, and fast or slow drying was applied; AFM images of the obtained collagen layers are presented in Fig. 5(a) and (b), respectively. The behaviour of MCF-7/6 cells placed in contact with these collagen layers presenting contrasted supramolecular organisations (smooth layer vs. net-like pattern) was then investigated.

On the one hand, the number of attached cells was counted after 4 h of contact in serum-free medium. Compared to a control PMMA substrate ($\sim 40$ cells/mm$^2$), cell attachment was reduced on the smooth and continuous collagen layer obtained by fast drying ($\sim 20$ cells/cm$^2$) but was enhanced on the net-like collagen structure ($\sim 55$ cells/cm$^2$) [7].

On the other hand, attached cells were imaged in culture medium using AFM, without fixation. Fig. 5(c) and (d) shows AFM images (in the deflection mode, that is, error signal from the feedback loop) of MCF-7/6 cells attached to a PMMA substrate coated with a smooth or a net-like patterned collagen layer, respectively. AFM height images (not shown here) of adherent MCF-7/6 cells showed that the cells had a rounded shape (height of about 2.5 µm) on the continuous collagen layer, and were more spread (height of about 800 nm) on the patterned collagen layer. The deflection images presented here give more details about the cell morphology. On the continuous collagen layers, the cell surface was subject to deformation by the AFM scanning tip, and the observed features changed with the scanning direction and the number of scans. On the patterned collagen layer, cytoskeletal structures could be visualised through the cytoplasmic membrane, as already observed before on other types of adherent cells [19]. In this case, the cells could be scanned many times without undergoing alteration.

These results indicate that proteins secreted by the cells may be more efficient than type I collagen in triggering cell adhesion. Furthermore, the attachment and the cytoskeletal organisation of MCF-7/6 cells were enhanced on the patterns compared to the smooth collagen layer. This could be due to the created nanorelief, since it was already reported that cells could react to nanometer-scale topographic features [20], or to the space distribution of the collagen

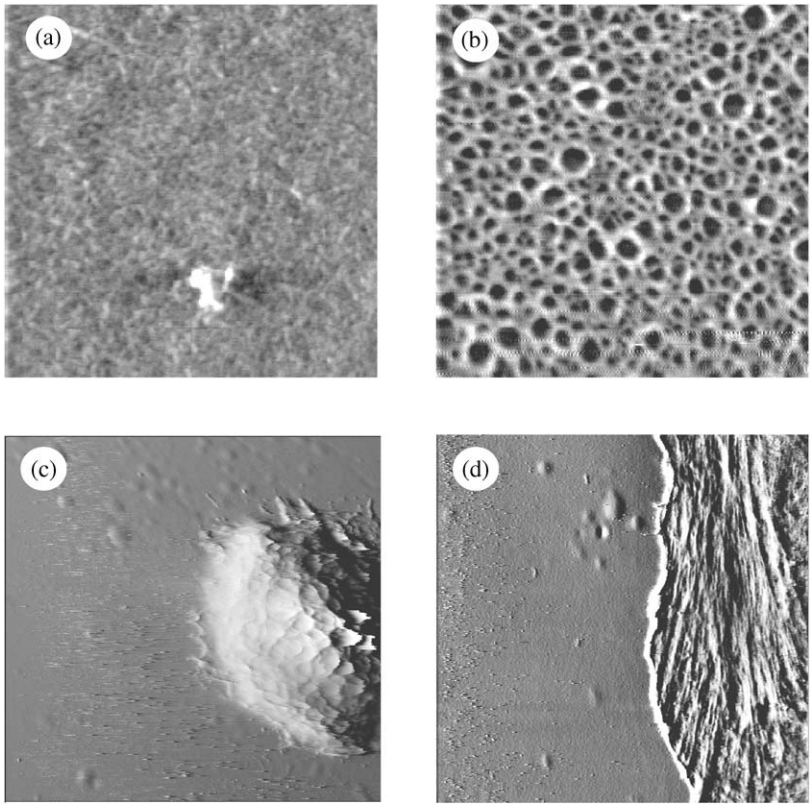


*Figure 5* AFM images ($2 \times 2\,\mu m^2$, $z = 7$ nm) acquired in air of collagen layers obtained by adsorption from a 7 µg/ml solution for 2 h on PMMA followed by fast (a) or slow (b) drying. AFM images (deflection mode) acquired in culture medium of MCF-7/6 cells attached to the collagen layer shown in (a) (c; $60 \times 60\,\mu m^2$) and in (b) (d; $50 \times 50\,\mu m^2$).

sequences which serve as signals for the cells. It has recently been observed that the morphology of smooth muscle cells was affected by the density of collagen fibrils present in the substratum [21]. In any case, this demonstrates that controlling the nanometer-scale organisation of adsorbed protein layers may allow a better control of cell–material interactions to be achieved.

## 4. Conclusions

The supramolecular organisation of collagen adsorbed on polymer surfaces can be modulated by changing the properties of the substrate and the characteristics of the collagen solution used for adsorption, as well as by adapting the preparation procedure. Elongated structures made at the interface by assembly of collagen segments protruding into the solution are readily formed on hydrophobic substrates. This requires surface smoothness, which is needed for the mobility of adsorbed collagen. The formation of these elongated structures is favoured by increasing the collagen concentration and thus the adsorbed amount; it is also better developed upon increasing the adsorption time, even beyond stages at which the adsorbed amount does no longer vary. The occurrence of collagen aggregation in solution has the same effect as adsorbing from a less concentrated solution: the adsorption process and the resulting supramolecular organisation are thus governed by free collagen molecules in solution rather than by preformed aggregates.

The collagen layers obtained by adsorption from low concentration solutions for short times on hydrophobic substrates reorganise strongly upon drying at low rate. A net-like structure is formed due to dewetting and collagen displacement by the water meniscus. This is prevented if collagen forms a mat (i.e. on hydrophilic substrates) or if the adsorbed amount is increased (i.e. high concentration and long adsorption time), which opposes collagen displacement along the surface plane.

Controlling the supramolecular organisation of adsorbed collagen opens the way to applications in biomaterials science and nanoengineering. A simple and versatile method allowing a polymer surface to be nanostructured, using a collagen template, was developed. On the other hand, it was shown that the attachment and the cytoskeletal organisation of MCF-7/6 cells was enhanced on a net-like patterned compared to a smooth collagen layer.

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