

I would like to acknowledge many people who made this thesis possible:

Professors Paul Rouzhet and Christine Dupont-Gillain for their enthusiasm, their inspiration, and their great efforts to explain things clearly and simply. Four years in CIFA taught me that the way to grow passes through the patience, care and good organization. Thank you for your hospitality. Special words go to Christine for her scientific, as well as human support.

Professors Patrick Bertrand, Christian Grandfils, Alain Jonas and Janos Vörös for their helpful comments, suggestions and instructive questions which allowed me to improve the content of this thesis.

John Booth for giving me the possibility to discover and work with quartz crystal microbalance technique.

Professor Fredrik Höök for valuable discussion regarding the quartz crystal microbalance data interpretation.

Professor Clive Roberts for the opportunity to work within his team. The stay at University of Nottingham although short, was very instructive for me. Particularly, I would like to thank Xinyong Chen for guiding me in the new aspects of the atomic force microscopy manipulations.

Françoise, Yasmine, Michel, Christophe, Sylvie, Rose-Anne and Pascal for providing logistics help, as well as for nice and equilibrate environment in the laboratory.

Samuel, Cristèle, Claire, Yann, Caroline, Matthieu, Emilienne, Maïté, Sandrine and all master's students for contributing interest and help on many occasions. Thank you also for the time spent outside the laboratory.

Ivana and Olivier, and their children for friendship and unforgettable time spent together. For the doors always opened to me.

Profesorowi Stanisławowi Błażewiczowi i Elżbiecie Pamule za okazaną mi pomoc i dyspozycyjność, mimo dzielącego nas dystansu.

Moim rodzicom, rodzinie mojego męża, dziadkom, braciom oraz siostrze z rodziną za wsparcie w trudnych chwilach, solidarność i poczucie domu do którego zawsze można wrócić. Tobie Karolku za uśmiech.

Lukaszowi, któremu tą pracę dedykuję.

Foreword

This dissertation is composed of two parts:

- An overview, including a general introduction, a short presentation of the thesis and a general conclusion (Part I).
- A detailed presentation of the thesis, consisting in 3 chapters in the form of publication and an appendix (Part II).

Chapter 1: E. Gurdak, J. Booth*, C.J. Roberts[†], Ch.C. Dupont-Gillain, P.G. Rouxhet. Resolution of the vertical and horizontal heterogeneity of adsorbed collagen layers by combination of QCM-D and AFM. *Langmuir* 21, 10684-10692 (2005).

Chapter 2: E. Gurdak, P.G. Rouxhet, Ch.C. Dupont-Gillain. Factors and mechanisms determining the formation of fibrillar collagen structures in adsorbed layers. *Colloids and Surfaces B*, in press.

Chapter 3: E. Gurdak, J. Booth, C.J. Roberts, P.G. Rouxhet, Ch.C. Dupont-Gillain. Influence of denaturation on the nanoscale organization of adsorbed collagen layers. *Journal of Colloid and Interface Science*, in press.

Appendix: E. Gurdak, N. Gadegaard[‡], P.G. Rouxhet, Ch.C. Dupont-Gillain. Supramolecular assembly of type I collagen upon adsorption on simultaneously micro- and nanostructured PMMA surfaces.

The author also contributed to the paper:

Ch.C. Dupont-Gillain, E. Gurdak, P.G. Rouxhet. Use of AFM and XPS to characterize the nanostructure of protein films adsorbed on solids. Submitted to *Encyclopedia of Surface and Colloid Science*.

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Abstract

The aim of this work is to better understand the factors and mechanisms leading to the supramolecular organization of collagen layers adsorbed on polymers.

Native collagen adsorption on polystyrene (PS) and plasma-oxidized polystyrene (PSox) substrates revealed that the adsorbed layer consists in two parts: a dense and thin sheet (~ 10 nm) in which fibrils are formed, as revealed by atomic force microscopy, and an overlying thick layer (~ 200 nm) which contains protruding molecules, as revealed by quartz crystal microbalance with dissipation monitoring. The protruding molecules are in low density but modify noticeably the local viscosity. Faster and enhanced fibril formation takes place on hydrophobic compared to hydrophilic substrate. As a result of drastic thermal denaturation, the ability of collagen to assemble into fibrils is lost and the number of protruding molecules responsible for higher viscosity is reduced.

Radiochemical measurements showed that collagen molecules are more easily displaced when adsorbed on a hydrophobic substrate compared to a hydrophilic substrate. This may explain why fibril formation occurs more readily on the more hydrophobic substrate, but is in contrast with higher surface affinity. The possible explanation of this paradox by the quick formation of a dense layer of collagen molecules having a smaller number of contact points with a very hydrophobic surface could not be demonstrated by a comparison of adsorption procedures.

Comparing different collagen sources revealed various modes of aggregation with different characteristics regarding size and order (large fibers in solution, smaller fibrils to featureless underneath layer in the adsorbed phase). Moreover, collagen aggregation in the solution is a process competing with adsorption: more aggregated solutions behave like less concentrated solutions regarding the adsorbed amount and fibril formation in the adsorbed phase.

It must be emphasized that interpretation of the QCM-D data, which is based on fitting physical quantities according to a model, has to be performed very carefully, and requires the examination of the sensitivity of the fitted data to the fitting parameters.

Part I

Overview

I.1 Introduction

I.1.1 Context of the research

Studying, and thereby understanding and controlling the supramolecular organization of adsorbed protein layers on synthetic surfaces is an area of research that recently gained enormous attention. Achieving spatial patterning of proteins has important implications in several fields of nanobiotechnology. The potential applications range from developing biomedical diagnostic assays and biosensors to tissue engineering and regulation of cell function.

When a material (biomaterial) or device is placed in contact with biofluids in the body, proteins from the fluid adsorb on the solid surface within seconds, forming a thin film [1]. This initiates the reaction between the material and the biological system. At the next stage, cells migrate toward the protein layer. They can attach to the surface, multiply and possibly organize into a tissue. Among all these active processes, cell adhesion is the first event which occurs.

Cells possess surface receptors able to recognize specific binding sites on proteins. Integrins, among the cell surface receptors (*e.g.*, selectins and cadherins) are the largest class of receptors forming a direct link between the cells and extracellular matrix (ECM) proteins, such as vitronectin, fibronectin, laminin and collagen [2]. Thus, integrins mediate cell-material adhesion using ECM protein, although some mediate also cell-cell adhesion. Integrins establish two-way communication between the inside and the outside of the cell. Outwards, integrins are recruited and concentrated to certain membrane locations. Inwards, integrin binding to ECM sites transduces signals into the cell to set off signaling cascades toward the nucleus, resulting in regulation of genome, protein expression and phenotypic modulation. Gene expression regulates normal cell functions: mobility, proliferation, cytoskeletal organization, survival and finally, protein production that cues signal to other cells upon release [3].

Integrins are formed by the noncovalent association of two subunits. The recognition between the cell and the protein is made through a key-lock interaction between the integrins and a small peptide sequence of the protein, which is often the RGD sequence (R: arginine, G: glycine, D: aspartic acid). The local accumulation of the integrins results in the formation of focal adhesion clusters and reorganization of the actin fibers of the cytoskeleton. The latter plays a

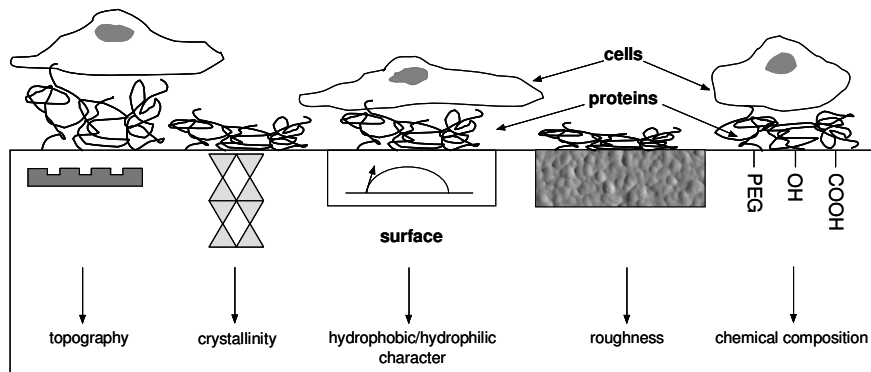


Figure I.1: Schematic illustration of specific materials surface-protein-cell interactions.

crucial role in the cell structure maintenance and cell adhesion, growth and mobility regulation. Thus, the control of the cell-material interactions may be achieved by the control of the focal contact formation [4].

A schematic illustration of material surface-cell interactions through a layer of adsorbed proteins is presented in Figure I.1. Material properties, such as surface topography and crystallinity, hydrophobicity and roughness, and finally chemical composition affect the organization of the adsorbed protein layer. This protein organization defined by molecules orientation, distribution and density may in turn interact with cells and direct their physiology and differentiation. Therefore, understanding and being able to control supramolecular organization of the adsorbed extracellular matrix protein layers is a challenging task in the field of nanobiotechnology.

The scope of this research is to study the factors and mechanisms leading to the supramolecular organization of adsorbed protein layers. It includes the study of the intrinsic properties protein solutions, *i.e.* degree of molecule association in solution and impact of solution thermal denaturation, that possibly influence further organization of the protein adsorbed layers.

I.1.2 Protein adsorption

Proteins are copolymers of about 22 different amino acids which are linked to each other to form a linear peptide chain. Amino acids varying hydrophobic-

ity makes proteins highly surface active, while the charge of their side group residues makes them positively or negatively charged [5,6].

Exposure of an aqueous protein solution to a solid surface almost always results in accumulation of protein molecules at the solid/liquid interface [7]. This accumulative tendency is determined by the nature of the proteins, of the surface as well as of the surrounding medium. Adsorption may be promoted by electrostatic interactions between protein and substrate, hydrophobicity of protein molecules and substrate, and conformational changes of the protein molecule [8]. However, to discuss the respective influence of these factors on the adsorption process separately is not possible, since electrostatic and hydrophobic interactions have a severe influence on protein structural stability.

Two sorts of proteins with regard to the structure stability may be distinguished: ‘soft’ ones with low internal stability (*e.g.* albumin and fibronectin), and ‘hard’ ones with higher stability (*e.g.* collagen and lysozyme) [7]. The former tend to adsorb on all surfaces, due to entropy gain resulting from structural rearrangements upon adsorption. The adsorption behavior of the latter is governed by the hydrophobic and electrostatic interactions between the protein and the surface. ‘Hard’ proteins adsorb readily on hydrophobic surfaces where dehydration is entropically favorable. On hydrophilic surfaces, they adsorb only if there are sufficiently favorable electrostatic interactions [6].

Despite the complexity of the adsorption process, intuitive behaviors, such as the increase of protein adsorbed amount with the adsorption time and with the protein concentration in solution are generally observed, until the protein adsorbed film approaches the monolayer coverage [9].

In the literature, protein adsorption on solid surfaces is often described as an irreversible process. This is because little or no desorption is detected when the surface with adsorbed protein molecules is brought into contact with a pure solvent [10,11]. However, protein molecules may be released from the surface by other surface active molecules added to the system. It is realized by an exchange mechanism in which the protein molecules are replaced from the surface in favor of adsorption of other molecules [12,13]. It has been demonstrated that, if the surface is brought into a contact with blood plasma, small molecules are the first to reach the surface and adsorb on it. Due to their lower diffusion coefficient, molecules of larger molecular weight arrive to the surface at later stage. The latter usually have a higher affinity for the surface and thus gradu-

ally replace the adsorbed smaller molecules. This exchange process between the adsorbed protein molecules and those present in the solution is known as Vroman effect [14]. The desorption and exchange between the adsorbed proteins on the substrate and those present in the solution may be studied in the light of physicochemical properties of the substrates. For example, protein exchange from a homomolecular system, *i.e.* fibronectin, indicated stronger anchorage to hydrophobic compared to hydrophilic maleic acid copolymer films used as substrates [15].

I.1.3 Collagen

In view of the research context, the focus is put on collagen. Detailed description of collagen is dictated by the complex character of its molecular structure as well as the multistage process of self-assembly. Therefore, collagen biosynthesis, *in vivo* fibrillogenesis, factors influencing collagen layers organization and the impact of *in vitro* created collagen layers on cell behavior are presented in the following paragraphs.

Molecular structure. Type I collagen is the major structural protein of the ECM in all higher vertebrates; it is especially abundant in skin, bones and tendons. It belongs to the family of the fibrillar collagens (till now, 20 different collagen types forming either fibrils, basement membranes, short chains or anchoring fibrils have been identified) [16, 17]. Collagen is composed of three polypeptide chains, called α chains, which fold together, giving the characteristic right-handed, helical structure (length about 290 nm, diameter about 1.5 nm; Figure I.2b). The common amino acid sequence of a single α chain is composed of Glycine-X-Y left-handed repeats in which the X and Y positions are frequently occupied by proline and hydroxyproline [18]. Each amino acid has a precise function. Glycine, as the smallest amino acid, is required every third residue to pack into the central core of the triple helix. Proline and hydroxyproline stabilize the helical conformation because of their ring structure (Figure I.2a). At the ends of the helical domain, nonhelical, short structures (length of about 5 nm) known as the N- and C-terminated telopeptides are found. Their participation in further collagen assembly seems to be catalytic rather than constitutive, as they would essentially facilitate achieving the ap-

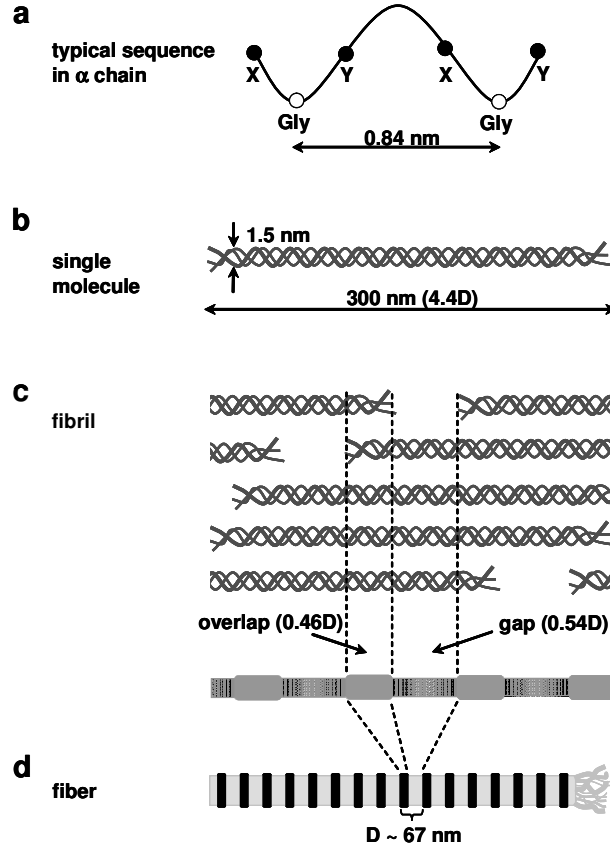


Figure I.2: Structural hierarchy in type I collagen: (a) basic sequence of an amino acid chain, (b) triple-stranded helical molecule with nonhelical ends, (c) side-by-side molecular packing with characteristic gap and overlap regions, (d) 67 nm banding pattern characteristic for bigger aggregates.

appropriate packing into fibers [19]. A widely accepted model of collagen axial and lateral molecular packing involves five molecules which fold together and form a characteristic band pattern, called D-period (Figure I.2c) [20]. Using electron microscopy, it was noticed that D-periodicity is a result of a 67 nm axial shift between the molecules in parallel array (Figure I.2d) [21]. Collagen molecule consists thus of four segments of length D and a fifth segment of length 0.46D, where the gap regions are 0.54D, while the overlap zones are 0.46D [22].

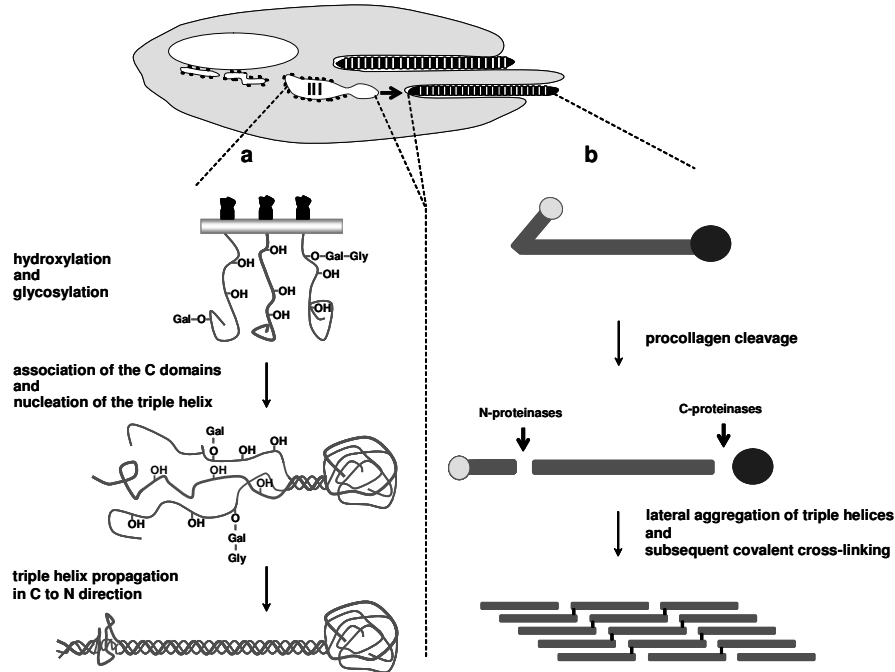


Figure I.3: Schematic representation of procollagen biosynthesis and fibers formation: (a) intracellular steps in the post-translational modification and assembly of the type I procollagen monomer, (b) extracellular steps in conversion of procollagen to collagen monomer and lateral assembly of the monomers into cross-linked collagen fibers.

Biosynthesis. The soluble precursors of collagens, called procollagens, are synthesized and secreted by various epithelial cells, *e.g.* fibroblasts and osteoblasts [16]. A schematic description of the biosynthesis processes is presented in Figure I.3a. Following transcription of the procollagen genes, procollagen synthesis starts with the translation of the pro α chains pre-mRNAs, on the rough endoplasmic reticulum (ER). As the growing α chains are translocated into the lumen of the ER, the collagenous domains are subsequently modified by hydroxylation. It concerns proline and lysine residues and leads to the formation of hydroxyproline and hydroxylysine, respectively. In addition, some hydroxylysine residues are glycosylated (galactose, glucose). Once the full length of α chain has been reached, the process of chain association takes place. It begins in the C-terminal propeptide regions and is rapidly followed by

intrachain disulfide bonds formation toward the N-terminal propeptide regions. It propagates in a zipper-like fashion. After the folding of the three polypeptide chains, the procollagen molecule is transported to the Golgi complex, and afterward is secreted out of the cell, where the extracellular processing takes place and leads to the collagen fiber formation.

Fibrillogenesis. Upon secretion from the cell, collagen molecules assemble to form macromolecular aggregates. These self-assembling processes take place *in vivo* as well as *in vitro* conditions. Once secreted, procollagen molecules are converted into collagen by specific enzymatic cleavage of terminal segments (N-propeptides and C-propeptides) by the procollagen metalloproteinases. Further, collagen assembly into fibrils is regulated and coordinated via lysine residues which are hydroxylated *in vivo* [23]. It has a character of covalent cross-linking and is initiated in the telopeptide (N- and C-telopeptide) end regions of the collagen molecules (Figure I.3b). Regions of cross-linking are repeated at regular intervals along the fibrils. At the level of the collagen triple helix, each α chain is strengthened via intramolecular water mediated hydrogen bonds, while the three adjacent chains are linked together via intermolecular, direct or water mediated, hydrogen bonds. As mentioned above, further collagen assembly involves five triple helical molecules which associate together to form collagen fibrils. In connective tissue, they assemble into bundles to form fibers as well as a large variety of macroassemblies [24, 25].

Established factors influencing collagen layer organization. Collagen adsorption at the interfaces has been studied for more than 20 years. The first studies were focused on quantification of adsorption (kinetics, isotherms, desorptions), which was usually performed using radiolabeled molecules. It was shown that higher adsorbed amounts with more pronounced affinity toward the surface, were observed for collagen on hydrophobic compared to hydrophilic substrates [26, 27]. Visualization of adsorbed layers with high spatial resolution, revealed that collagen molecules form fibrous structures on hydrophobic [28] and after longer adsorption time also on hydrophilic surfaces [29]. *In situ* force-distance measurements between two collagen layers adsorbed on mica showed that molecules interact at a distance as large as 300 nm from the surface [30]. Similar range of repulsion forces (300-400 nm) were recorded on collagen layers

adsorbed on hydrophobic substrate by means of an atomic force microscopy (AFM) [31], suggesting that the adsorbed molecules may protrude into solution. The process of collagen molecules assembly was also studied in relation with substrate topography. Therefore, substrates with the same chemical composition, either smooth or nanostructured (~ 15 nm high protrusions separated by a distance of 50-150 nm), were created. The presence of nanometer-scale protrusions hindered the formation of collagen fibrous structures [32].

Collagen layer organization: influence on cell behavior. Collagen, due to its filamentous shape, rigidity and associative properties is a promising molecule for creation of 3D (scaffolds) [33] as well as 2D (adsorbed or deposited films) [34,35] micro- and nanostructures. Indeed, adsorption from solutions on solid substrates, combined with influence of mentioned factors such as surface topography and chemistry or preparation conditions (pH, ionic strength, temperature, mode of layer drying), leads to a variety of organized layers. Concentric ring patterns [36], discontinuous structures [37] or aligned, dense collagen tracks surrounded by less dense and less regular collagen aggregates [38] may be created. The design of organized layers, and the understanding of such layers formation is of great importance, since it may affect the cell behavior. Type I collagen, as an adhesive protein may be recognized by cell receptors, integrins. As mentioned, recognition takes place via, *e.g.* RGD or RGE (E: glutamic acid) amino acid sequences.

The adhesion of human umbilical vein endothelial cells was observed on both discontinuous and smooth continuous collagen films. Further cellular processes, *i.e.* spreading and cytoskeletal organization were clearly enhanced on discontinuous collagen networks. On the other hand, a high density of collagen fibrillar structures in the continuous layers resulted in reduced cell spreading [35]. Smooth muscle cells spread more extensively on adsorbed layers of denatured collagen (lack of fibrils) than on adsorbed layers of native fibrillar collagen [34]. These studies emphasized that the presence of the fibrils plays a determining role in cell responses to collagen films. Cells can move directionally along the axis of fibrous structures [39]. In the case of mouse dermal fibroblast cells, it was shown that the D-periodicity of the collagen fibers indeed directed cell orientation and mobility. One of the explanations proposed by the authors is that the cellular adhesion apparatus has its own periodicity and that cell

orientation and directed mobility require alignment of the cellular and acellular structures [40]. It is worth mentioning that cells themselves may reorient the collagen fibers as well [41].

I.1.4 Aim and research strategy

The aim of this research is to **better understand the supramolecular organization of collagen layers** adsorbed on polymers. This involves different facets:

- a better knowledge of the **nanometer-scale structure** of the adsorbed collagen layer,
- a broader information on the **factors** affecting this structure,
- a deeper insight into the **mechanisms** in terms of molecular interactions at the interface.

The surface properties of the substrates are expected to play a central role in the organization of the adsorbed protein layer. A crucial factor determining the supramolecular organization is the surface hydrophobicity. Therefore, the choice of polystyrene (PS) and plasma oxidized polystyrene (PSox; variety of oxygen containing functions) as model hydrophobic and hydrophilic substrates, respectively, is relevant. In order to obtain a detailed picture of the adsorbed phase, two techniques, were given the priority: atomic force microscopy (AFM) and quartz crystal microbalance with dissipation monitoring (QCM-D).

Deeper insight into mechanisms which lead to the nanostructuration of the adsorbed phase was searched by: (i) examining polymer surfaces which possess polar groups of better defined nature and concentration compared to PSox; (ii) investigating the influence of different adsorption procedures; and finally, (iii) monitoring the ability of adsorbed molecules to desorb. X-ray photoelectron spectroscopy (XPS) and radiochemical measurements were used in order to gain information about chemical composition of the surface and about the adsorbed/desorbed collagen amount.

In order to hopefully clarify the interplay between collagen-collagen and collagen-surface interactions, the influence of the properties of the collagen solutions was examined by changing the collagen source and using denatured collagen solutions.

I.1.5 Polymer substrates

Polymers are materials widely used in fundamental research as well as in industrial applications. Due to the incredible range of characteristics displayed by polymeric materials, their potential applications appear to be unlimited. They are used as contact lenses, artificial organs, food packaging and photoresist material during microfabrication process. Moreover, properties of polymers can be easily and in an efficient way altered by different processes namely, laser ablation, magnetron sputtering, ion bombardment, *etc.*

Two factors dictated the choice of the polymer substrates for collagen adsorption in this research, namely the relevance of these materials in the biomaterial context and the selection of the particular surface properties. Thus, polymers used as collagen adsorption substrates were: (i) polystyrene (PS) and plasma oxidized polystyrene (PSox), and (ii) polystyrene derivative characterized by a substitution of all repeat units with hydroxyl groups, poly(4-vinylphenol) (PSOH), and finally (iii) poly (methyl methacrylate) (PMMA). PS and PSox were selected as model supports owing to their differentiated influence on the nanoorganization of adsorbed collagen layers as recalled in section I.1.3. PSOH and PMMA were also used as polymers carrying better defined polar functions. The structural formula of PS, PSOH and PMMA is recalled in Figure I.4.

Polystyrene and plasma oxidized polystyrene. PS is an amorphous polymer. Tissue culture polystyrene (TCPS), obtained by plasma oxidation of the PS surface, is widely used for *in vitro* cell cultures. Plasma treatment allows changing the surface properties of the materials, while the bulk properties remains intact [42]. Oxygen plasma treatments are performed by submitting oxygen gas to an electrical discharge; this creates a variety of oxygenated functions which are incorporated into the surface, increasing its wettability.

Polystyrene homologue. In order to work with defined oxygen functions (contrary to PSox) at the surface and to gain better insight into their influence on the organization of protein layers, PS homologous polymer with hydroxyl groups, PSOH was used. It is also an amorphous polymer.

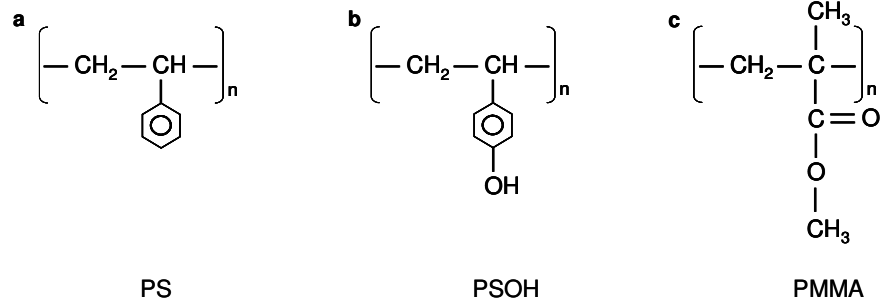


Figure I.4: Structural formula of: (a) polystyrene, (b) poly(4-vinylphenol), and (c) poly(methyl methacrylate).

Poly(methyl methacrylate). PMMA is also an amorphous polymer. It is used as bone cement in orthopedics and as a permanent bone substitute in the treatment of fractures of the vertebrae and skeleton [3]. PMMA combined with other plastics (in order to increase the oxygen permeability) is also used in production of hard contact lenses [43]. Moreover, PMMA presents a specific interest for the design of nanostructured surfaces by embossing or electron-beam lithography [44, 45].

I.1.6 Surface characterization at the nanometer-scale

Two techniques, atomic force microscopy (AFM) and quartz crystal microbalance with dissipation monitoring (QCM-D), are described in detail here owing to their particularity and to their major importance in this work.

I.1.6.1 Atomic force microscopy

Principle. Atomic force microscopy (AFM) belongs to the family of scanning probe microscopies (SPM) and is used to probe sample morphologies and physico-chemical properties. The merit of AFM relies on ability to probe the samples under physiological conditions with very high spatial resolution (< 1 nm) [46]. The working principle of this method is illustrated in Figure I.5. Briefly, a sharp probe (tip) mounted at the end of a flexible cantilever scans over the sample surface in a series of horizontal sweeps. Deflections of the cantilever caused by attractive or repulsive forces sensed by the probe on a sample surface are detected by the reflection of a laser beam toward a segmented

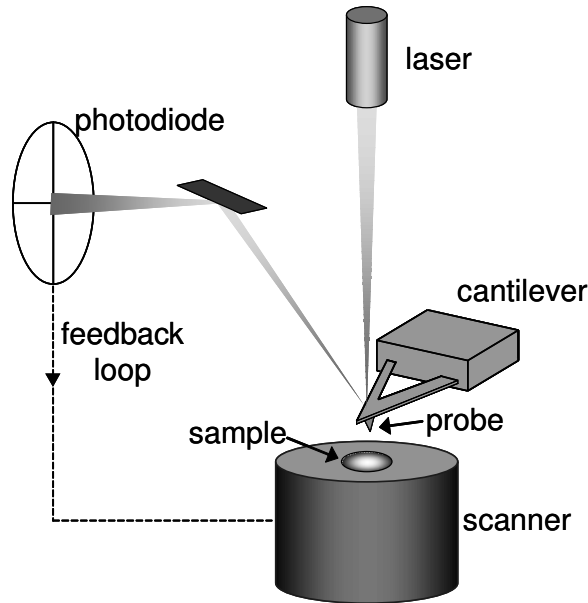


Figure 1.5: Schematic representation of atomic force microscope working principle.

photodiode. Several AFM imaging modes are available.

AFM in contact mode. In contact mode (CM) AFM, the scanning probe is in direct contact with the surface sample. The images are created by bringing the tip and the sample into a contact and scanning the probe across the surface while the sample height is adjusted using a feedback loop to keep the bending or deflection of the cantilever constant. This yields the topographic image. Actually, small cantilever deflections occur because the feedback loop is not perfect, and the resulting error signal can be used to generate a ‘deflection image’, which may be useful in revealing sample surface details [47].

AFM in tapping mode. In tapping mode (TM, called also dynamic or intermittent mode) AFM, an oscillating tip is scanned over the surface sample, *i.e.* the cantilever must first be excited to oscillate close to its resonant frequency (free amplitude of oscillation), and then when brought close to the surface, it interacts with the latter, changing its oscillation (amplitude of oscillation while scanning). The ratio between those two amplitudes is kept constant by adjust-

ing the sample height. This leads to the generation of the sample height map. Due to the intermittent sample contact, tip-sample interaction time, and hence lateral forces between tip and sample are significantly reduced. Therefore, TM compared to CM is more suitable for imaging delicate, biological samples.

AFM in tapping mode offers also the possibility to record the phase signal which is the shift between the oscillation of excitation of the cantilever and that resulting from the contact with the sample. Its character is however very complex because of the variety of involved interactions, being of chemical, electrostatic, viscoelastic and topographic origin, which may account for the signal at the same time [48, 49]. However, the attempts to get valuable information about the molecular interactions from the phase signal are progressing [50].

A tool to measure molecular forces. Besides being applied as a microscope, the AFM can be used to measure molecular interactions between the probe and the sample. This is achieved by the analysis of the force-distance curves, *i.e.* by recording the cantilever deflection as a function of vertical displacement of the piezoelectric scanner (sample). Force-distance curves may be recorded either at a single, well defined location on the sample or at multiple locations giving a so-called ‘force-volume image’. The sensitivity of the measured forces is in the range of piconewtons.

Technique applications. Owing to its high resolution, AFM in tapping mode under aqueous conditions allowed imaging single proteins in purple membrane and hexagonally packed intermediate layers with a lateral resolution of 1.1 to 1.5 nm and a vertical resolution of about 0.1 nm [51]. *In situ* also, and real time AFM imaging of rodlike protein C hordein allowed visualizing the earliest stages of monolayer formation and to observe orientation of the molecules on the surface [52]. Another study revealed that the orientation of collagen microfibrils adsorbed on mica under hydrodynamic flow could be controlled via AFM tip manipulation in a very precise manner. The forces required to align collagen molecules were between 100 and 300 pN [53]. The intermolecular forces associated with ligand-receptor interactions were in the same range for biotin-streptavidin system and were up to 170 pN [54]. Using functionalized probes it was possible to map the distribution of individual adhesins, concentrated into nanodomains on the surface of living bacteria [55]. The application

of AFM to image, sense and probe at the nanometer-scale is thus a relevant way to characterize alive, biological samples.

I.1.6.2 Quartz crystal microbalance with dissipation monitoring

Principle. The QCM-D technique is an electromechanical device. It is a surface sensitive method that via simultaneous registration of frequency (Δf) and dissipation (ΔD) changes *in situ* and real time conditions, and application of a conversion model, may give information about mass-uptake and viscoelastic properties of the adsorbed/deposited matter. The core component of the device is a piezoelectric, particularly cut quartz crystal (SiO_2), called AT-cut which is sandwiched between two thin, metal (usually gold) electrodes (Figure I.6a) [56]. One of the electrodes serves as a ‘sensing’ surface and is in the contact with the measured sample, while the second serves in an electric circuit and is in contact with air. Commonly, such piezoelectric quartz crystals are called thickness shear mode resonators [57]. The sensing principle of the QCM-D technique is based on the piezoelectric property of the quartz. Applying an electric field over the two electrodes, the quartz crystal deforms laterally, *i.e.* along the surface plane (Figure I.6b). When the static electric field is replaced by alternating electric field, the crystal can be made to oscillate at its resonance frequency, f_0 (Figure I.6c).

Relationship between the frequency and the mass changes. In the late 50s, Sauerbrey [58] has shown that the change in the resonance frequency (Δf) of the crystal is proportional to the oscillating mass (Δm), *i.e.* the added mass on the crystal:

$$\Delta f = -\frac{f_0}{h_q \rho_q} \Delta m = -n \frac{2f_0^2}{v_q \rho_q} = -n \frac{1}{C} \Delta m \quad (\text{I.1})$$

where $\Delta m = \Delta h_a \rho_a$, h_a and ρ_a are the thickness and the density of the added mass, respectively; $C = 17.7 \text{ ng/cm}^2\text{Hz}$ and is the mass sensitivity (for AT-cut 5 MHz crystal oscillating at its fundamental frequency, f_0); n is the overtone number; h_q , ρ_q ($= 2650 \text{ kg/m}^3$) and v_q ($= 3340 \text{ m/s}$) are the thickness, specific density and the shear wave propagation velocity in quartz, respectively.

Sauerbrey relation (I.1) is valid only if the added mass is: (i) small compared

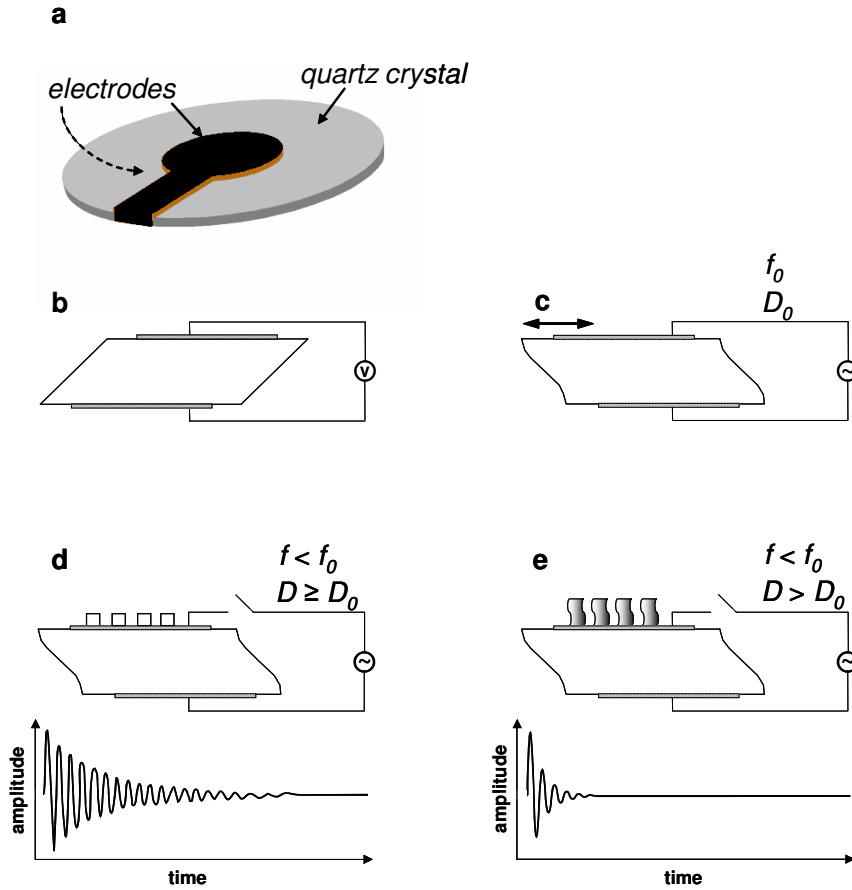


Figure I.6: Schematic representation of the QCM-D technique working principle: (a) a quartz crystal sensor sandwiched between two metal electrodes, (b) the sensor mechanical deformation while a static electric field is applied, (c) the sensor oscillating deformation (mechanical back and forth movements) at a resonance frequency, while an alternating electric field is applied, (d) and (e) attachment of a respectively rigid and viscoelastic mass to the oscillating sensor surface and subsequent monitoring of a freely decaying amplitude of oscillation after power switching off. The frequency (f) and dissipation (D) responses compared to the initial state (f_0 and D_0) of the system are indicated.

to the weight of the quartz crystal, (ii) rigidly attached to the sensor surface (no slip or deformation of the mass is imposed by the oscillating surface) and (iii) evenly distributed over the active area of the crystal. QCM became a powerful tool to measure non-specific gas adsorption [59], oxidation processes [60], catalytic reactions [61] as well as thin film deposition [62]. However, initially it could be only performed in vacuum and gaseous environments.

Overtone. A characteristic property of the AT-cut piezoelectric quartz is that, apart from the oscillation at fundamental frequency ($n = 1$), higher harmonics (overtone) can also be excited. In QCM-D technique, usually 5 MHz ($n = 1$) crystals are used. Rapid switching between the higher frequencies, as 15, 25 and 35 MHz ($n = 3, 5$ and 7) relies on applying alternating pulses corresponding to the overtone frequencies [63]. The resonance frequency of a crystal is mainly determined by the crystal thickness; it increases when the crystal thickness decreases, *e.g.* a 5 MHz quartz crystal exhibits a thickness of 330 μm , while a 30 MHz crystal is only 55 μm thick [56]. The possibility to operate at different overtones helps in interpretation of the data and produces a set of results which may be comparable with theoretical representation (this will be reviewed in paragraph ‘Viscoelastic model’).

Dissipation factor. QCM-D technique makes use of the fact that the sensors which are mass loaded induce dissipative losses while oscillating. A parameter describing such energy losses is called dissipation factor, D and is represented as:

$$D = \frac{E_{dissipated}}{2\pi E_{stored}} \quad (\text{I.2})$$

where $E_{dissipated}$ is the energy dissipated during one period of oscillation and E_{stored} is the average energy stored in the oscillating system. The factor D thus expresses the effect of all mechanisms that dissipate energy while the system oscillates (inherent crystal imperfections, resistive losses coming from crystal-electrical circuit contacts and losses induced from surrounding medium, and loaded mass). It was noticed that the crystal becomes significantly damped when it is moved from air to water environment. However, even after immersion in liquid it could be still excited to a stable oscillation [64]. The free decay of the crystal oscillation (measured as voltage or current) is recorded when the

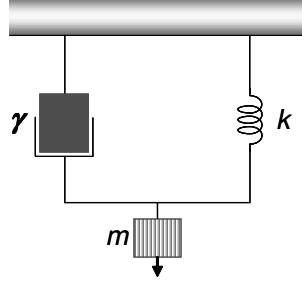


Figure I.7: Schematic illustration of a Voigt-based mechanical model used in QCM-D modeling. It is represented by the equivalent mass, m connected to a spring with a spring constant k and a dashpot with a damping γ .

driving power in the system is switched off. Simultaneous measurements of dissipation and frequency changes are thus achieved by numerically fitting the decay curve which is an exponentially damped sinusoidal [65]. In water, the measurements are achieved with a sampling rate of about 1 point/s and the resolution in frequency and in dissipation better than 0.2 Hz and 1×10^{-7} , respectively [66]. Quartz crystal oscillating with an attached mass couples it to its own motion. The nature of the added mass determines then a damping of the crystal oscillation. If a mass is rigidly coupled to the sensor, *i.e.* it follows the movement of the oscillator, the recorded decay would be progressive in function of time (Figure I.6d). An opposite situation takes place when the crystal is covered by a ‘soft’ mass. In this case, the damping of the crystal would be much faster due to viscoelastic losses within the mass contacting the surface of the crystal (Figure I.6e).

Viscoelastic model. Protein adsorption from aqueous solution often induces a significant change in frequency as well as in energy dissipation, indicating that there is a meaningful mass-uptake and moreover that, this mass acts as a dissipative adlayer. Thus, the assumptions underlying equation (I.1) may not be strictly fulfilled. The adsorbed mass has to be considered as a viscoelastic adlayer where viscous and elastic behaviors are interrelated. Mechanically, this situation may be represented by the Voigt-based model for viscoelastic solids, which consists of a spring and a dashpot in parallel (Figure I.7) [67]. Applying oscillation to the system, a mass would vibrate causing the motion of a spring which is responsible for purely elastic behavior and of a dashpot

Overview

which corresponds to the purely viscous behavior. Accordingly, the viscoelastic adlayer may be characterized by a complex shear modulus:

$$G = G' + iG'' = \mu_a + i2\pi f\eta_a = \mu_a(1 + i2\pi f\tau_a) \quad (\text{I.3})$$

where G' is the storage modulus, G'' is the loss modulus, μ_a is the elastic shear modulus, f is the oscillation frequency, η_a is the shear viscosity, and τ_a is the characteristic relaxation time of the adlayer. If the adlayer is situated between the sensor and a semi-infinite Newtonian liquid under no-slip conditions, and has a uniform thickness (h_a) and an uniform density (ρ_a), the Δf and ΔD [68, 69] become:

$$\Delta f = \frac{\text{Im}(\beta)}{2\pi h_q \rho_q} \quad (\text{I.4})$$

and

$$\Delta D = -\frac{\text{Re}(\beta)}{\pi f h_q \rho_q} \quad (\text{I.5})$$

where

$$\beta = \xi_1 \frac{2\pi f \eta_a - i\mu_a}{2\pi f} \frac{1 - \alpha \exp(2\xi_1 h_a)}{1 + \alpha \exp(2\xi_1 h_a)}, \quad \alpha = \frac{\xi_1 \frac{2\pi f \eta_a - i\mu_a}{2\pi f \eta_l} + 1}{\frac{\xi_1 \frac{2\pi f \eta_a - i\mu_a}{2\pi f \eta_l} - 1}$$

$$\xi_1 = \sqrt{-\frac{(2\pi f)^2 \rho_a}{\mu_a + i2\pi f \eta_a}}, \quad \xi_2 = \sqrt{i \frac{2\pi f \rho_l}{\eta_l}}$$

and where ρ_l and η_l are the bulk liquid density and viscosity, respectively. A schematic illustration of this situation is presented in Figure I.8. In order to gain mass and dissipative information coming only from the protein adlayer (together with the liquid trapped inside the adlayer), the influence of the bulk liquid on Δf and ΔD (Δf_l and ΔD_l) may be withdrawn from equations (I.4) and (I.5), respectively, by subtracting the following terms [70, 71]:

$$\Delta f_l = -\frac{1}{2\pi h_q \rho_q} \sqrt{\pi f \rho_l \eta_l} \quad (\text{I.6})$$

$$\Delta D_l = \frac{1}{h_q \rho_q} \sqrt{\frac{\rho_l \eta_l}{\pi f}} \quad (\text{I.7})$$

Each measured data point yields two measurements (f and D). According

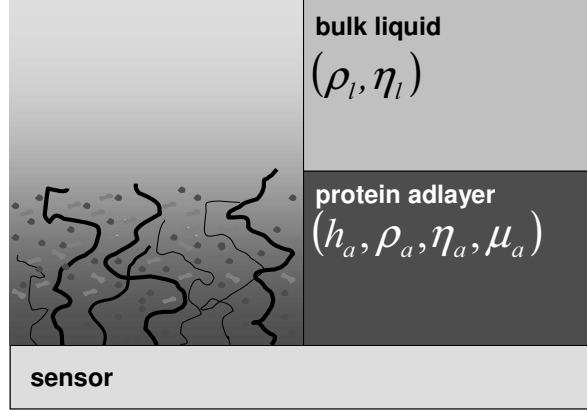


Figure I.8: Schematic illustration of the geometry and the parameters used to simulate the QCM-D sensor covered with a viscoelastic protein adlayer with a thickness h_a , being in contact with a sensor surface and semi-infinite Newtonian liquid. The protein adlayer is represented by the density, ρ_a , viscosity, η_a and shear modulus, μ_a . The bulk liquid is represented by a density, ρ_l and a viscosity, η_l .

to the model, Δf and ΔD are thus influenced by three unknown parameters, *i.e.* adlayer thickness h_a , viscosity η_a and shear modulus μ_a . Three other parameters, adlayer density ρ_a , liquid viscosity η_l and liquid shear modulus μ_l are imposed and considered to be constant through the whole adlayer) [72]. This means that the output is not unique for one measurement, since several combinations of h_a , η_a and μ_a can account for the experimental results. Operating at different overtones ($n = 1, 3, \dots$) offers the possibility to obtain values fitting the raw data. However, best fitting does not mean that the obtained adlayer characteristics are the real ones. Thus, examining the sensitivity of the fitted quantities (Δf and ΔD) to the fitting parameters (h_a , η_a and μ_a) should always be considered.

Technique applications. Using the QCM-D in a liquid phase creates new experimental opportunities. Nowadays, it is a widely used method in biomedical science to study processes such as: protein adsorption, antibody-antigen binding [66], lipid vesicle formation [73] as well as cell adhesion [74]. A combination of optical methods with QCM technique running in liquid phase led to the determination of the amount of coupled water in human serum albumin,

fibrinogen and hemoglobin adlayers. It was estimated to be up to 70 % of the total detected mass [75]. Independent study revealed that the amount of water taking part to the protein adsorbed layer is higher as the molar mass of the protein increases [76]. In another study, QCM was used to follow the effect of the cross-linking in mussel adhesive protein Mefp-1 adlayers [69]. In comparison with the initial, water-rich and hydrogel-like adlayer, a more compact and less water-rich adlayer was obtained after cross-linking. Mono- and polyclonal fibrinogen antibody adsorption on preadsorbed fibrinogen adlayers put in evidence that the structure of the protein adlayer is surface-chemistry dependent [77].

1.1.6.3 Outlook of techniques used in this research

Although AFM is a well established method for imaging adsorbed proteins, the poor resolution of the collagen images obtained in contact mode did not provide a detailed map of the layer organization. Thus, in this research, imaging of the surface was realized using AFM in tapping mode.

Beside AFM and QCM-D, two methods were used to characterize the adsorbed collagen layers and to study their properties. X-ray photoelectron spectroscopy (XPS), was used in order to determine the chemical composition of the surface, keeping in mind that XPS data are influenced by the organization of the interfaces. Radiolabeling of collagen allowed the adsorbed amount to be determined.

1.2 Collagen organization as a response to surface chemistry

1.2.1 Model hydrophobic vs hydrophilic polymer surfaces

In previous works concerning collagen adsorption on polystyrene (PS) and plasma oxidized polystyrene (PSox), as a model hydrophobic and hydrophilic surfaces respectively, higher adsorbed amounts with more pronounced affinity toward the surface were observed on PS compared to PSox [29]. Formation of elongated, fibrillar structures in the adsorbed phase was observed on PS. Sim-

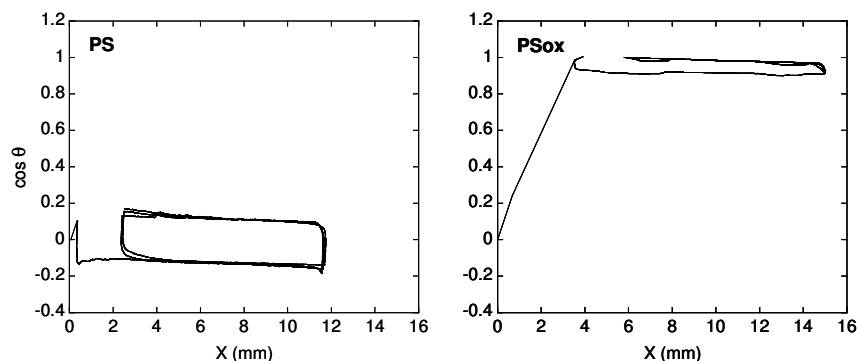


Figure I.9: Variation of the cosine of the water contact angle (θ) as a function of the three-phases contact line position X during repeated immersion-emersion cycles in water (pH ~ 5.6) for PS and PSox. Three following cycles were run without any stop.

ilar collagen fibrils were also noticed on PSox surface but they appeared more slowly and to a lesser extent [29]. The present study investigates more deeply the interaction between collagen and hydrophobic/hydrophilic support, with the main interest put on *in situ* and real time collagen adsorption monitoring. Therefore, the collagen adsorption was monitored using QCM-D technique. In a complementary fashion, AFM in tapping mode under water provided visualization of collagen layers for different solution concentrations. The results shown in this section were obtained at a collagen concentration of 40 $\mu\text{g/ml}$. Results obtained with concentrations of 7 and 100 $\mu\text{g/ml}$, and further details are presented in Chapter II.1.

In order to verify the hydrophobic and hydrophilic characters of PS and PSox, respectively, the wetting properties of both surfaces were investigated in dynamic conditions using the Wilhelmy plate method (details in Chapter II.2). Figure I.9 presents the wetting curves of both polymers recorded in water (pH ~ 5.6). Three successive immersion-emersion cycles were recorded. The hysteresis loop was small and remained almost constant upon repeating cycles on PS, with advancing contact angles of $92-96^\circ$ and receding contact angles of 82° . PSox showed hydrophilic properties, as reported before [78]. A very small hysteresis loop was observed. The first advancing contact angle was equal to 23° and did not exceed 15° in the second and the third cycle. Static contact angles (sessile drop method) measured on PS and PSox were about 90° and

20 °, respectively.

Representative topographic images recorded on collagen layers adsorbed on PS and PSox are presented in Figure I.10. On PS, fibrillar structures of collagen were observed. On PSox, collagen features up to 5 nm were present but they did not form well contrasted fibrils. As elongated structures they appeared only at high concentration (100 $\mu\text{g/ml}$; details in Chapter II.1, Figure II.1.3). AFM observations revealed thus that collagen fibril formation in the adsorbed phase was favored on PS compared to PSox. Additionally, it appeared that collagen fibril formation on the latter could be enhanced either by a solution concentration increase (details in Chapter II.1, Figure II.1.3) or, according to the literature, by longer adsorption times [29].

Raw QCM-D data, *i.e.* frequency (f) and energy dissipation (D) changes as a function of adsorption time (details in Chapter II.1, Figure II.1.2) indicated higher shifts upon collagen adsorption on PS than on PSox. Keeping in mind that f changes are related to the adlayer mass changes (Introduction, equation I.1), while f and D changes are related to the variation of the adlayer viscoelasticity (Introduction, equations I.4 and I.5), raw records suggested that a thicker and more viscous adlayer is formed by collagen adsorbed on PS than on PSox substrate. In order to extract values of adlayer thickness h , viscosity μ and elasticity η , a best fitting procedure based on viscoelastic model was applied. However, as described in Introduction (paragraph ‘Viscoelastic model’) one set of adlayer thickness, viscosity and shear modulus is not an unique solution. Therefore, a more appropriate approach was to compute ΔD vs Δf charts (Introduction, equations I.4 and I.5), by which the sensitivity of the experimental data to the parameters of adlayer h , μ and η could be examined. Such a chart (third overtone) is shown in Figure I.11. The experimental ΔD vs Δf curves recorded upon adsorption of collagen on PS and PSox are presented in color. Each of the computed curves represents a range of thicknesses (from 0.1 to 200 nm; 8 dots), and corresponds to a given viscosity (from 1×10^{-3} to 5×10^{-3} Pa.s; differentiated by the shape of the dots) and a given shear modulus (between 10 and 100 kPa; open, black and gray symbols). On the one hand, the chart shows that the value of the shear modulus cannot be determined accurately; *e.g.* the values of 10 kPa (open dots) or 100 kPa (black dots) could fit the data, with close values of thickness and viscosity. Accordingly, the shear modulus was excluded from further consideration. On the other hand, the val-

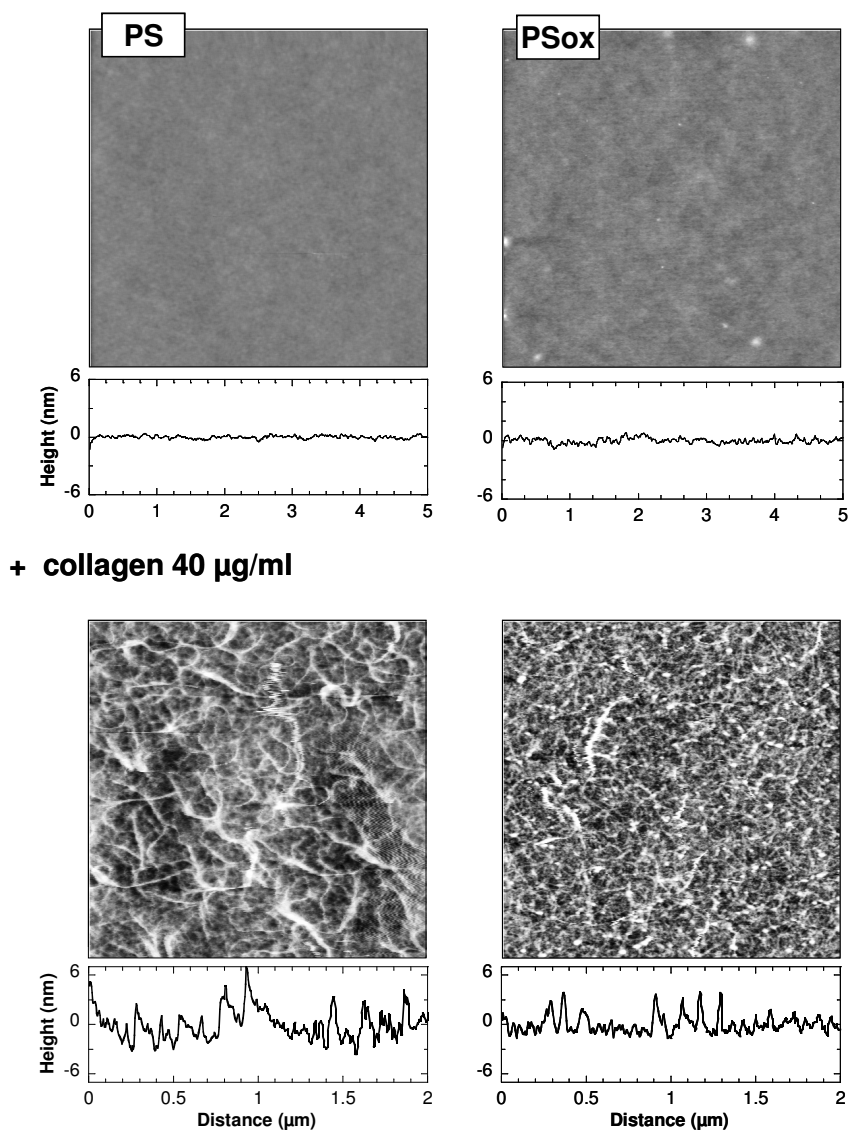


Figure I.10: Representative AFM tapping mode topographic images ($5\ \mu\text{m} \times 5\ \mu\text{m}$, $z\text{-range} = 10\ \text{nm}$, top; $2\ \mu\text{m} \times 2\ \mu\text{m}$, $z\text{-range} = 10\ \text{nm}$, bottom) recorded in air on bare PS and PSox substrates, and under water on collagen layers adsorbed after 24 h on PS and PSox from solutions at $40\ \mu\text{g/ml}$. The cross-sections were taken in the middle of the images. The images were flattened using a third order polynomial (details of flattening procedure in Appendix II.A, Figure II.A.6).

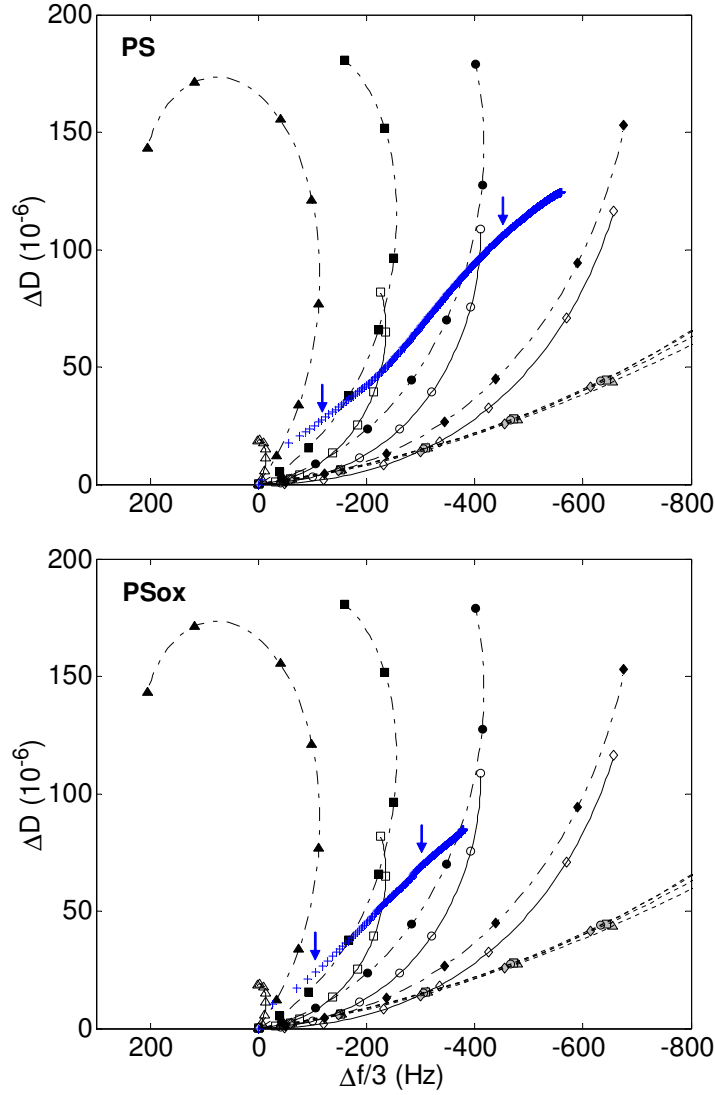


Figure I.11: ΔD vs Δf charts computed for an adlayer, considering a density of 1100 kg/m^3 , a thickness of 0.1, 10, 25, 50, 75, 100, 150 and 200 nm, (set of 8 dots in the increasing order on each spiral curve), a viscosity of $1 \times 10^{-3} \text{ Pa.s}$ (triangles), $2 \times 10^{-3} \text{ Pa.s}$ (squares), $3 \times 10^{-3} \text{ Pa.s}$ (circles), $5 \times 10^{-3} \text{ Pa.s}$ (diamonds) and a shear modulus of $1 \times 10^4 \text{ Pa}$ (open symbols, continuous line), $1 \times 10^5 \text{ Pa}$ (black symbols, long and short strokes), $1 \times 10^6 \text{ Pa}$ (gray symbols, short strokes). In blue color (crosses), plots of experimental data for collagen adsorption on PS and PSox (overtone 3) for concentrations of $40 \mu\text{g/ml}$. The arrows indicate adsorption times of 6 minutes and 60 minutes. Each curve ends after 300 minutes of adsorption.

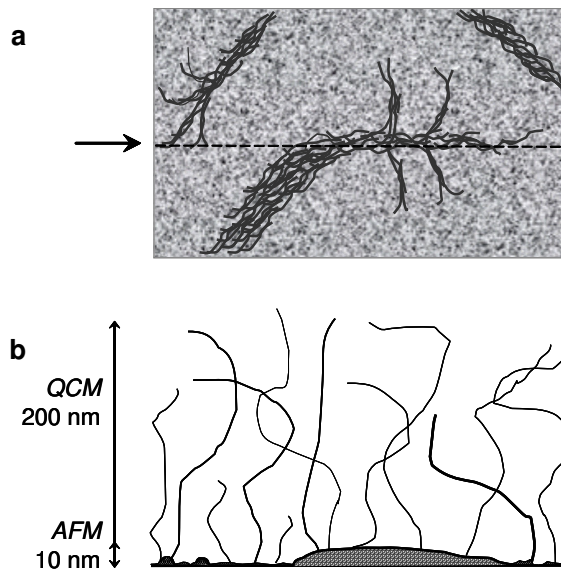


Figure I.12: Schematic representation of the collagen adlayer on PS and PSox substrates: (a) top view, (b) cross-section taken at the position indicated by the arrow. The thickness probed by each technique is indicated.

ues of adlayer thickness and viscosity which fit a given experimental point on the chart (*e.g.* after 5 h of adsorption) are close to that found by best fitting.

It is clear from Figure I.11 that, when adsorption time increased (arrows on the raw data), the increase of the adlayer thickness was accompanied by a simultaneous increase in viscosity, both on PS and PSox. At the end of the adsorption (5 h), the adlayer thickness reached values of the order of 150-200 nm on PS and 100-150 nm on PSox. These thickness ranges were in agreement with the thickness evaluated by viscosimetry for chick skin collagen adsorbed on glass [79]. In parallel, the viscosity of the adlayer was close to 5×10^{-3} Pa.s on PS and about 3×10^{-3} Pa.s on PSox. Keeping in mind that the mass adlayer (thickness) detected by QCM-D contains not only the mass of dry matter but also the mass of trapped liquid, the thickness of 150 nm corresponds to about 4 vol.% of collagen and 96 vol.% of liquid (details in Chapter II.1).

The collagen layer adsorbed on PS and PSox at sufficiently high concentration and/or long adsorption time [29] may be described as schematized in Figure I.12. AFM indicated that molecules bound to the surface (height of

about 10 nm) were aggregated at different scales; moreover their density and size increased with time and collagen concentration. In a complementary fashion, QCM-D revealed a thick (up to 200 nm) collagen adlayer containing about 96 % of water. The high thickness is due to molecules or bundles which protrude into the solution, modifying noticeably the local viscosity. Again, as the collagen concentration and, or adsorption time increase, the adlayer thickness and viscosity increase simultaneously. After about 1 h of adsorption, both parameters are close to stationary conditions (Figure I.11, second arrow). The fibril formation is indeed faster and progresses to a larger extent on PS compared to PSox, in agreement with a higher adsorbed amount (details in Chapter II.1) [29]. For the first time, the complexity of adsorbed collagen layers was revealed by the combination of AFM and QCM-D.

I.2.2 Extension to polymer surfaces with defined surface polar groups

According to the literature and to the present results, surface hydrophobicity strongly influences the supramolecular collagen layer organization [38]. Keeping in mind collagen adsorption on PS and PSox, and the differences in fibril formation resulting from different surface hydrophobicities, other substrates, with better defined polar groups, were studied. Collagen was adsorbed from 40 $\mu\text{g}/\text{ml}$ solutions on PSOH, and from 100 $\mu\text{g}/\text{ml}$ solutions on PMMA (details in Chapter II.2 and Appendix II.A, respectively). The adsorbed layer organization was visualized in air, using AFM in tapping mode, while surface chemical composition of the substrate prior to and after collagen adsorption was determined by XPS. The wetting properties of PSOH and PMMA were investigated in dynamic conditions using the Wilhelmy plate method.

Figure I.13 presents the wetting curves of PSOH and PMMA recorded in water (pH $\sim$ 5.6). Three successive immersion-emersion cycles were recorded. In both cases, the hysteresis loop remained almost constant upon repeated wetting cycles.

In order to compare wetting characteristics of PSOH and PMMA, on the one hand, and of PS and PSox, on the other hand, the values of contact angles measured in dynamic and static conditions are presented in Table I.1. According to the static contact angle results, surface hydrophobicity of considered substrates

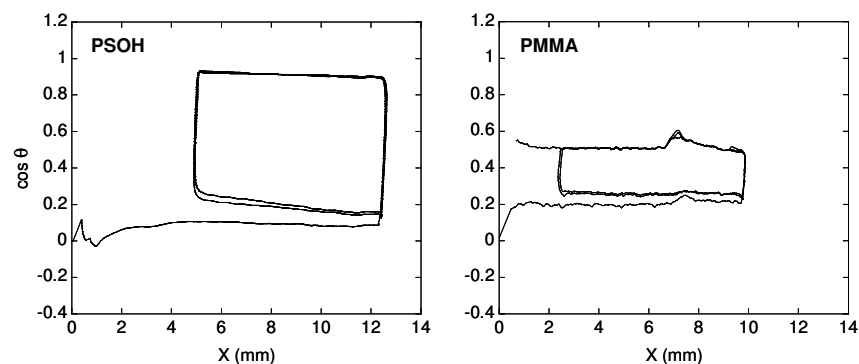
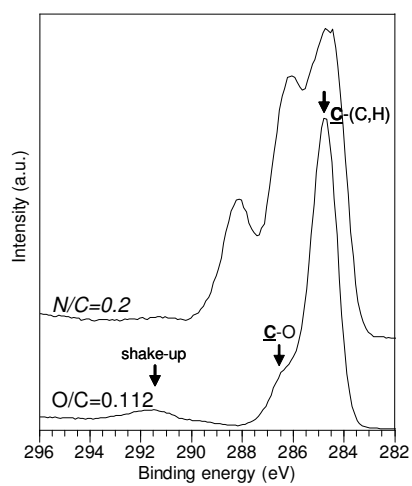
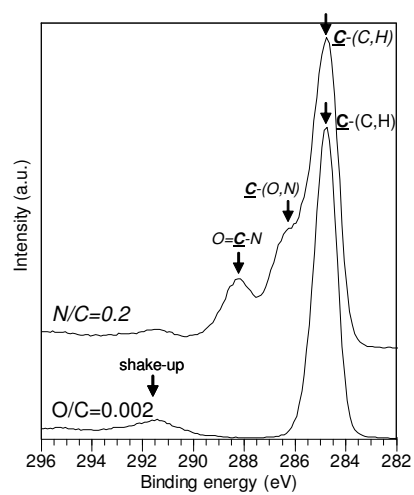
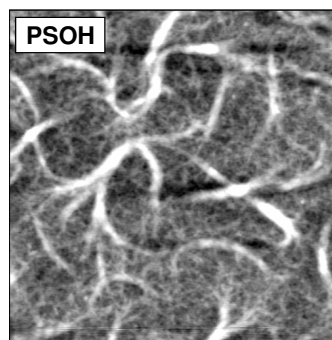
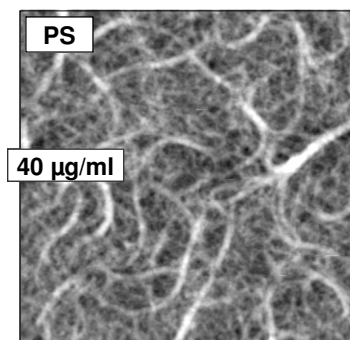


Figure I.13: Variation of the cosine of the water contact angle (θ) as a function of the three-phases contact line position X during repeated immersion-emersion cycles in water (pH ~ 5.6) for PSOH and PMMA. Three following cycles were run without any stop.

Table I.1: Water contact angle values measured in static (sessile drop method) and dynamic (Wilhelmy plate method) conditions for PS, PSOH, PSox and PMMA surfaces.

	polymer substrates			
	PS	PSOH	PSox	PMMA
static condition				
contact angle ($^{\circ}$)	90 ^a	82 ^a	20 ^a	74 ^a
dynamic condition				
advancing contact angle ($^{\circ}$)	96-92	84-76	23-15	78-74
first receding contact angle ($^{\circ}$)	82	23	less than 15	54
hysteresis loop	medium	large	small	medium

^a Average standard deviation did not exceed 3 $^{\circ}$



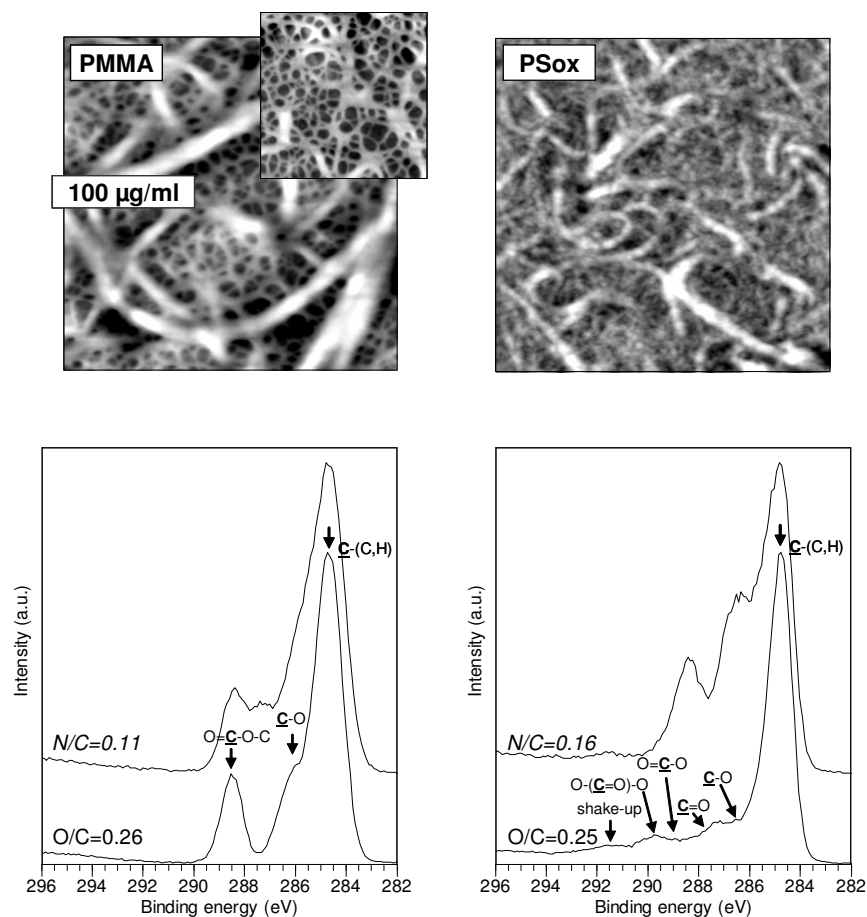


Figure I.14: Representative AFM tapping mode topographic images ($1 \mu\text{m} \times 1 \mu\text{m}$, $z = 7 \text{ nm}$) recorded in air on collagen layers adsorbed from: (i) $40 \mu\text{g/ml}$ solution on PS and PSOH during 24 h, (ii) $100 \mu\text{g/ml}$ solution on PMMA during 2 h (insert $1 \mu\text{m} \times 1 \mu\text{m}$, $z = 7 \text{ nm}$) and (iii) $100 \mu\text{g/ml}$ solution on PSox during 24 h. C_{1s} peaks recorded on bare substrates (bottom curves) and on the substrates covered by the collagen layers (top curves) adsorbed for 24 h (except 2 h on PMMA). The ordinate scale was adjusted to obtain the maximum of the main peak component (284.8 eV) at the same height.

could be classified in the following order: PSox < PMMA < PSOH < PS. However, wetting curves recorded in dynamic conditions revealed that polymer surface responses are more complex. PS, as an apolar polymer showed typical, hydrophobic surface properties. Close values of advancing contact angles indicated that the surface is not perturbed by swelling in water. Significant hysteresis loop could be attributed to the surface irregularities and/or surface contaminations [78]. In the case of PSOH and PMMA surfaces, the advancing contact angles were comparable. The difference appeared in receding contact angles; PSOH showed more hydrophilic character compared to PMMA with angle values of about 23° and 54° , respectively. The difference could be attributed to the enhanced rearrangements of polar groups on PSOH compared to PMMA surface. Also, the occurrence of a large hysteresis in the former could be due to several phenomena, among which a reorientation of the polar groups which tend to get into a contact with water but are buried in the polymer to reduce the interfacial energy in contact with air [80]. The PSox surface with a variety of the polar functions showed hydrophilic character [78].

XPS analysis of bare substrates (Figure I.14, bottom curve in each C_{1s} graph) indicated a presence of chemical functions containing oxygen. Their nature was defined for PSOH and PMMA, and showed a variety of functions for PSox. The measured O/C molar concentration ratio was close to expected on PSOH, and lower than expected on PMMA, possibly due to surface contamination.

After collagen adsorption, components typical of the protein appeared as indicated for PS in Figure I.14. The N/C molar concentration ratios calculated from XPS data showed the same values on PS and PSOH (about 0.2) and lower values on PMMA and PSox (about 0.11 and 0.16, respectively). The lower collagen adsorption on more hydrophilic substrates is in agreement with previous studies [28,29]. Also the adsorbed amount determined by radiolabeling was comparable on PS and PSOH ($\sim 1.1 \mu\text{g/ml}$ [29] and results not shown, respectively), lower on PSox ($\sim 0.8 \mu\text{g/ml}$ [29]) and much lower on PMMA ($\sim 0.13 \mu\text{g/ml}$ [81]) after 24 h adsorption from $40 \mu\text{g/ml}$ solutions. Similar trends of lower collagen adsorbed amount on more hydrophilic substrates were reported in the literature [26].

The collagen supramolecular organization created on the collection of polymer substrates, analyzed from AFM high resolution images, revealed the com-

plex character of the adsorbed layers (Figure I.14). The uppermost layer consisted of fibrillar structures present on all substrates. The underneath layer appeared as continuous on PS, PSOH and PSox and clearly had a perforated character on PMMA; the same perforated relief was observed for the layers adsorbed from lower concentrations as well (10 and 40 $\mu\text{g/ml}$; not shown). The cross-sections taken through the holes revealed a layer thickness of about 6 nm (details in Appendix II.A).

PS and its analogues (PSOH and PSox) led to similar uppermost and underneath layer organization. However, the tendency of collagen to form fibrils in the adsorbed phase varied in the order $\text{PSox} < \text{PSOH} < \text{PS}$. The difference between PS and PSox is marked at low collagen concentration and/or short adsorption time. It vanishes in stationary conditions, *i.e.* longer adsorption times and/or higher solution concentrations.

The observations pointed to a perforated underneath collagen layer on PMMA, and smooth continuous underneath layer appearance on PS, PSOH and PSox. This difference may be due to a global difference of hydrophobicity, or to more specific aspects, such as the nature and concentration of the oxygen functions at substrate surface. Interestingly, the large collagen fibrils which took their roots from the underneath layer seemed not to be influenced by the continuous or perforated character of the latter. Further studies, using more substrates with well-defined oxygen functions on the surface, may give more information about the collagen specific arrangement in the adsorbed phase.

I.3 Mechanisms controlling collagen fibril formation in the adsorbed phase

I.3.1 Study of exchanges between adsorbed phase and solution

Previous studies concerning collagen adsorption on polystyrene (PS) and plasma-oxidized polystyrene (PSox) revealed that higher collagen adsorbed amounts were detected on PS compared to PSox substrates [29]. At early stages of adsorption, fibrils were observed on PS but not on PSox, pointing to faster and more extensive molecular assembly on the former. Fibril network on PS

Table I.2: Amount of collagen transferred between the solution and the layer adsorbed on PS and PSox, between 30 min and 2 h, express as % of the amount adsorbed after 30 min. The collagen concentration was 40 $\mu\text{g/ml}$. Absolute experimental and computed values in Chapter II.1, Table II.1.1.

	PS %	PSox %
	I/II	I/II
desorption due to exchange	17/26	-0.5/-7
amount not subject to desorption	54/54	71/80

^I First set of data.

^{II} Second set of data obtained independently.

appeared however, to be less resistant to AFM probe damage, pointing to weaker collagen-PS compared to collagen-PSox interactions. These observations were tentatively explained in terms of higher collagen molecules mobility on PS compared to PSox [82]. We have thus tried to evaluate the exchange between molecules adsorbed on PS and PSox substrates and those present in the solution (details in Chapter II.2).

The exchange between the collagen molecules adsorbed on PS and PSox substrates, and collagen molecules present in the solution was quantified using radiochemically labeled collagen (*coll**). In a first step, either *coll** or unlabeled collagen (*coll*) was adsorbed on the substrate during 30 min. In a second step, the previously adsorbed collagen was exposed to the solution of *coll**, *coll* or to pure buffer for the next 1.5 h. Thus, the desorption via exchange (*coll** molecules adsorbed on the substrate *vs coll* molecules present in the solution, or inversely) or dilution (*coll** molecules adsorbed on the substrate *vs* pure buffer) lasted 1.5 h.

Table I.2 presents the relative amount of collagen desorbed via exchange and the relative amount not subjected to desorption. Collagen desorption via exchange between the adsorbed molecules and those present in the solution took place on PS but was negligible on PSox. The proportion of collagen adsorbed after 30 min which is not subject to subsequent desorption (whether by dilution or exchange) is higher on PSox compared to PS.

The radiochemical measurements (Table I.2) help to understand the difference between PS and PSox. While the amount of adsorbed collagen is higher on PS compared to PSox, the collagen molecules adsorbed on the former are

more readily involved in an exchange with molecules present in the solution. This reveals a higher molecule mobility which explains the enhanced tendency to form fibrils in the adsorbed phase. This observation, together with faster adsorption [29] and higher apparent collagen affinity [83] on PS compared to PSox, can be tentatively rationalized by considering that collagen molecules are bound to PSox through a higher number of contact points than to PS. The possible role of interactions between collagen molecules and PSox chains which become hydrated in an aqueous environment [78, 84] should also be kept in mind.

1.3.2 Influence of the rate of collagen supply to the interface

Keeping in mind previous considerations on hydrophobic substrates, collagen mobility at the liquid/solid interface, collagen affinity toward the surface, as well as collagen ability to form layers with fibrillar character are always enhanced compared to hydrophilic substrates. As an attempt to determine mechanisms which may be responsible for such collagen behavior on hydrophobic substrate, two different ways of supplying collagen to the interface, either slow and progressive or quick and massive were realized. In both procedures collagen was adsorbed from 0.1, 0.4, 7, 40 and 100 $\mu\text{g/ml}$ solutions on PS substrate (details in Chapter II.2).

In the first way, called ‘one step’ (‘os’) method, five PS samples were immersed in a collagen solution of a given concentration for 24 h. In a second way, called ‘step by step’ (‘sbs’) method, the concentration of the collagen solution was progressively increased. Therefore, all samples were incubated at the lowest investigated collagen concentration for 24 h. One sample was then withdrawn and the other samples were incubated at a consecutive collagen concentration for the next 24 h. One sample was then withdrawn and the procedure was repeated, increasing the collagen concentration at each step till the last investigated concentration was reached (details in Chapter II.2).

It was expected that adsorption of collagen on PS, following the ‘sbs’ procedure could favor a stronger attachment of collagen molecules to the surface (more time and space for molecules to reorganize) compared to the ‘os’ procedure, and thus could impede the formation of fibrils in the adsorbed phase,

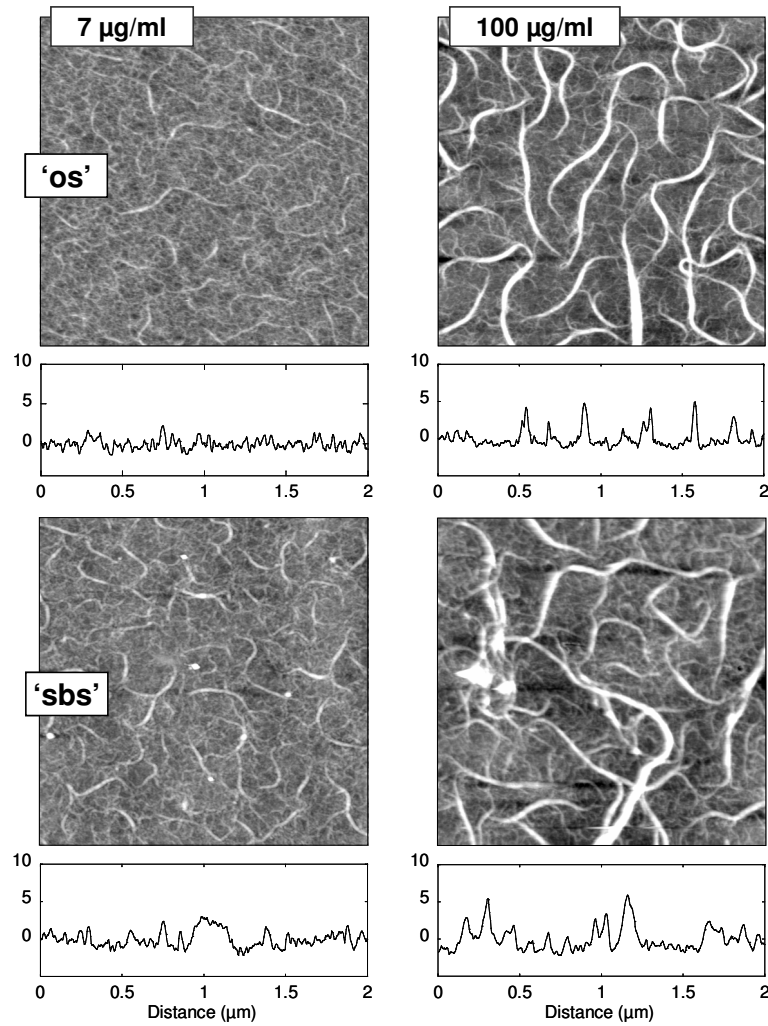


Figure I.15: Representative AFM tapping mode topographic images ($2\ \mu\text{m} \times 2\ \mu\text{m}$; z-range 7 nm) recorded in air on PS substrates after 24 h of collagen adsorption from solutions at a final concentration of 7 and 100 $\mu\text{g}/\text{ml}$. The adsorption procedure was either 'one step' (top) or 'step by step' (bottom). The cross-sections were taken in the middle of the non-flattened images.

mimicking the collagen behavior on PSox substrate.

Representative AFM images recorded in air on collagen layers adsorbed according to the ‘os’ and ‘sbs’ methods are presented in Figure I.15. Using a $7\text{ }\mu\text{g/ml}$ collagen concentration, small fibrillar structures (height about 3.5 nm) were observed. At high collagen concentration ($100\text{ }\mu\text{g/ml}$), broader, longer and higher (about 7 nm) fibrils appeared. For the $0.1\text{ }\mu\text{g/ml}$ concentration, the image differed from that obtained on the bare substrate by very small filamentous features. The concentrations of 0.4 and $40\text{ }\mu\text{g/ml}$ showed intermediate morphologies. The AFM images of the collagen layers revealed a similar topography for both adsorption procedures. Also results of power spectral density (details in Chapter II.2, Figure II.2.9) performed on topographic images obtained according to the ‘os’ and ‘sbs’ adsorption methods, providing surface roughness as a function of the spatial wavelength, did not reveal a significant difference between ‘os’ and ‘sbs’ adsorption procedures. In a complementary fashion, XPS surface analysis of adsorbed collagen layers confirmed that no distinct difference was observed according to the procedure (at $100\text{ }\mu\text{g/ml}$, the N/C ratio reached the value of 0.15 and 0.17 for the ‘os’ and ‘sbs’, respectively).

The supply of collagen molecules to the interface in a slow and progressive way (‘sbs’) thus led to the same surface layer organization as the supply provided by a quick and massive procedure (‘os’). The ‘sbs’ procedure, contrary to the expectations, did not hinder the formation of fibrils in the adsorbed phase. The reorganization within the adsorbed phase on PS was probably too quick to be differentiated by the two different adsorption procedures. It would have been preferable to compare ‘os’ and ‘sbs’ using PSox rather than PS, since the mobility of the molecules is lower on PSox compared to PS. The question raised in this study, why collagen exchanges and reorganizes more readily on more hydrophobic surface is not understood yet.

I.4 Influence of the nature of collagen solutions

I.4.1 Collagen source

In order to investigate the interplay between collagen-collagen and collagen-substrate interactions, the influence of the properties of the collagen solution was examined by changing the collagen source. Three different sources of type I collagen (from calf skin) were examined with a particular attention to the formation of collagen aggregates in solution and upon a contact with the substrate (details in Chapter II.2). All collagens (called RD, AB and USB) were purchased as solutions in acetic acid. The rate of collagen aggregation in solution upon aging at 37 °C was monitored by recording absorption spectra and measuring the absorbance at 400 nm [85]. The adsorbed collagen layer organization (after 10 min and 24 h adsorption) was probed by means of AFM in tapping mode in air conditions, using PS as a substrate.

AFM images (Figure I.16) revealed the collagen layer organization to be comparable in the case of RD and AB collagen and drastically different in the case of USB collagen. After 10 min adsorption, fibrillar structures with a thickness of about 5 nm were observed for RD and AB collagens, while a perforated layer with a hole depth of 1 to 2.5 nm was created with USB collagen; no fibrils were observed at this stage. After 24 h adsorption, RD and AB collagen fibrils showed a small evolution compared to the structures formed after 10 min adsorption. A drastic change was noticed in the case of USB collagen. Large fibers (height of about 80 nm) surrounded by a rather smooth layer with small aggregates appeared (Figure I.16, right bottom image). Additionally, cross-sections taken through such a single fiber revealed a periodicity with a distance between two valleys in the range of 62 to 74 nm. The optical density of collagen solutions (Table I.3) was comparable for RD and AB, and was much higher for USB collagen. Additional studies, where the USB collagen fibers were either adsorbed (40 $\mu\text{g}/\text{ml}$ for 24 h) or deposited (40 $\mu\text{g}/\text{ml}$ solution aged for 24 h; the collagen suspension was collected and delivered on a substrate) onto a substrate from the same conditions resulted in very similar appearance of large fibers (details in Chapter II.2, Figure II.2.5). From these observations it may be concluded that the large fibers observed for USB collagen were formed in the

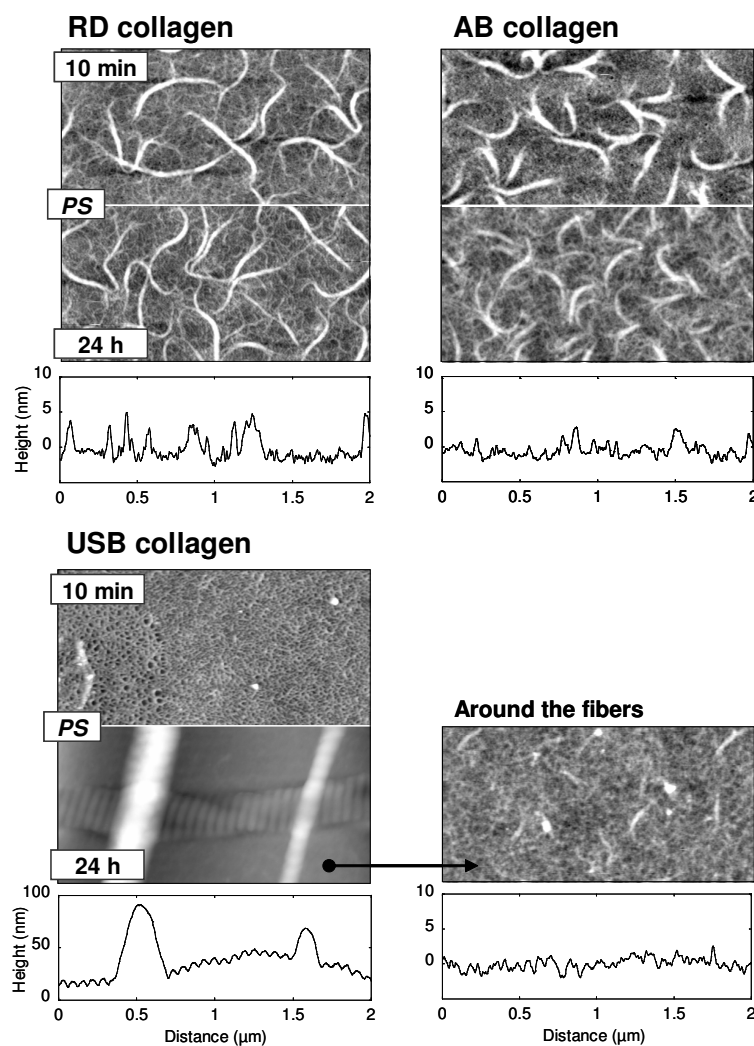


Figure I.16: Representative AFM tapping mode topographic images ($2\ \mu\text{m} \times 1\ \mu\text{m}$; z-range 7 nm except for USB collagen fibers, z-range 60 nm) recorded in air after adsorption of RD, AB and USB collagen ($40\ \mu\text{g}/\text{ml}$) on PS surfaces for 10 min (top half image) and 24 h (bottom half image). The cross-sections were taken in the middle of the non-flattened images. Right bottom side of the figure: detailed image of the background around the collagen fibers.

Table I.3: Absorbance values measured at 400 nm for RD, AB, and USB type I collagen from calf skin; the solutions (40 and 150 $\mu\text{g/ml}$) were aged for 15 min, 30 min, 6 h, 1 day, 2 days and 7 days.

concentration ($\mu\text{g/ml}$)	aging time	absorbance at 400 nm		
		RD ^a	AB	USB
40	0.5 hour	-	0.01	0.01
	6 hours	-	0	0.01
	1 day	-	0.01	0.12
150	0.25 hour	0.01	-	0.10
	0.5 hour	-	0.01	0.31
	6 hours	-	0.01	b
	1 day	-	0.10	-
	2 days	0.06	-	-
	7 days	0.13	-	-

^a From Dupont-Gillain et al. [85]; measured at pH 7.5.

^b The measurements were not reproducible; large, elongated collagen aggregates were observed in solution.

aqueous phase, and settled onto the substrate afterward. Moreover, collagen aggregation in solution competed with adsorption, *i.e.* more aggregated solutions behaved like less concentrated solutions, regarding the adsorbed amount and fibril formation at the interface (Figure I.16, right bottom image). The same trend was reported before for RD collagen under conditions provoking aggregation [85].

Despite the fact that they shared the same origin (calf skin), the intrinsic characteristics of the different collagen sources and thus the tendency to form aggregates in aqueous and/or adsorbed phase strongly differed (RD and AB *vs* USB collagen). The properties of collagen solutions may be influenced by the different factors.

(i) The collagen extraction process. According to the literature, collagen extraction from human or animal species may be performed in three solvents: in neutral salt, in diluted organic acid or in enzymatic solutions [86]. For RD [86] and USB [87] collagen, the extraction was performed in diluted acetic acid. Its role is to hydrolyze and solubilize aggregates. However, the proportion of monomers with respect to aggregates made by covalent cross-linking, depends not only on the extraction and purification process. The tissue age and organ from which it is extracted also plays an important role. Thus, the solution composition in regard to monomers *vs* aggregates content could differ from a

collagen solution to another, since pure monomer solutions are very difficult to obtain [88].

(ii) The proportion of the collagen monomers with intact nonhelical ends (telopeptides). It was demonstrated that the lack of telopeptides slows down the collagen aggregation *in vitro* [89,90].

(iii) The content of type III collagen. The *in vitro* self-assembly study of a collagen mixture containing type I and type III species, indicated that the fibril diameter decreased when the participation of type III collagen in the mixture increased [91]. The content of type III collagen was known for RD ($\sim 5\%$) and AB ($\sim 10\%$), but was unknown for the USB specimen.

A significant difference was observed between collagen fibrils formed at the interface and the fibers formed as a result of aggregation in solution, and subsequently deposited onto the surface. The latter were always large fibers (height ~ 100 nm) and possessed a striking regular band pattern with a valley to valley periodicity of the order of 62 to 74 nm and a valley to peak depth of 3 to 6 nm, as shown in Figure I.17 (right images, sections c and d). The 67 nm periodicity on collagen fibers was first observed by electron microscopy [21] and more recently was also visualized by AFM [92]. The observation of the necks with a well defined periodicity, marked by a 3 to 6 nm lower height may be attributed to the combination of an ordered lateral association of the molecules due to acid-base interactions, and to the gap between molecules aligned axially (schematic representation in Introduction, Figure I.2c), according to the model proposed by Hodge [93]. In contrast, the fibrils formed on the surface were always small aggregates with a height of about 10 nm (Figure I.17, left image) made by assembly of smaller entities anchored at the surface (highlighted by the dotted circle). This observation, as well as the importance of branching between the fibrils, indicates that small structures are formed parallel to the surface. Detailed observation of the cross-over points (Figure I.17, sections a and b) indicated an intermingling of crossing fibrils. The relief observed along a given fibril and the morphology of fibril ends reveal that they are far from hair-like but rather are the result of an aggregation process at different scales, started from molecules lying along the surface. Moreover, no periodicity along the fibrils could be observed. The axial and lateral organization of collagen molecules was too low to allow such periodicity to be created, while the small size of the fibrils produced in the adsorbed phase could be attributed to the

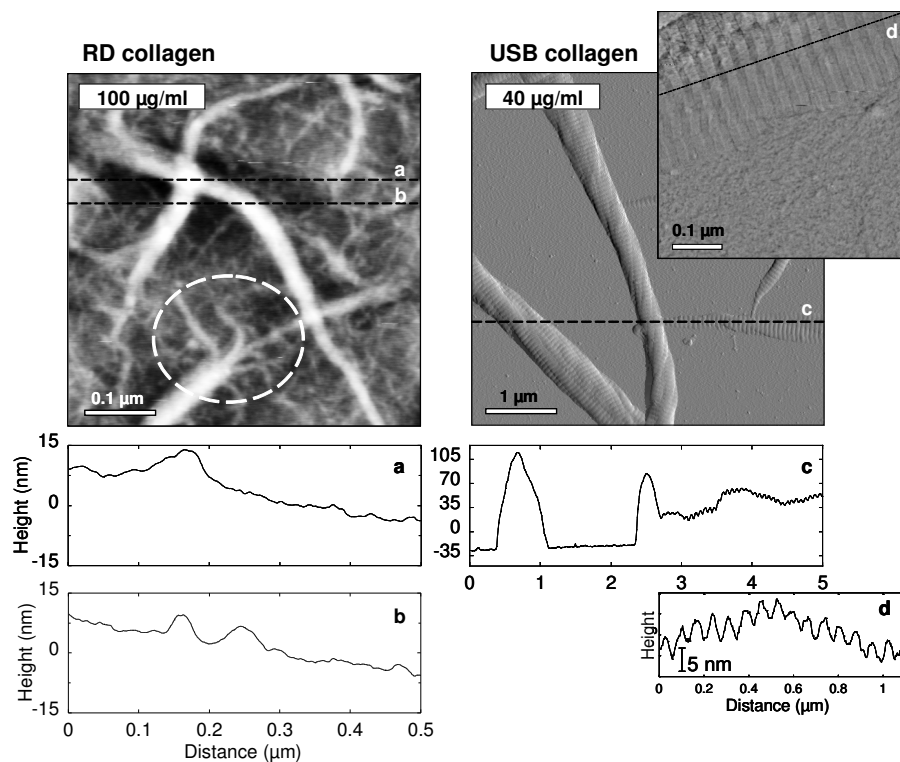


Figure I.17: Representative AFM tapping mode images (scale bars indicated on each image) recorded in air on PS, either after 24 h of RD collagen adsorption from a 100 $\mu\text{g}/\text{ml}$ solution (left, topography image) or after 24 h contact with a USB collagen at 40 $\mu\text{g}/\text{ml}$ solution (right, amplitude images). Collagen fibers are presented using AFM amplitude images, in order to better visualize the periodicity along their length. All cross-sections were taken from height images as indicated by the dashed lines.

reduced mobility of adsorbed collagen in comparison with molecules of solution.

Very distinct behaviors have thus been detected for solutions of type I collagen from different suppliers. One of them lead to formation of large fibers in solution (USB), whereas two others showed the formation of fibrils at the surface (RD and AB).

I.4.2 Collagen thermal denaturation

The ‘resistance’ of collagen solutions to denaturation upon temperature rise is dictated by the properties of extracted solutions, *i.e.* is dependent on collagen species [94]. In this work, the influence of heat-denaturation on the adsorbed collagen layer organization was investigated using collagen solutions which were preheated at a temperature of 40 °C (*den40*), 60 °C (*den60*) and 90 °C (*den90*) for 30 min (details in Chapter II.3). For comparison, layers created using native collagen (*native*) are also presented. The *in situ* kinetics of collagen adsorption from 40 $\mu\text{g/ml}$ solutions on PS was followed using QCM-D; the results were modeled to determine the layer thickness as well as its viscoelastic properties. AFM was used to observe the surface nanometer-scale collagen organization. X-ray photoelectron spectroscopy (XPS) allowed determining the surface chemical composition.

The AFM images (Figure I.18) showed that the tendency to form fibrils in the adsorbed phase was very marked for native collagen and decreased when the pretreatment temperature of the solutions increased: *native* > *den40* > *den60* > *den90*. In absence of pretreatment, the formed fibrils created a dense pattern with a fibril thickness up to 6 nm, in agreement with previous observations [29, 31]. Moderate heating of collagen solutions (40 and 60 °C) caused a diminution of fibrillar structures and appearance of small, short globules instead (20 to 30 nm in diameter and about 1 nm thick). For *den90* collagen, only small, globular structures were visible. AFM analysis under water on native and denatured collagen layers revealed structures shape and density similar to those observed in air (details in Chapter II.3, Figure II.3.1). Drying the heat-denatured collagen layers (*den40* and *den90*; in a smaller extent *den60*) resulted in the formation of discontinuous films. The cross-sections taken through the holes showed a thickness of the adsorbed layers being about 4 nm (details in Chapter II.3, Figure II.3.2).

The evolution of the N/C molar concentration ratio as a function of adsorption time, measured by XPS on collagen native and denatured layers adsorbed on PS, is shown in Figure I.19. Note that the nitrogen signal comes only from the collagen, while the carbon signal originates from both the collagen layers and the support. In all cases, the N/C ratio reached a plateau after about 30 min adsorption and was the highest for native collagen (0.18) and the low-

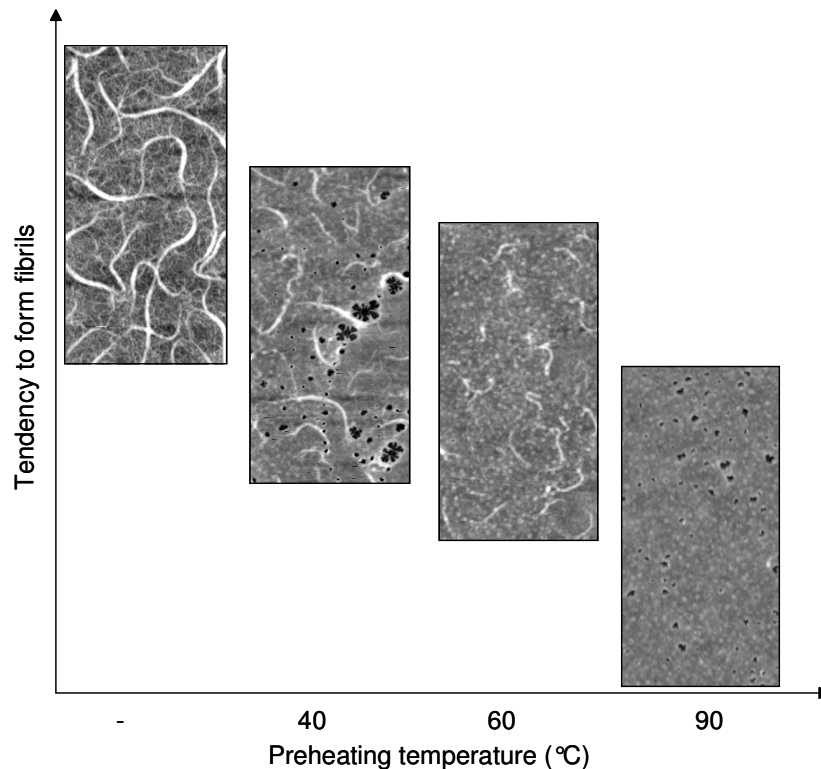


Figure I.18: Representative AFM tapping mode topographic images ($1\ \mu\text{m} \times 2\ \mu\text{m}$, $z = 7\ \text{nm}$) recorded in air on PS substrate after 2 h of adsorption of collagen not preheated or preheated at 40° , 60° and 90°C , using a concentration of $40\ \mu\text{g/ml}$.

est for collagen *den90* (0.10). Intermediate values were reached for collagen *den40* and *den60*. The O/N ratios determined at different adsorption times for the four types of collagen solutions were in the range of 1.10 to 1.16. As points of comparison, the N/C and O/N ratios measured on a thick film of native collagen (made by drop deposition) were 0.26 and 1.01, respectively. Thick films of denatured collagen showed similar values: $\text{N/C} = 0.25$ and $\text{O/N} = 1.03$. Molar ratios computed from the amino acid composition of type I calf skin collagen are $\text{N/C} = 0.32$, $\text{O/N} = 1.09$ [88]. The high resolution C_{1s} peaks (details in Chapter II.3, Figure II.3.4) recorded on native and denatured collagen adsorbed layers confirmed that the contribution of the peptide link decreased in the following order: *native* $>$ *den40* $\geq$ *den60* $>$ *den90*, in agreement with

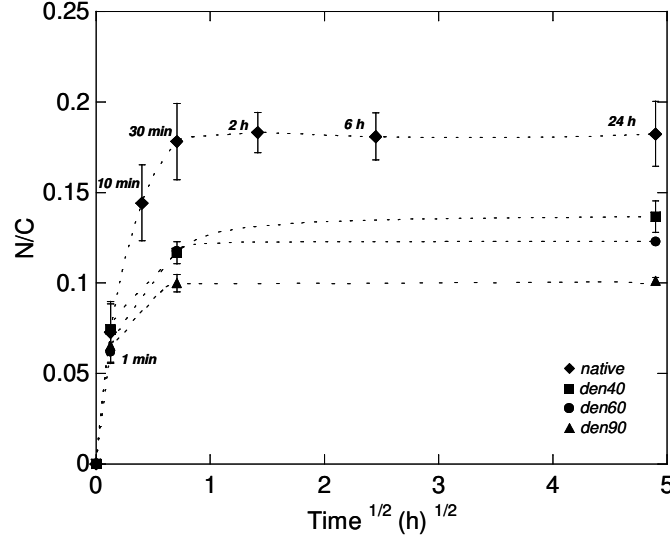


Figure I.19: N/C ratio determined by XPS on PS substrates after adsorption for increasing time at a collagen concentration of 40 $\mu\text{g/ml}$: native collagen (diamonds), collagen preheated at 40 °C (squares), at 60 °C (circles) and at 90 °C (triangles). The presented results are the average from four (native collagen) or three (preheated at 40 °C and 90 °C collagen) independent sets of data. The error bars show the standard deviation. One set of data is presented for collagen preheated at 60 °C.

the N/C ratio. Thus, the XPS surface analysis of the heat-denatured collagen layers, together with AFM imaging showed that the denaturation had a strong impact on the collagen layer organization and, or on the adsorbed amount.

The effect of thermal denaturation on adsorbed collagen layers was also investigated using the QCM-D technique. Raw data, *i.e.* frequency (f) and energy dissipation (D) shifts as a function of adsorption time (details in Chapter II.3, Figure II.3.5) indicated a clear difference according to preheating temperature. After 10 h adsorption, $\Delta f/3$ and ΔD values (measured at third harmonic) were about -560 Hz, -230 Hz, -80 Hz and 125×10^{-6} , 55×10^{-6} , 17×10^{-6} , respectively for *native*, *den40* and *den90* collagen. As a point of comparison, much lower values of Δf and ΔD were measured for human serum albumin and for hemoglobin (about -11 Hz and 0.6×10^{-6} , and about -52 Hz and 1.4×10^{-6} , respectively), at fundamental harmonic [66]. Keeping in mind that the f changes may be related to the adlayer thickness changes, while f

Table I.4: Three ways to estimate the maximum thickness (h), viscosity (η) and shear modulus (μ) reached by the adlayer of collagen (native and denatured at 40 and 90 °C): (i) Sauerbrey relation, (ii) best fitting based on the Voigt model and (iii) chart shown in Figure I.20^c.

	native			den40			den90		
	h (nm)	η (10 ⁻³ Pa.s)	μ (kPa)	h (nm)	η (10 ⁻³ Pa.s)	μ (kPa)	h (nm)	η (10 ⁻³ Pa.s)	μ (kPa)
<i>Sauerbrey relation</i>									
$n=3$	95	-	-	42	-	-	13	-	-
$n=5$	77	-	-	33	-	-	12	-	-
$n=7$	65	-	-	28	-	-	11	-	-
<i>best fitting^a</i>									
$n=3$ and 5	165	4.3	130	140	2.1	7	56	1.4	8
<i>chart^b</i>									
$n=3$	220	4.2	10	135	2.2	10	47	1.6	10
	175	4.4	100	80	2.5	100	26	2.4	100

^a The best fitting values were obtained with the data of overtones 3 and 5.

^b The chart was generated for overtone 3; h and η were evaluated for μ set at 10 and 100 kPa.

^c Collagen adsorption was performed on PS substrates from 40 $\mu\text{g/ml}$ solution and the considered adsorption duration was 10 h. In all computations, the collagen adlayer density was set at 1050 kg/m³.

and D shifts may be related to the variation of the adlayer viscoelasticity, raw Δf and ΔD records thus indicated that a thicker and more viscoelastic adlayer is created by native compared to heat-denatured collagen. Moreover, the collagen layers formed upon drastic denaturation (*den90*) were closer to the layer characteristics of smaller proteins, as *e.g.* albumin ($3 \times 8.2 \times 8.5 \text{ nm}^3$) [95] or hemoglobin ($4.5 \times 4.5 \times 6.4 \text{ nm}^3$) [96]. The denatured collagen thus behaves more like a globular protein, in line with AFM observations.

QCM-D data were analyzed using the three approaches described in section I.2.1. and the results are summarized in Table I.4. They were thus: (i) calculated on the basis of proportionality equation between the frequency shift and the mass-uptake (Sauerbrey relation), (ii) best fitted on the basis of the viscoelastic model, where simultaneous changes in f and D are related to the changes in adlayer thickness, h , viscosity, η and shear modulus, μ (best fitting), and finally (iii) evaluated by examining the sensitivity of the measured Δf and ΔD to the adlayer parameters, namely h , η , and μ (charts).

The adlayer thickness for *native*, *den40* and *den90* collagens calculated from

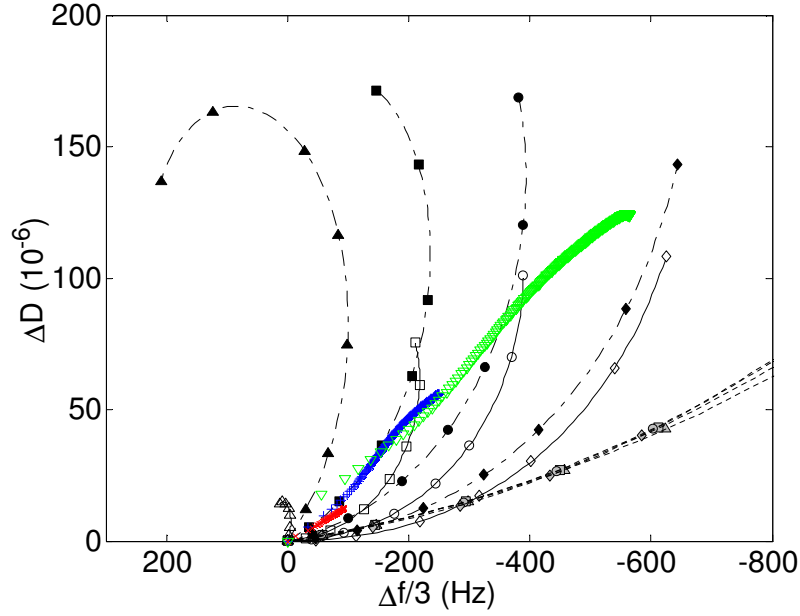


Figure I.20: ΔD vs Δf charts computed for a collagen adlayer, considering a density of 1050 kg/m^3 , a thickness of 0.1, 10, 25, 50, 75, 100, 150 and 200 nm (set of 8 dots in the increasing order on each spiral curve), a viscosity of $1 \times 10^{-3} \text{ Pa.s}$ (triangles), $2 \times 10^{-3} \text{ Pa.s}$ (squares), $3 \times 10^{-3} \text{ Pa.s}$ (circles), $5 \times 10^{-3} \text{ Pa.s}$ (diamonds) and a shear modulus of $1 \times 10^4 \text{ Pa}$ (open symbols, continuous curve), $1 \times 10^5 \text{ Pa}$ (black symbols, long and short strokes), $1 \times 10^6 \text{ Pa}$ (gray symbols, short strokes). In colors, plots of experimental data obtained by QCM-D for native collagen (green downward triangles), collagen denatured at 40°C (blue crosses) and collagen denatured at 90°C (red x-marks) adsorbed on PS from $40 \mu\text{g/ml}$ solution during 10 h.

the third overtone on the basis of Sauerbrey equation was about 95, 42 and 13 nm, respectively. On the other hand, results of best fitting showed similar tendency regarding adlayer thickness as well as viscosity: *native* > *den40* > *den90*, while the shear modulus values seemed to be higher for native collagen and comparable for collagen *den40* and *den90*. However, the results of best fitting have to be taken with caution, since they are not unique. Therefore, the sensitivity of Δf and ΔD to the parameters of h , η , μ was examined (details in Chapter II.3). Figure I.20 presents a chart of dissipation shift (at the third overtone) as a function of frequency shift computed on the basis of model

equations (Introduction, equations I.4 and I.5), with a thickness ranging from 0.1 to 200 nm, a viscosity varying from 1×10^{-3} to 5×10^{-3} Pa.s and a shear modulus between 10 and 100 kPa (see Figure caption). The experimental data for native and denatured collagens are presented in colors. As emphasized in section I.2.1, chart presentations pointed to the non unique solution of adlayer thickness, viscosity and shear modulus obtained from a given experimental pair Δf - ΔD . The determination of the shear modulus values of the adlayer appeared to be highly imprecise.

Table I.4 showed that, whatever the approach used to interpret the QCM-D data, the heat-denaturation led to a strong decrease of the adlayer thickness at the end of the kinetic run. This is in agreement with the work of Silberberg and Klein [97], who also observed a significant decrease of the adsorbed layer thickness of calf skin collagen at pH 7.4, after heat-denaturation. By surface force measurements, where the force acting between two mica plates covered by collagen was measured, they found a collagen layer thickness of 212 nm and 53 nm for native and denatured collagen, respectively. The viscosity of the adlayers obtained at the end of the kinetic run decreased correlatively, as a result of denaturation. This process did however not appreciably affect the relationship between ΔD and Δf over a whole kinetic run, indicating that the relationship between viscosity and thickness was not appreciably modified either.

In situ monitoring of collagen layers revealed that, in native state, collagen formed a layer of a few nm in which fibrils were lying on the surface, as seen by AFM. On the other hand, QCM-D analysis revealed another picture of the collagen layer, sensing a thickness up to about 200 nm, which was attributed to protrusion of molecules or bundles. Their density was probably very low, but high enough to modify noticeably the local viscosity. Note that collagen layers, as seen by QCM-D, are probed together with liquid which is trapped inside the collagen structures. Drastic preheating of the collagen solution (*den90*) prevented fibril formation in the adsorbed phase (AFM) and reduced significantly the molecule protrusion and, thereby, the adlayer thickness and viscosity. The relative decrease of apparent thickness found by QCM-D was higher than the relative decrease of the adsorbed amount deduced from XPS and AFM. Moderate heating (*den40* and *den60*) of collagen solutions led to a partial denaturation. The adsorbed layer properties, with regard to the thickness and

viscosity as well as the AFM images were intermediate between native and completely denatured collagen.

I.5 Conclusions

I.5.1 Main achievements

Adsorbed collagen. This work provides a better characterization of the supramolecular organization of the collagen layers adsorbed on polymers and an information on factors which affect collagen layer organization. It also contributes to a deeper understanding of the mechanisms which lead to such organization.

Collagen adsorption on polystyrene (PS) and plasma oxidized polystyrene (PSox), as model hydrophobic and hydrophilic substrates, respectively, revealed that formation of the adsorbed phase had a complex character. At an initial stage of adsorption (*i.e.* short time and/or low solution concentration) the adsorbed layers consist of nanofibrillar structures on PS, and have a rather globular character on PSox. The increase of time and concentration causes the evolution of the collagen layer organization into bigger fibrils. According to the methods used and their respective sensitivity, collagen layers adsorbed on PS and PSox consist in two parts:

- AFM reveals the presence of a mat of collagen in which fibrils are formed. The density and size of fibrils increase with time and collagen concentration. Fibril formation occurs more readily on PS compared to PSox.
- QCM-D reveals a thick (up to 200 nm) collagen adsorbed layer containing more than 96 % water. As the collagen concentration or adsorption time increases, both the adlayer thickness and viscosity increase. This adlayer develops faster and to a greater extent on PS compared to PSox, in agreement with a higher adsorbed amount measured by radiochemical measurements.

The combination of AFM and QCM-D thus showed that the collagen adlayer consists of (i) a thin mat in which fibrils develop depending on substrate, concentration and time, (ii) a thick overlayer containing protruding molecules or bundles which are in very low concentration but modify noticeably the local viscosity and play a major role in the interaction with an approaching surface.

Radiolabeling measurements showed that collagen molecules are more easily displaced when adsorbed on a hydrophobic substrate compared to a hydrophilic substrate. This may explain that fibril formation occurs more readily on the more hydrophobic substrate but is in contrast with the apparent higher affinity of collagen with such hydrophobic surface. The possible explanation of this paradox by the quick formation of a dense layer of collagen molecules forming a small number of contact points with a hydrophobic surface could not be demonstrated by a comparison of adsorption procedures leading to a progressive adsorption, and to a quick and massive adsorption, respectively. Further investigations where collagen mobility at the surface is directly probed, are needed to get more insight into the influence of surface hydrophobicity on collagen molecules assembly in the adsorbed phase.

The tendency of collagen to form fibrils when adsorbed on poly(4-vinyl phenol) (PSOH) is intermediate between PS and PS_{ox}, in agreement with the sequence of overall substrate hydrophobicity, as revealed by static and dynamic wetting measurements. On the other hand, observations of the underneath adsorbed layer with PMMA as a substrate, point to the possible influence of the nature and concentration of particular polar functions.

Comparing collagen sources revealed different modes of aggregation in the adsorbed phase or in the solution. Three different forms of collagen features may be distinguished:

- Structures formed in the solution appear as large fibers with the characteristic banding pattern, typical for the family of fibrillar collagens.
- Structures assembled in the adsorbed phase, appear as rather small fibrils without a characteristic periodicity.
- Smaller features, which give rise to the growth of the fibrils are formed in close contact with the substrate and allow the fibril anchoring to the substrate.

Collagen aggregation in the solution is a process competing with adsorption. More aggregated solutions indeed behave like less concentrated solutions regarding the adsorbed amount and fibril formation in the adsorbed phase.

As a result of drastic thermal denaturation, the ability of collagen to assemble into fibrils is lost and the number of protruding molecules responsible for a local higher viscosity is strongly reduced. The adsorbed layer appears essentially as a monolayer of flattened coils. Moderate denaturation leads to

an adsorbed phase which show properties intermediate between those observed with native and extensively denatured collagen, regarding both the ability to form fibrillar structures and the layer thickness and viscosity.

Methodological developments. This work shows that the combination of radioassays, AFM monitoring under water and QCM-D sensing *in situ* and in real time gives a detailed picture of the adsorbed phase: (i) adsorbed amount (layer thickness in air), (ii) organization of the layers, and (iii) layer thickness including trapped water, and viscosity. In a complementary fashion, analysis of collagen layers in the dry state by XPS and AFM in tapping mode gives precise information about surface chemical composition and adsorbed layer organization. The combination of different methods is needed to get an image approaching the reality of complex interfaces.

Construction of ΔD vs Δf charts in QCM-D data modeling permitted to evaluate the sensitivity of the data to the parameters characterizing the adlayer, *i.e.* thickness, viscosity and shear modulus. It must be emphasized that interpretation of the QCM-D data, which is based on fitting physical quantities according to a model, has to be performed very carefully and requires the examination of the sensitivity of the fitted data to the fitting parameters.

I.5.2 Future prospects

Directing collagen assemblies on a substrate. The literature reports that factors such as substrate chemistry and topography or controlled conditions of adsorption may specifically trigger collagen assembly on the solid/liquid interface. For instance, substrates which were heterogeneous with respect to the surface hydrophobicity (tracks of methyl-terminated alkylsilanes drawn in an oligo(ethylene glycol)-terminated matrix), forced adsorbed collagen molecules to align along the hydrophobic tracks, owing to their width (30-90 nm) which is small compared to the length of collagen molecules [38]. On the other hand, surfaces modified by nanometer-scale protrusions hindered the collagen assembly into fibrous structures [32]. Adsorption under a hydrodynamic flow aligned collagen microfibrils in a given direction on a mica substrate [98]. The methods and know how established in the present work have may be used, as a contribution to design polymer substrate that could interfere with collagen self-assembly

process.

Preliminary results were obtained following this line of thinking by using, topographically micro- and nanostructured poly(methyl methacrylate) (PMMA) substrates provided by N. Gadegaard (University of Glasgow) obtained by embossing (details in Appendix II.A). The aim of the substrate microstructuration was to guide the collagen molecules to align along the grooves, decreasing the possibilities of lateral movements. The second level of sample structuration, *i.e.* nanogrooves, was created in order to encourage collagen molecules to form fibrils with characteristic banding pattern.

AFM topographic images recorded in air on PMMA substrate are presented in Figure I.21 (top images). The large scale image (top left) recorded with scanning direction perpendicular to the microgrooves, revealed that the distance between two adjacent grooves was around $5\text{ }\mu\text{m}$, the depth of the grooves was 70 to 80 nm, while the width of one microgroove at the level of the surface was 1.6 to $1.8\text{ }\mu\text{m}$. The microgrooves appeared thus as troughs, with very gentle slope of the edges. Moreover, their dimension and the shape were repetitive and regular over a large area. The small scale image (top right) recorded parallel to the microgrooves revealed the presence of the nanogrooves located in the bottom of the microgrooves. Their depth and periodicity, measured on averaged cross-sections (right bottom) were about 5 nm and 76 nm, respectively. The length of the nanogrooves was of the order of 200 to 300 nm. The depth of the nanogrooves was about 5 nm; their periodicity ($\sim 76\text{ nm}$) was comparable to the periodicity observed along collagen fibers (67 nm [92], Figure I.21; bottom image).

AFM topographic images recorded in air on collagen layers adsorbed (solution concentration $300\text{ }\mu\text{g/ml}$; extended to 100 and $600\text{ }\mu\text{g/ml}$ in Appendix II.A) on structured PMMA substrate are presented in Figure I.22 together with cross-sections. The top image was recorded scanning perpendicular to the groove direction; the bottom image was obtained by scanning parallel to the groove direction. Both images were flattened using 3-order polynomial (Appendix, Figure II.A.6) in order to gain information of the collagen layer organization inside the groove. The results pointed to the almost unchanged depth of the microgrooves (70 to 80 nm as on bare substrate) after collagen adsorption. The presence of the microgrooves showed no effect on the collagen organization. No discontinuity in the adsorbed phase nor alignment of colla-

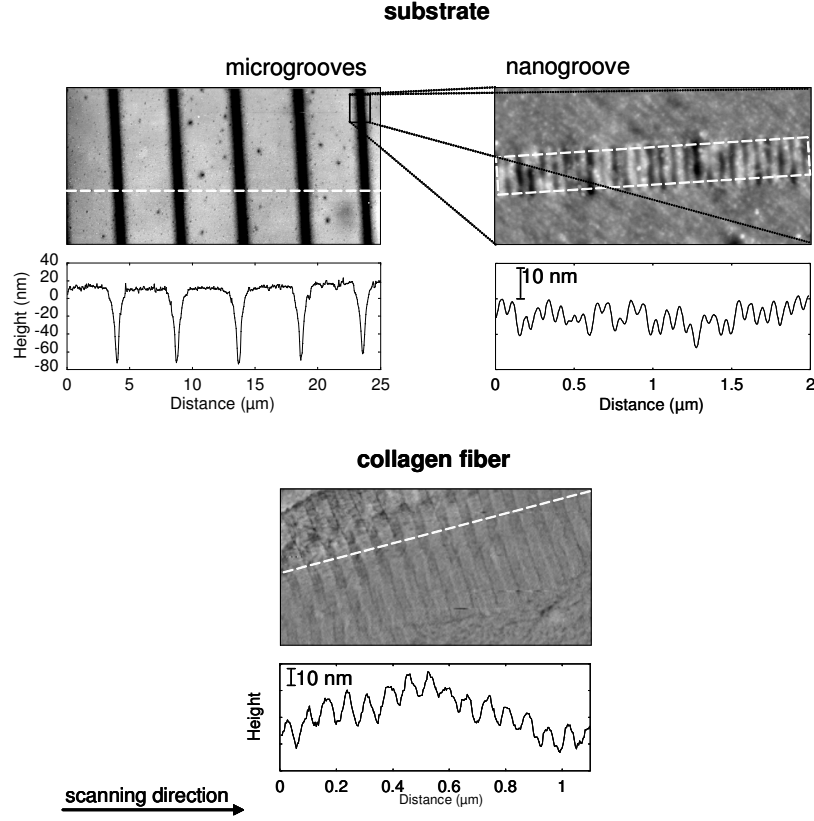


Figure I.21: Representative AFM tapping mode images recorded in air on grooved PMMA substrate (top) and along collagen fibers (bottom). Images size: $25\ \mu\text{m} \times 12.5\ \mu\text{m}$, $z = 60\ \text{nm}$ (top left; topography); $2\ \mu\text{m} \times 1\ \mu\text{m}$, $z = 20\ \text{nm}$ (top right; topography); $1\ \mu\text{m} \times 0.5\ \mu\text{m}$ (bottom; amplitude). The cross-sections were taken from the topographic, non-flattened images as indicated by the dashed lines. The cross-section shown in the right top image is the average of all cross-sections contained in the area indicated by the dashed rectangle. Scanning direction as indicated.

gen fibrils in a preferred direction was observed. Aggregates of crossing fibrils were observed as on flat PMMA surface areas, covering almost entirely the nanogrooves located in the bottom of the microgrooves (dashed rectangle on the bottom image).

These preliminary results of collagen adsorption on micro- and nanostructured PMMA substrate showed that collagen layers adsorbed from high solution

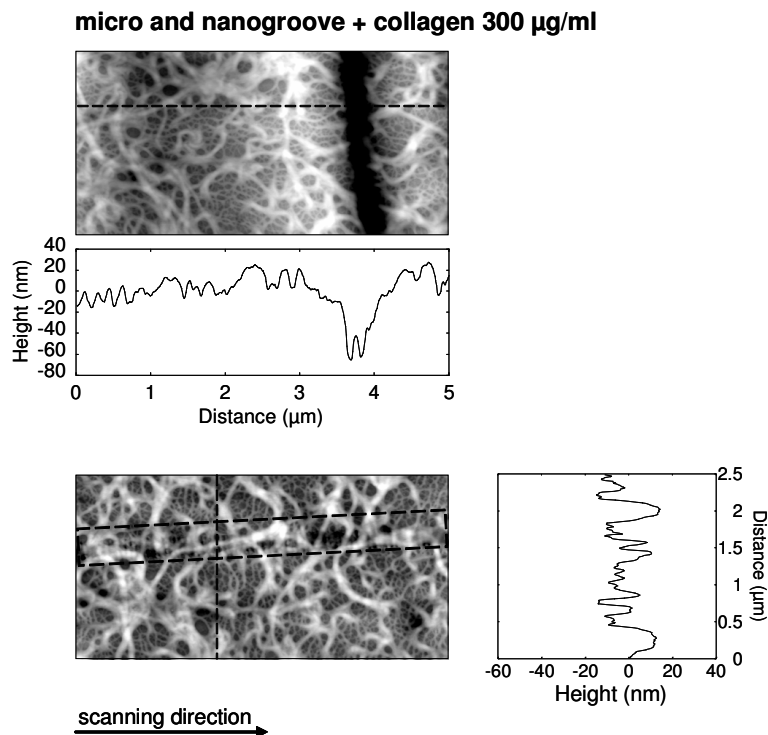


Figure I.22: Representative AFM tapping mode third-order flattened topographic images ($10\ \mu\text{m} \times 5\ \mu\text{m}$; $z = 60\ \text{nm}$ for height image) recorded in air of collagen layers adsorbed from $300\ \mu\text{g/ml}$ solution on grooved PMMA substrate. The top image was recorded while scanning perpendicular to the grooves direction. The bottom image was recorded while scanning parallel to the groove direction. The cross-sections were taken as indicated by the dashed lines. The rectangle shows the position of the microgroove with the nanostructuration on the bottom.

concentrations (100, 300 and $600\ \mu\text{g/ml}$; Appendix II.A) were not affected by the substrate topography. In the future, it would be interesting:

- to modify the shape of the microgrooves: $\sqcup$ -shaped grooves with sharper edges would favor surface discontinuity and alignment of the molecules along the microgrooves;
- to perform collagen adsorption from lower solution concentrations: the nanostructuration of the substrate may indeed have an effect only at the onset of molecules assembly; this may be critical for creating a periodicity

along the collagen fibrils;

- to replace the PMMA substrate by a more hydrophobic polymer (family of fluoropolymers), in order to favor molecular mobility and to facilitate the process of molecular assembly.

Probing molecules mobility on a substrate. One of the questions raised in this research was the collagen molecular mobility. The exchange measurements between the radiolabeled molecules adsorbed and those present in the solution showed a higher exchange in the case of collagen molecules adsorbed on PS compared to PSox. In the selected experimental conditions (solution concentration of $40\mu\text{g/ml}$; time of exchange 1.5 h), the adsorbed collagen was already assembled in fibrils. It would be interesting to monitor the molecular mobility of smaller collagen entities. Adsorption from very diluted collagen solutions may result in smaller collagen aggregates, possibly single molecules [99], and collagen mobility could be measured at the onset of the self-assembly process. Fluorescence recovery after photobleaching may be applied for that purpose [100].

Organization of the adsorbed phase on chemically defined substrates. Studying the collagen layer organization on PS and its homologues (PSOH, PSox), as well as on PMMA, showed that the adsorbed layers have a complex character. On all substrates, collagen assemblies were observed in the form of well-contrasted fibrils. However, the underneath layers were different (smooth on PS, PSOH and PSox, and perforated on PMMA). Thus, a large range of different substrates with well defined nature and concentration of polar groups deserve further comparison. Time-of-flight secondary ion mass spectrometry (ToF-SIMS) may give information about the adsorbed molecule conformation or orientation at the surface in relation with the chemical composition of the latter. Again, studying collagen adsorption from low solution concentrations (*e.g.* $0.05\text{--}7\mu\text{g/ml}$) on such well chemically defined substrates may give better insight into adsorbed phase formation at the onset of molecules aggregation.

Influence of the adsorbed phase organization on cell behavior. This study showed that collagen fibrils formed in the adsorbed phase are the result of aggregation at different scales. The size of the features created upon contact

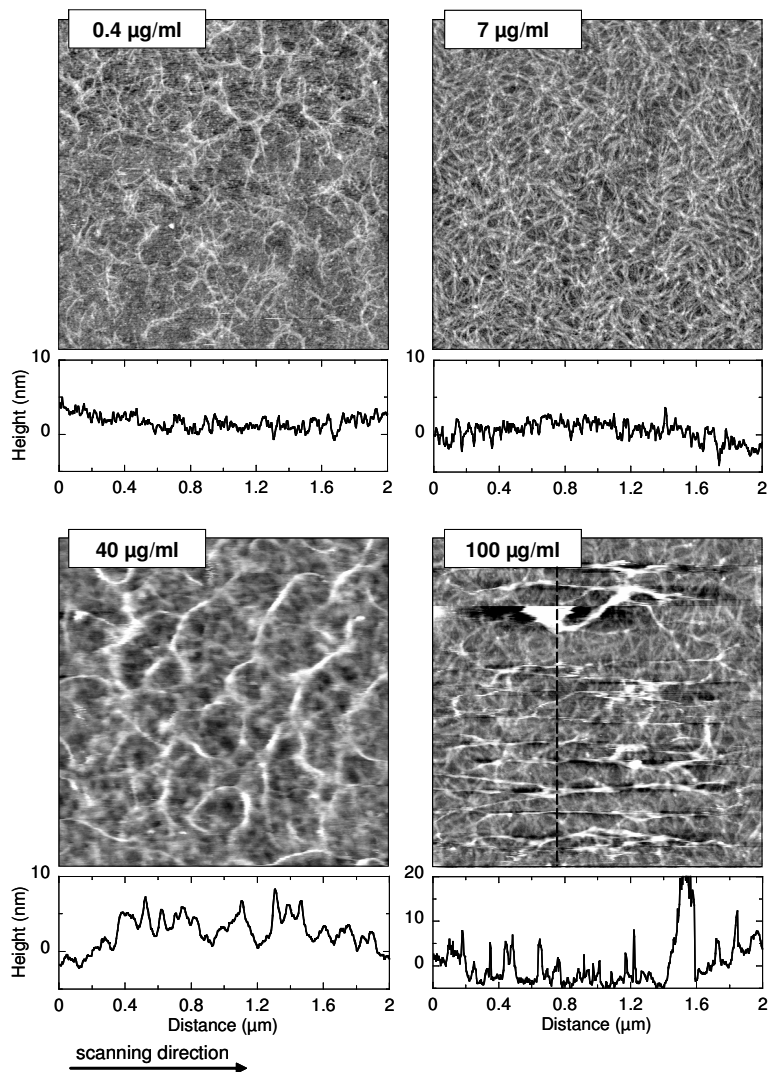


Figure 1.23: Representative AFM tapping mode topographic images ($2\ \mu\text{m} \times 2\ \mu\text{m}$; $z = 5\ \text{nm}$ for top images, and $z = 10\ \text{nm}$ for bottom images) recorded under water after collagen adsorption on a PS sample, increasing the solution concentration ‘step by step’ (30 min each; 0.4, 7, 40 and 100 $\mu\text{g/ml}$). Adsorption was performed at room temperature. The images were taken after rinsing with water. The cross-sections were taken in the middle of the non-flattened images if not indicated.

with the substrate (fibrils) was found to be always smaller than the size of the structures created by aggregation in solution (fibers). Adsorption conditions such as longer time [29], higher solution concentration as well as more hydrophobic character of the substrate, enhanced collagen molecular assembly. Using AFM and QCM-D techniques, it was demonstrated that collagen in the adsorbed phase formed a thin and dense sheet in which molecules were lying along the surface (thickness of few nm), on the one hand, and thick and more diluted overlayer in which molecules or bundles of molecules were protruding into the solution (thickness ~ 200 nm), on the other hand. Such complexity of the collagen adsorbed phase encourages to examine its influence on cell behavior.

In order to distinguish the respective roles of lying and protruding molecules, two kinds of samples could be investigated. Collagen layers with molecules lying on the substrate may be created using relatively low collagen solution concentrations, while the overlayers in which collagen molecules are standing-up may be obtained using higher concentrations. With this idea in mind, preliminary experiments were performed with PS, using an adsorption protocol inspired by the ‘step by step’ method described in section I.3.2. (30 min with $0.4 \mu\text{g/ml}$; same, then 30 min with $7 \mu\text{g/ml}$; same as preceding, then 30 min with $40 \mu\text{g/ml}$; same as preceding, then 30 min with $100 \mu\text{g/ml}$). Figure I.23 presents high magnification tapping mode images recorded under water on samples collected at each adsorption step. In comparison with results obtained following the ‘one step’ procedure (section I.2.1; Figure I.10; details in Figure II.1.3), the results obtained at final concentrations of 7 and $40 \mu\text{g/ml}$ are similar; however, the adsorbed layer obtained with the ‘step by step’ procedure with a final concentration of $100 \mu\text{g/ml}$ was strongly perturbed by the AFM probe (Figure I.23), in contrast with the adsorbed layer examined after 24 h adsorption from a $100 \mu\text{g/ml}$ solution. It thus appears that the images acquired at low collagen concentration show a smooth and thin layer, with a rather robust organization whatever the adsorption protocol, while images recorded after a short adsorption from higher concentrations reveal a thicker layer, very sensitive to AFM probe damage.

A question to be answered concerning cells placed in contact with such collagen layers is: Would the different types of collagen arrangement in the adsorbed phase (lying *vs* standing molecules) influence RGD sequence acces-

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sibility, and thus the density and distribution of sites recognized by cell integrins? This again emphasizes the possible importance of dynamic properties of the adsorbed layer, and its influence on the cell response. AFM-based force spectroscopy (recording force *vs* distance curves) and adhesion mapping [101] using adequately modified probes could provide information at different levels: compressibility of the adsorbed collagen layer, nature of specific adhesion events (rupture length, adhesion force [31]), distribution of adhesion events.

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Part II

Detailed presentation

Chapter II.1

Resolution of the vertical and horizontal heterogeneity of adsorbed collagen layers by combination of QCM-D and AFM

Summary

Collagen (type I from calf skin) adsorption on polystyrene (PS) and plasma-oxidized polystyrene (PSox) was studied, using a quartz crystal microbalance with energy dissipation measurements (QCM-D) and atomic force microscopy (AFM) in tapping mode. Radiolabeled collagen was used to measure the adsorbed amount and the ability of adsorbed collagen to exchange with molecules in the solution. The results show that the collagen adlayer consists in two parts: a dense and thin sheet in which fibrils are formed (directly observed by AFM) and an overlying thick layer (up to 200 nm) containing protruding molecules or bundles which are in very low concentration but modify noticeably the local viscosity. The thickness and viscosity of the semi-liquid adlayer both increase with adsorption time and collagen concentration. Fibril formation near the surface also increases with time and collagen concentration and occurs more readily on PS compared to PSox. Radiochemical measurements show that this may be related to the larger mobility of molecules adsorbed on PS, presumably owing to a smaller number of binding points.

II.1.1 Introduction

Adsorption of proteins on solid surfaces is an important process for many applications such as biomaterial development and biosensor design. In the field of biomaterials, proteins at the interface are the mediators between the solid (implant) and the body (living tissue). Most studies of protein adsorption are concerned with the adsorbed amount and the adsorption kinetics [1–4]. Recent works have emphasized the importance of spatial organization and the conformation of proteins in the adsorbed phase as well as the influence of the substrata in terms of surface topography and chemical composition [5–8].

Type I collagen is an extracellular matrix protein; the molecule is a semi-flexible helix formed by three polypeptide chains (molar mass ~ 300 kg/mol, length ~ 300 nm, diameter ~ 1.5 nm). Non helical parts are found at each end of the molecules (~ 5 nm) [9]. Collagen has the property to assemble and form fibrils in vivo as well as in vitro [10, 11]. Each polypeptide chain contains aminoacid sequences which may be recognized by specific cell receptors [12]. This recognition plays a role in cell adhesion, and further spreading. Furthermore, the organization of collagen in adsorbed phases influences cell adhesion [13, 14]. It was reported that adsorption of collagen from rat tail tendon at low pH was faster on substrates of increasing hydrophobicity (glass, siliconized glass, Teflon) and lead to increasing adsorbed amounts [15]. The amount adsorbed on polyethylene (PE) was higher compared to mica but lower compared to poly(maleic acid) grafted PE [16, 17]. The initial slope of the adsorption isotherms of type I collagen from calf skin is steeper on polystyrene (PS) compared to tissue culture polystyrene (TCPS) [18] and plasma oxidized polystyrene (PSox) [19]; the initial rate of adsorption is also higher on PS than on PSox. AFM study of collagen adsorbed on PS [20], poly(ethylene terephthalate) (PET) [21] and self-assembled monolayers (SAM) of CH_3 -terminated alkanethiols [6] revealed the presence of fibrillar structures. These were not observed on PSox and OH-terminated SAM in the same conditions. The fibrillar structures still develop on PS at adsorption times for which the adsorbed amount is no longer increasing significantly. Moreover, collagen self-assembly also takes places on PSox, if the adsorption time is long enough [19]. It was also demonstrated that the fibrillar structures observed on PS were formed at the surface and did not result from adsorption of aggregates formed in the so-

lution [22]. The adsorption isotherms of type I collagen on different polymers show a plateau at adsorbed amounts in the range of 0.6 to 1.1 $\mu\text{g}/\text{cm}^2$, which corresponds to a dry collagen thickness in the range of 4 to 8 nm. In contrast, time-of-flow viscosimetry indicated that the film of chick skin collagen adsorbed on glass was about 140 nm thick [23]. The possibility to align collagen fibrils on mica under the influence of a liquid flow was demonstrated recently [24].

The quartz crystal microbalance (QCM) has been shown to be a sensitive and practical tool to quantify adsorption *in situ* and in real time [25–27]. A recent version of the QCM with energy dissipation monitoring (QCM-D) enables the viscoelastic properties (viscosity and shear modulus) of the adsorbed films to be analyzed [28,29]. This technique showed that adsorption of hemoglobin took place in two phases, one associated with strong bridging and the other one associated with formation of a second layer of more loosely bound proteins [30]. The adsorbed mass measured by QCM-D includes both the protein and the water which is hydrodynamically coupled to the protein [31].

In this paper, we used QCM-D and atomic force microscopy (AFM) in tapping mode together with radioassays, to investigate the spatial organization (both along the surface and perpendicular to the surface) of collagen films adsorbed on polymer substrates differing according to hydrophobicity. Application of QCM-D and AFM techniques, allowed the monitoring of the adsorption kinetics *in situ*, avoiding artefacts due to dehydration, and hence to address contradictions from the literature.

II.1.2 Materials and methods

II.1.2.1 Polymer substrates

Polystyrene (PS) (Mw ca. 230 000 g/mol, Mn ca. 140 000 g/mol, Tg = 94 °C; Sigma-Aldrich, Bornem, Belgium, ref. 43,010-2) was spin-coated (acceleration = 20,000 rpm/s, speed = 6000 rpm, time = 30 s) on 15 and 12 mm diameter glass coverslips (VWR, Belgium) using a 10 % (w/w) solution in toluene (Vel, Leuven, Belgium). The samples with a diameter of 15 mm were spin-coated on one side with 40 μl of polymer solution and used for AFM examinations, whereas those of 12 mm were spin-coated on both sides with 30 μl of polymer solution and used for radiocounting experiments. Polymer coating of

quartz crystals for QCM study is described below. Surface-oxidized polystyrene (PSox) was prepared by plasma discharge in oxygen. Therefore, PS samples were placed in the reactor chamber and were exposed to a plasma discharge for 1 min at a power of 150 W under an oxygen flow. Substrates for AFM and QCM experiments were prepared by oxidation in a plasma barrel etcher (Bio-Rad, Hemel Hempstead, UK), whereas those for radiocounting experiments were exposed to oxidation in a plasma cleaner 300 (TePla, Atlanta, USA). X-ray photoelectron spectroscopy (Kratos Axis Ultra, Kratos Analytical Ltd., Manchester, UK) analysis and water contact angle measurements confirmed the similarity of surface chemical composition and wetting properties of the PSox obtained using these two reactors. After oxidation, the samples were stored in a desiccator containing P_2O_5 and used within 5 days.

II.1.2.2 Collagen

Type I collagen from calf skin (Roche Diagnostic, Mannheim, Germany) was received in solution (3 mg/ml at pH 3.0). To obtain the desired concentrations of 7, 40 and 100 $\mu\text{g/ml}$, the collagen solution was diluted in phosphate buffered saline (PBS) (pH ~ 7.2 ; 137 mM NaCl, 6.44 mM KH_2PO_4 , 2.7 mM KCl, 8.0 mM Na_2HPO_4 , (Vel, Leuven, Belgium)). The deionized water used in all experiments was obtained using a Milli-Q system from Millipore (Molsheim, France). Collagen solutions at concentrations of 7, 40 and 100 $\mu\text{g/ml}$ (referred to as *coll7*, *coll40*, *coll100*, respectively) were prepared at 4°C and placed in a water bath for 15 min at the desired temperature before being used for adsorption measurements.

II.1.2.3 Radioassays

Collagen was labeled by reductive methylation of amino groups using sodium borohydride ($[\text{}^3\text{H}]$ hydride (Amersham Bioscience, UK; ref. TRK663). The labeling procedure was adapted from Means [32] and was described in detail by Dewez et al. [18]. The concentration of the labeled collagen solution was 2.4 mg/ml as determined using the Lowry method (Kit, Reagents for Micro Protein Determination, Sigma Diagnostics, ref. 690-A). The solution of labeled collagen was stored at 4°C and used within 6 months.

The exchange between adsorbed molecules and collagen in the solution was

quantified using labeled collagen ($coll^*$) and unlabeled collagen ($coll$). In parallel, desorption in PBS was determined. The collagen concentration used was $40\text{ }\mu\text{g/ml}$. The time of adsorption was chosen according to the results of Pamula et al., [19] who showed that the adsorbed amount did not increase appreciably after 2 h of adsorption, on PS and PSox from a $40\text{ }\mu\text{g/ml}$ solution. Two independent sets of measurements were performed as follows, using one polymer sample for each individual measurement:

1. adsorption of $coll^*$ for 30 min / rinsing 5 times with water, referred to as $coll^* 30\text{ min}$;
2. adsorption of $coll^*$ for 2 h / rinsing 5 times with water, referred to as $coll^* 2\text{ h}$;
3. adsorption of $coll^*$ for 30 min / rinsing 5 times with PBS / adsorption of $coll$ for 1.5 h / rinsing 5 times with water, referred to as $coll^* 30\text{ min} / coll 1.5\text{ h}$;
4. adsorption of $coll^*$ for 30 min / rinsing 5 times with PBS / 1.5 h in PBS / rinsing 5 times with water, referred to as $coll^* 30\text{ min} / PBS 1.5\text{ h}$;
5. adsorption of $coll$ for 30 min / rinsing 5 times with PBS / adsorption of $coll^*$ for 1.5 h / rinsing 5 times with water, referred to as $coll 30\text{ min} / coll^* 1.5\text{ h}$.

The polymer-coated slides were placed in polypropylene round-bottom tubes (Falcon, VWR, USA; ref bdaa 2059) containing 2 ml of collagen solution; these tubes were preferred to ensure homogeneous adsorption on both sides of the samples. After the desired adsorption time at 37°C , rinsing was performed without removing the sample from the liquid. This was done by adding 2 ml of PBS or deionized water, removing 3 ml of the solution, adding 3 ml of PBS or deionized water again, and repeating these two last steps still four times. The rinsed samples were deposited wet into glass vials. Seven ml of scintillation liquid (Pico-Fluor 40, Packard Instruments, IL, USA; ref. 6013349) were added to the samples, which were then left dissolving overnight (Pico-Fluor is a solvent of PS). The glass supports remained at the bottom of the counting vials; it was checked that they did not interfere with counting. Counting was performed in triplicate for each individual sample, using a liquid scintillation

analyzer (Tricard 1600 CA from Packard Instrument Company, IL, USA). The activity recorded on the samples after adsorption was converted into collagen adsorbed amount using a calibration curve established with known amounts of collagen deposited on polystyrene samples by evaporation of the solution. The conversion factor was found to be 618 cpm/ μg . In addition, the activity of the solutions collected upon rinsing was measured; therefore 3 ml of these solutions were mixed with 14 ml of Pico-Fluor. The balance of the amounts of labeled collagen recovered in the rinsing solutions and measured on the substrates fitted the amount of labeled collagen introduced in the system within better than 90 %. The small discrepancy may be attributed to adsorption on the vessel walls.

II.1.2.4 Atomic force microscopy

In order to allow AFM examination in water, the glass slides were glued (epoxy bisphenol glue; Araldite, Sodiema, France) to the sample pucks of the AFM (Digital Instruments, Santa Barbara, CA, USA) and left overnight in a dessicator before performing the adsorption on the samples glued to the AFM pucks. The collagen solutions (4 ml) were poured into the wells of a tissue culture plate (Falcon, NJ, USA) where the slides had been placed. The samples were incubated at 37 °C for 24 h. The samples were then rinsed 5 times without being removed from the solutions. This was done each time by removing 2 ml of the liquid and adding 2 ml of deionized water. Samples to be analyzed in water were examined immediately. Samples to be analyzed in air were quickly dried by flushing with nitrogen and examined within 48 h. The AFM measurements were performed at ambient temperature, under water or in air, with a Nanoscope IIIa MultiMode AFM (Digital Instruments, Santa Barbara, CA, USA). Tapping mode AFM (intermittent contact between the scanning tip and the surface) was used to limit lateral deformation and to enhance the resolution of the images. The imaging force was kept as low as possible.

Under water, NP-S oxidation-sharpened silicon nitride probes (Digital Instruments) were used. To image the layers obtained at low collagen concentration (7 $\mu\text{g}/\text{ml}$), cantilevers with a low spring constant (~ 0.01 N/m) were used, whereas for the layers obtained at higher collagen concentration (40, 100 $\mu\text{g}/\text{ml}$), cantilevers with a higher spring constant (~ 0.6 N/m) were used.

The scan frequency was about 2-3 Hz for $2\ \mu\text{m} \times 2\ \mu\text{m}$ images. The working resonant frequency was always chosen close to 10 kHz. The samples were analyzed immediately after rinsing.

In air, tapping mode etched silicon probes (Digital Instruments) were used; the spring constants ranged between 20 and 100 N/m and the resonant frequency between 300 and 362 kHz. The scan frequency was about 2.5 Hz for $1\ \mu\text{m} \times 1\ \mu\text{m}$ images.

II.1.2.5 Quartz crystal microbalance experiments

II.1.2.5.1 Experimental set-up

The measurements were performed with a Q-Sense D300 System (Göteborg, Sweden). The QCM-D sensors (Q-Sense AB) with gold electrodes were polished crystals with a fundamental frequency of about 5 MHz. The gold-coated crystal (14 mm in diameter) was covered with polystyrene by spin-coating using 30 μl of the PS solution. The quartz crystals were reused after cleaning by sonication in a mixture of toluene : water (1:1) for a few minutes, rinsing with pure toluene, drying in a nitrogen flow and storage in a desiccator. After spin-coating with polystyrene, the quartz crystals were stored in a desiccator at least 12 h before use. The QCM-D measurements were performed in static conditions at 25 °C. Before adsorption, the cell was filled with PBS to record a baseline. The geometry of the cell design provided a fast replacement of PBS by the collagen solution [31], and the response of the QCM sensor was monitored as a function of time. All the measurements were performed for four harmonics ($n = 1, 3, 5$ and 7); some of them were repeated independently.

II.1.2.5.2 Theoretical background and data analysis

The QCM-D technique allows the simultaneous measurement of changes of resonance frequency (Δf) and changes of energy dissipation (ΔD) provoked by the formation of an adsorbed layer on the crystal surface. Sauerbrey [33] has shown that the mass adsorbed (Δm) on an oscillating crystal is proportional to the recorded frequency shift (Δf):

$$\Delta m = \left(\frac{C_{QCM}}{n} \right) \Delta f \quad (\text{II.1.1})$$

where C_{QCM} is the mass sensitivity constant ($= 17.7 \text{ ng/cm}^2 \text{ Hz}$ at $f = 5 \text{ MHz}$) and n is the overtone number (1, 3, 5 and 7). However, this simple proportionality law is valid only when the added material is rigid, sufficiently thin and evenly distributed over the active area of the crystal. Protein adsorption often induces a significant change in energy dissipation, indicating that the added mass acts as a soft and dissipative adlayer. In this case, a Voigt-based viscoelastic model may tentatively be used to fit the QCM-D data. Briefly, this model describes the behavior of the system on the basis of two simple components: a spring (elastic contribution) and a dashpot (viscous contribution) in parallel arrangement [34, 35]. Accordingly, the adlayer may be characterized by a complex shear modulus:

$$G = G' + iG'' = \mu + i2\pi f\eta = \mu(1 + i2\pi f\tau) \quad (\text{II.1.2})$$

where G' is the storage modulus, G'' is the loss modulus, μ is the elastic shear modulus, f is the oscillation frequency, η is the shear viscosity, and τ is the characteristic relaxation time of the system. The Voigt-based model describes the propagation and the damping of shear-bulk acoustic waves in a homogeneous viscoelastic adlayer which is in contact with a semi-infinite bulk Newtonian fluid under no-slip conditions [36, 37]. In the model, four parameters of the adlayer are involved: h and ρ , the adlayer thickness and density, as well as η and μ , the adlayer viscosity and shear modulus. The parameters of the semi-infinite Newtonian liquid must be used in modeling: $\rho_l = 1000 \text{ kg/m}^3$, $\eta_l = 0.001 \text{ Pa.s}$. The values of Δf and ΔD were related to the adlayer parameters [37] (equations A5, A6 within ref [37]), assuming that the contribution of the adlayer is additive to the contribution of the polymer coating and of the bulk solution, which determine the baseline. This is acceptable due to the small thickness of the polymer coating (about 100 nm). It was indeed checked by Sakti et al. that the addition of a PS coating on the crystal does not contribute to the damping of the crystal if the coating thickness is below $3 \text{ }\mu\text{m}$ [38]. Experimental values of Δf and ΔD were used to obtain a best fitting value of the adlayer parameters, using the harmonics 3 and 5 (Q-Tools software, Q-Sense D300, Göteborg, Sweden). The records for the first harmonic were not used because these data were perturbed by the O-ring which ensures cell tightness.

Table II.1.1: Amount of radiolabeled collagen adsorbed on PS and PSox samples (**experimental data**) and computed **difference** between the indicated samples (variation between 30 min and 2 h). The collagen concentration was 40 $\mu\text{g/ml}$.

		PS		PSox	
		(mg/m ²)	%	(mg/m ²)	%
	experimental data (one set)				
1	coll* 30 min	4.07		2.21	
2	coll* 2 h	4.62		2.85	
3	coll* 30 min/coll 1.5 h	2.65		1.68	
4	coll* 30 min/PBS 1.5 h	3.36		1.67	
5	coll 30 min/coll* 1.5 h	2.42		1.29	
	difference				
	meaning		a/b		a/b
2-1	additional adsorption	0.55	14/16	0.64	29/34
1-4	desorption by dilution	0.71	17/27	0.54	24/33
1-3	desorption by dilution and exchange	1.42	35/53	0.53	24/26
4-3	desorption due to exchange	0.71	17/26	-0.01	-0.5/-7
5-(2-1)	uptake due to desorption by dilution and to exchange	1.87	46/46	0.65	29/20
2-5	amount not subject to desorption	2.20	54/54	1.56	71/80

^a Computed from the preceding column, with respect to the amount adsorbed at 30 min; one set of data.

^b Values from a second set of measurements performed independently.

II.1.3 Results

II.1.3.1 Radiochemical measurements

The collagen amount adsorbed on PS and PSox after the different experiments is presented in Table II.1.1, as well as the computed variations due to longer adsorption, to dilution or to exchange between labeled and unlabeled collagen. The repeatability of counting was characterized by a confidence interval below $\pm 9\%$ (probability level 95 %). The same trend was found for the two sets of independent experiments. The amounts adsorbed after 30 min and 2 h were always higher on PS compared to PSox. Moreover, the increment of adsorbed amount measured between 30 min and 2 h (2 - 1) was relatively higher on PSox compared to PS, confirming that after 30 min, PS is closer to stationary conditions than PSox [19]. The difference (1 - 3) represents the desorption of labeled species (*coll**) resulting from dilution and exchange with the unlabeled species (*coll*) in the solution. The difference 5 - (2 - 1) represents the uptake of *coll**

which results from the desorption of coll due to dilution and from exchange between adsorbed *coll* and *coll** in the solution. For both substrates, there is an agreement between (1 - 3) and 5 - (2 - 1), demonstrating the consistency of the data. They represent the amount which can be exchanged between the surface and the solution, due to dilution plus readsorption and to displacement of one molecule by another at the surface. This is appreciably higher for PS than for PSox. The contribution of desorption by dilution to these results (1 - 4) is in the same range for the two substrates but the contribution of replacement of one molecule by another (4 - 3) is appreciable on PS and not significant on PSox. It turns out that the amount of collagen adsorbed after 30 min, which is not subject to desorption (2 - 5), is lower in absolute value but higher in relative value on PSox compared to PS.

II.1.3.2 QCM-D data

Typical records obtained by QCM-D at three harmonics as a function of adsorption time are shown in Figure II.1.1 for adsorption on PS and PSox from *coll100*. Values of Δf and ΔD no longer changed appreciably on PS after 2 h of adsorption for high collagen concentrations (40 and 100 $\mu\text{g/ml}$). The variations were more progressive on PS at 7 $\mu\text{g/ml}$ and on PSox at all concentrations (not shown). The relationships between ΔD and $\Delta f/n$ within a kinetic run are presented in Figure II.1.2 for the two adsorbents and the three collagen concentrations, using harmonic 3 (15 MHz). The scale of adsorption time is indicated by arrows. It appears from Figure II.1.2 that the data follow the same trend on PS and on PSox; however the values of Δf and ΔD at a given adsorption time for concentrations of 40 and 100 $\mu\text{g/ml}$ are higher for PS compared to PSox. This type of data presentation will be extended to other harmonics in the discussion (Figure II.1.8).

II.1.3.3 AFM examination

Representative height images recorded under water on collagen layers adsorbed on PS and PSox for concentrations of 7, 40 and 100 $\mu\text{g/ml}$ after 24 h of adsorption are presented in Figure II.1.3. On PS, fibrillar structures of collagen were observed at all concentrations; their density and thickness increased with the concentration. On PSox, elongated features were only apparent for a concen-

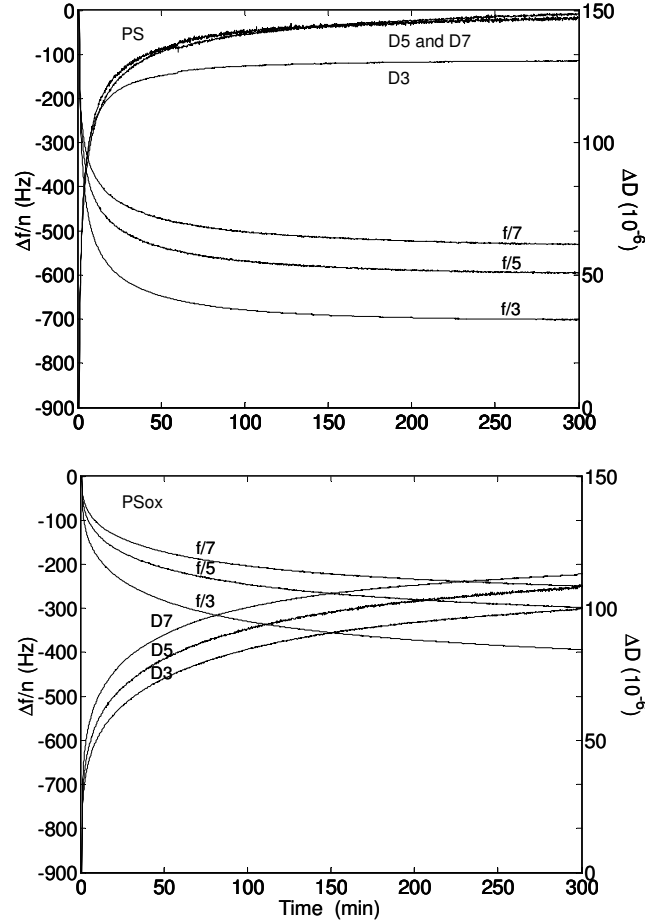


Figure II.1.1: Frequency shift divided by the harmonic number ($\Delta f/n$) and dissipation shift (ΔD) at harmonics 3, 5 and 7 as a function of time for adsorption of collagen on PS and PSox substrates from a 100 $\mu\text{g}/\text{ml}$ solution.

tration of 100 $\mu\text{g}/\text{ml}$; in that case, the images were similar to images obtained on PS after collagen adsorption from a 7 $\mu\text{g}/\text{ml}$ solution. For collagen concentrations of 7 and 40 $\mu\text{g}/\text{ml}$, structural features up to 5 nm high were observed but they did not form well contrasted fibrils. High resolution images recorded under water and in air (Figure II.1.4) confirmed that the morphologies formed at 7 $\mu\text{g}/\text{ml}$ on PS and 100 $\mu\text{g}/\text{ml}$ on PSox were similar. At higher concentrations on PS, the collagen fibrillar structures were more developed as illustrated

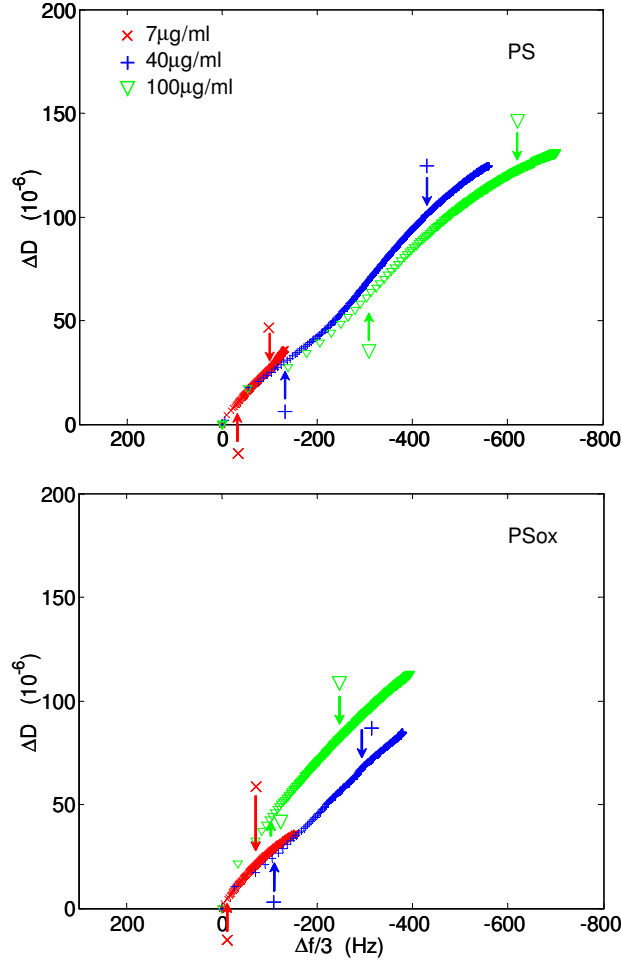


Figure II.1.2: ΔD vs $\Delta f/n$ plots using QCM-D data for harmonic 3. Adsorption on PS and on PSox substrates from collagen solutions at concentrations of: 7 $\mu\text{g/ml}$ (red x-marks), 40 $\mu\text{g/ml}$ (blue crosses) and 100 $\mu\text{g/ml}$ (green downward triangles). The end of each curve corresponds to adsorption for 5 hours. The arrows indicate two lower durations of adsorption: upward, 2 minutes; downward, 30 minutes.

for 100 $\mu\text{g/ml}$ after 24 h and even for 40 $\mu\text{g/ml}$ after 30 min of adsorption. Some fibril ends showed clearly a root-like morphology (highlighted by the dotted circles). The cross sections shown in Figure II.1.5, recorded in the highest resolution image from Figure II.1.4, reveal more details on the fibrillar

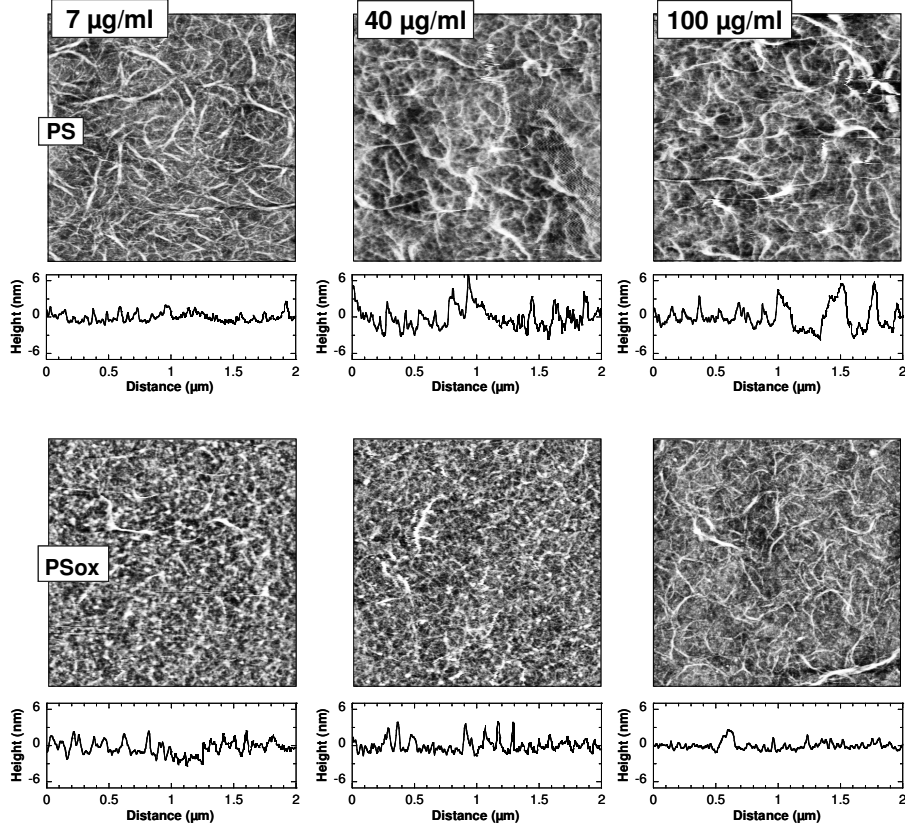


Figure II.1.3: AFM topographic images ($2\ \mu\text{m} \times 2\ \mu\text{m}$, $z\text{-range} = 10\ \text{nm}$) recorded under water after 24 hours of collagen adsorption on PS (top) and on PSox (bottom) from solutions at 7 (left), 40 (middle) and 100 $\mu\text{g/ml}$ (right). The cross-sections were taken in the middle of the images. The images were flattened using a third order polynomial algorithm (see Appendix II.A, Figure II.A.6).

structures. Section c ($0.10\text{--}0.38\ \mu\text{m}$) illustrates the increase of fibril thickness resulting from the association of thinner structures, which is in agreement with the observation of the root-like morphology. While the height is higher at the cross point of fibrils (which appears at the following positions: a, $0.17\ \mu\text{m}$; c, $0.39\ \mu\text{m}$; d, $0.19\ \mu\text{m}$; e, $0.36\ \mu\text{m}$), this is not just the superposition of smooth fibrils (shown separated in section b) but seems to involve intermingling of the crossing fibrils.

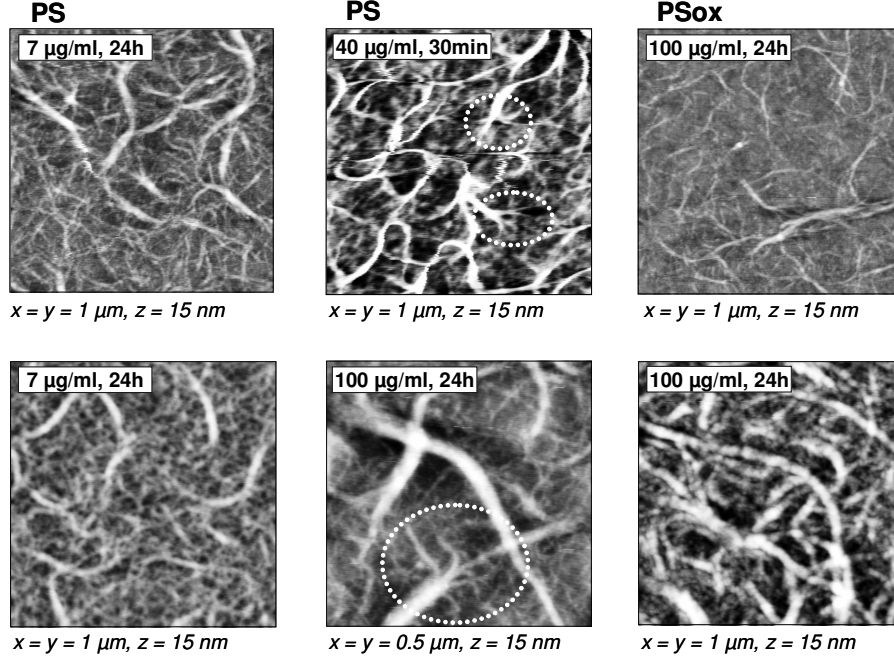


Figure II.1.4: High resolution AFM images recorded under water (top line) and in air (bottom line) after collagen adsorption on PS (left and middle column) and PSox (right column). Adsorption was performed from 7, 40 and 100 $\mu\text{g/ml}$ solution on PS and from 100 $\mu\text{g/ml}$ solution on PSox substrata. The adsorption time was 24 h or 30 min, as indicated. The images were flattened using a third order polynomial algorithm.

II.1.4 Discussion

II.1.4.1 Characteristics of the adsorbed layer seen by QCM-D

The frequency (Δf) and dissipation (ΔD) shifts recorded for collagen (Figure II.1.1) were much higher than those reported for other proteins. For collagen adsorbed on PS, Δf was about -888 Hz and ΔD was about 148×10^{-6} at the fundamental frequency (not shown); for human serum albumin, values of $\Delta f = -11$ Hz and $\Delta D = 0.6 \times 10^{-6}$ were measured; these values were -52 Hz and 1.4×10^{-6} for hemoglobin [39]. It may be noted that the size of these molecules is about $1.5 \times 1.5 \times 300 \text{ nm}^3$ for collagen, $3 \times 8.2 \times 8.5 \text{ nm}^3$ for albumin [40],

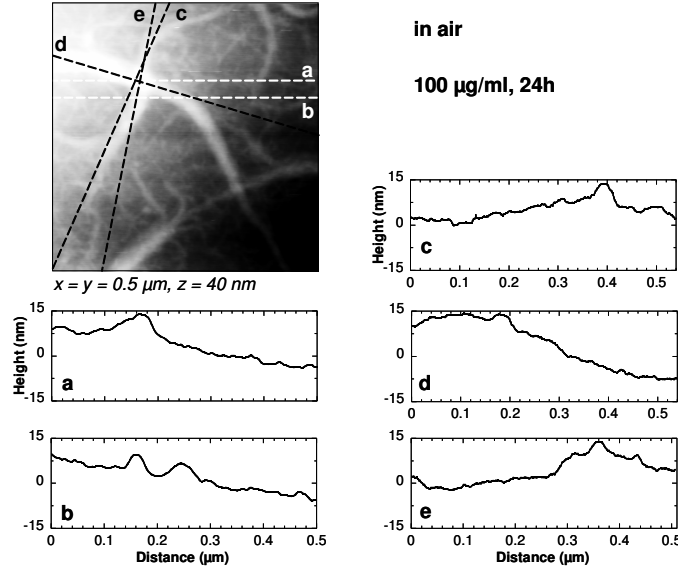


Figure II.1.5: High resolution AFM image recorded in air after 24 h collagen adsorption on PS from a 100 $\mu\text{g}/\text{ml}$ solution. Cross-sections extracted as indicated on the non-flattened image (from left to right) also shown flattened in Figure II.1.4.

and $4.5 \times 4.5 \times 6.4 \text{ nm}^3$ for hemoglobin [41].

According to the Sauerbrey relation (II.1.1), the collagen adsorbed amount would be twice as high on PS than on PSox after 5 h adsorption from *coll100*. However, the Sauerbrey relation supposes that the adlayer is a rigid solid, which is in contradiction with the observation of large energy dissipation. The data recorded with harmonics 3 and 5 were used to compute the best fitting values of the thickness, viscosity and shear of the adlayer. Therefore, the density of the adlayer had to be fixed. Figure II.1.6 shows, for data recorded on PS and PSox after 5 h adsorption from *coll40*, the dependence of the thickness (A), viscosity (B) and shear modulus (C) obtained by best fitting with respect to the density chosen for the adlayer, in the range from the density of pure water to that of ‘pure protein’. It appears that the selected density affects only slightly the value of the best fitting parameters. In particular, differences between PS and PSox regarding viscosity and shear modulus are larger than differences which may be due to uncertainties in the density. Thus, a density of $1100 \text{ kg}/\text{m}^3$ was used for the adlayer in all computations described below.

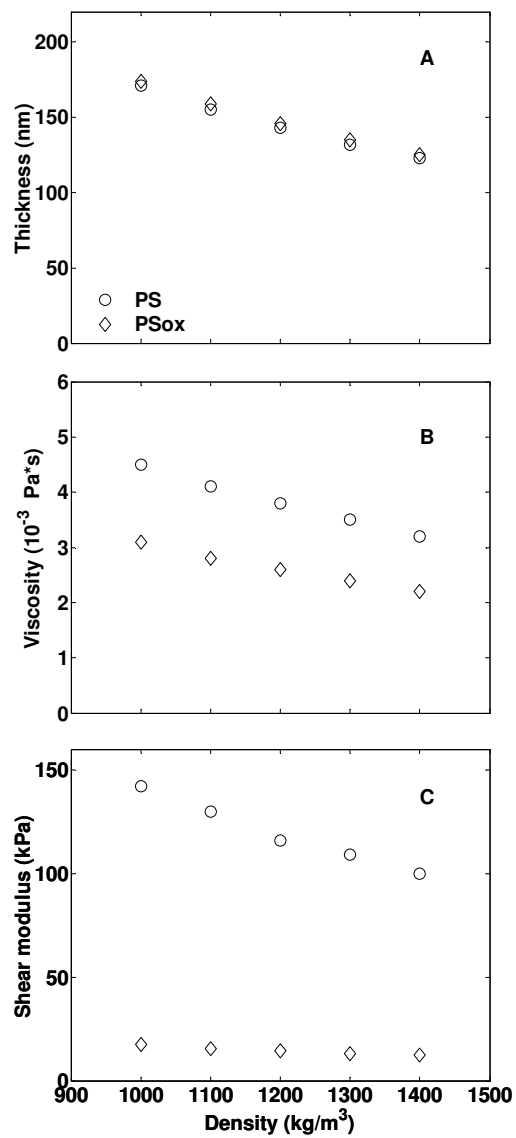


Figure II.1.6: Best fitting values of thickness (A), viscosity (B) and shear modulus (C) of the adlayer *vs* selected density (1000-1400 kg/m³). The adsorption was performed during 5 hours on PS (circles) and PSox (diamonds) substrates from a concentration of 40 μ g/ml.

The data obtained after adsorption for 2, 30 and 300 min were used to compute the best fitting values of the adlayer thickness, viscosity and shear modulus, which are presented in Figure II.1.7. A thick adlayer was already formed after 2 min of adsorption for all collagen concentrations and both substrates (Figure II.1.7.A). The thickness increase was still appreciable between 2 and 30 min but was small after 30 min. After 5 h of adsorption, the thickness of the adlayer was comparable on PS and PSox substrata and equal to about 190 nm for *coll100*. Figure II.1.7.B indicates that the viscosity of the adlayer on PS was higher than on PSox for adsorption from *coll40* and *coll100*. The shear modulus (Figure II.1.7.C) was also much higher on PS compared to PSox.

It must be kept in mind that the best fitting procedure involves adjusting three parameters on the basis of Δf and ΔD measured on two harmonics. The best fitting values are not necessarily the most meaningful values. Therefore, ΔD versus Δf charts were generated to examine the sensitivity of the experimental data to the parameters characterizing the adlayer. Figure II.1.8 presents the charts obtained for the three overtones, using a density of 1100 kg/m³, a thickness varying from 0.1 to 200 nm, a viscosity between 1×10^{-3} and 5×10^{-3} Pa.s and a shear modulus between 1×10^4 to 1×10^6 Pa. Similar charts were published by Höök et al. for a viscous polymer film examined in air and in liquid [37]. Figure II.1.8 also presents the experimental ΔD vs $\Delta f/n$ plots obtained for PS using three collagen concentrations and three harmonics, and for PSox using three collagen concentrations and one harmonic ($n = 3$). These plots all follow the same trend, whatever the harmonic and the substrate. This was also the case for measurements performed with harmonics 5 and 7 on PSox (not shown).

The charts presented in Figure II.1.8 illustrate that a set of fitting parameters (thickness, viscosity, shear modulus) is not unique for a given experimental pair Δf - ΔD . The range of experimental data indicates that the shear modulus is in the range of 1×10^4 to 1×10^5 Pa, over which it does not affect strongly the ΔD vs Δf relationship (closeness between curves with black and white dots). The difference in shear modulus between PS and PSox, although quite clear in Figure II.1.7, is thus not meaningful. This example shows that the results obtained by best fitting should be taken with care as long as the sensitivity to fitting parameters is not examined. Finally, Figure II.1.8 reveals that, as adsorption time increases (set of identical experimental points), the increase

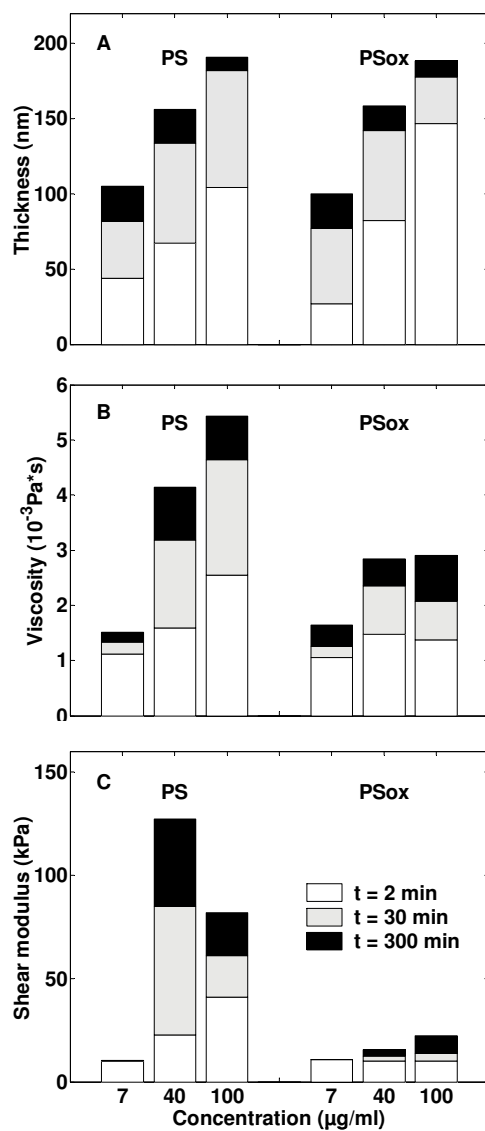


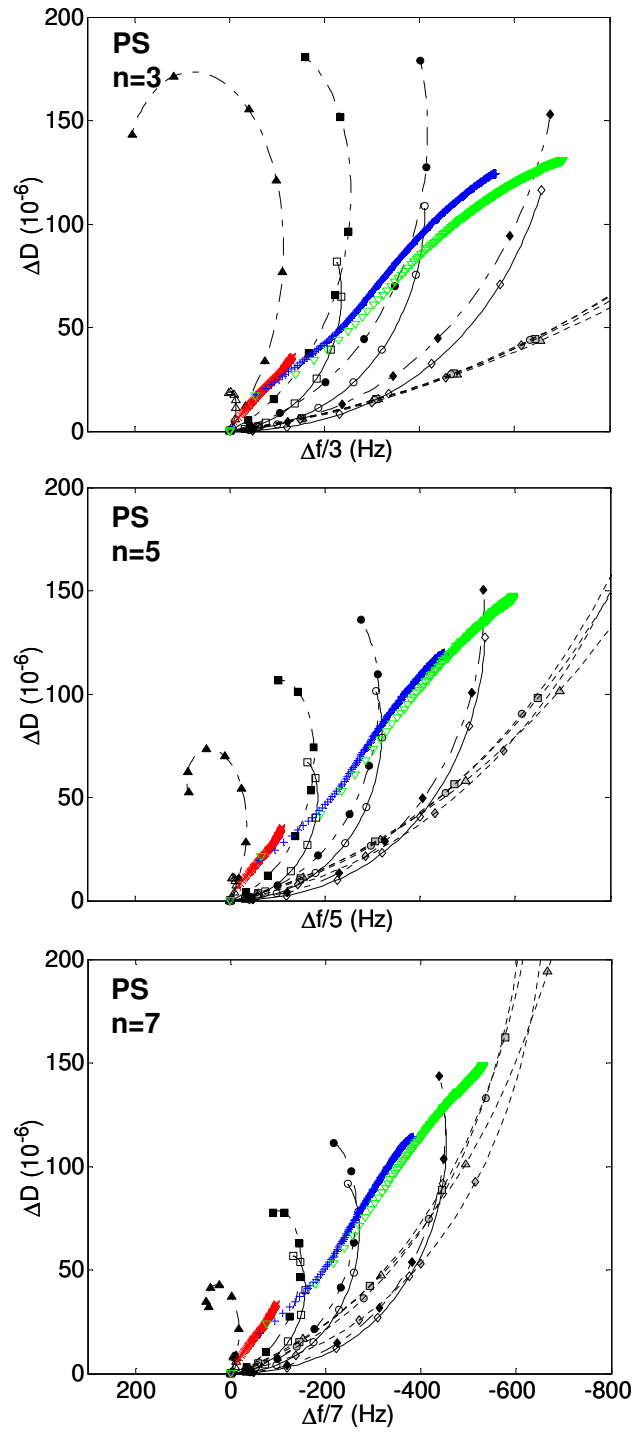
Figure II.1.7: Best fitting values of thickness (A), viscosity (B) and shear modulus (C) (from harmonics 3 and 5) of the layer adsorbed on PS (left) and PSox (right) substrates from different collagen concentrations (7, 40 and 100 $\mu\text{g/ml}$) for increasing adsorption times: $t = 2$ min (open squares), $t = 30$ min (open + grey squares), $t = 300$ min (open + grey + black squares). The layer density was fixed at 1100 kg/m^3 in all cases.

of the adlayer thickness is accompanied by an increase in its viscosity. The trend is the same whatever the harmonic, the collagen concentration and the substrate. However, Figure II.1.2 shows that the collagen concentration influences the extent of Δf and ΔD changes in a given period of time. Moreover, for a given adsorption time, the extent of Δf and ΔD changes is larger on PS compared to PSox.

It thus appears that, as the collagen concentration increases and/or the adsorption time increases, the adlayer thickness increases to reach values of the order of 150-200 nm on PS and 100-150 nm on PSox. In parallel, the viscosity of the adlayer increases by a factor of 5 and 3, respectively. This is in agreement with the thickness evaluated by viscosimetry for chick skin collagen adsorbed on glass [23]. If the adlayer mass detected by QCM was constituted of non-hydrated collagen, a thickness of 150 nm would correspond to an adsorbed amount of 170 mg/m². In contrast, the adsorbed amount measured on the plateau of the adsorption isotherms for *coll100* is about 8.5 mg/m² (Gurdak, in preparation). Accordingly, if the adlayer had a homogeneous composition, it would consist of about 4 vol.% of collagen and 96 vol.% of trapped water.

II.1.4.2 Characteristics of the absorbed layer seen by AFM

The AFM observations provide a picture of the adlayer which is very different from QCM. As shown in Figure II.1.3, collagen adsorbed on PS forms fibrils at all concentrations after 24 h. These observations are in agreement with those made before on PS and other hydrophobic substrates [6, 20, 21]: however, the images obtained in tapping mode show a higher resolution. Similar patterns were recorded as the background of much larger fibrils described in the literature [13]. Images obtained at high enough concentrations (*coll40*, *coll100*) reveal fibril-end morphologies (Figure II.1.4 and II.1.5), which indicate that they are made by assembly of smaller entities anchored at the surface. The AFM images recorded in air after fast drying are similar to those recorded in water, while allowing a higher resolution to be achieved. This observation, as well as the importance of branching between fibrils, suggests that these are formed parallel to the surface and are not traces of fibrils grown perpendicular to the surface and folded down during AFM examination. This interpretation



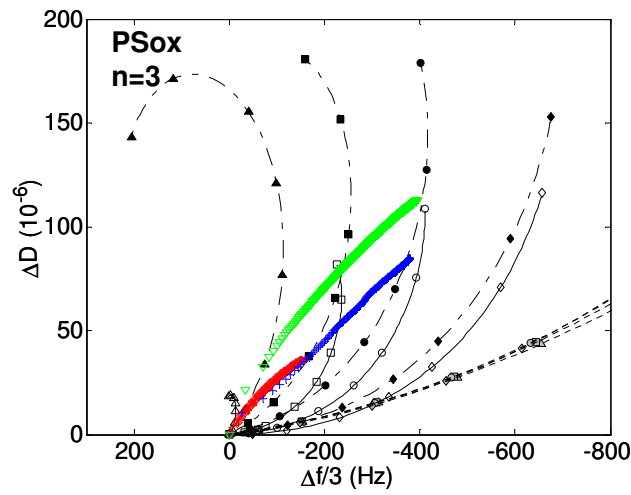


Figure II.1.8: ΔD vs Δf charts computed for an adlayer, considering a density of 1100 kg/m^3 , a thickness of 0.1, 10, 25, 50, 75, 100, 150 and 200 nm, (set of 8 dots in the increasing order on each spiral curve), a viscosity of $1 \times 10^{-3} \text{ Pa.s}$ (triangles), $2 \times 10^{-3} \text{ Pa.s}$ (squares), $3 \times 10^{-3} \text{ Pa.s}$ (circles), $5 \times 10^{-3} \text{ Pa.s}$ (diamonds) and a shear modulus of $1 \times 10^4 \text{ Pa}$ (open symbols, continuous line), $1 \times 10^5 \text{ Pa}$ (black symbols, long and short strokes), $1 \times 10^6 \text{ Pa}$ (grey symbols, short strokes). In colors, plots of experimental data for collagen adsorption during 5 hours on PS (overtones 3, 5 and 7) and PSox (overtone 3) for concentrations of $7 \mu\text{g/ml}$ (red x-marks), $40 \mu\text{g/ml}$ (blue crosses) and $100 \mu\text{g/ml}$ (green downward triangles).

is also supported by the detailed observation of cross-over points (such as in Figure II.1.5) which suggests an intermingling of crossing fibrils. The relief observed along a given fibril and the morphology of fibril ends indicate that they are far from hair-like but are rather the result of an aggregation process at different scales, started from molecules lying along the surface.

It has been reported [19] that the development of the fibrillar structures still progresses between 2 and 24 h of adsorption, although the adsorbed amount no longer increases significantly; the increasing resistance of the film to scraping with the AFM probe also was highlighted. Figure II.1.3 indicates that the increase of the collagen solution concentration and the resulting rise of the amount of adsorbed material also favors the development of the fibrillar structures.

On PSox, fibrillar structures are also formed. They are similar to those observed on PS; however for an adsorption time of 24 h, they appear only at high collagen concentration. It was reported before [19] that at a 40 $\mu\text{g/ml}$ collagen concentration, the fibril formation occurs after longer adsorption times on PSox compared to PS. When the adsorption time and, or the collagen concentration are too low to lead to the observation of well contrasted fibrils on PSox, the adsorbed layer has a rough morphology, with peaks up to 5 nm, which evokes aggregation with coherence at a shorter distance. It must be noted that this may not be explained in terms of collagen denaturation; adsorption measurements performed using solutions of denatured collagen do not show the appearance of fibrils (Gurdak, in press).

II.1.4.3 Global vision and understanding

The collagen layer adsorbed on PS, and PSox at sufficiently high concentration and long adsorption time, may be described as illustrated by Figure II.1.9. (i) AFM indicates that molecules bound to the surface are involved in aggregation at different scales, leading to the build up of fibrils, up to 7 nm in thickness. (ii) In addition, bundles of molecules protrude into the solution, creating a thick adlayer with a slightly higher viscosity. This was not observed by AFM in the present work but explains the repulsion (300-400 nm) at long distance observed by AFM with collagen adsorbed on PET [42] and CH_3 -terminated SAM [6] or observed between two mica surfaces covered with adsorbed collagen [16].

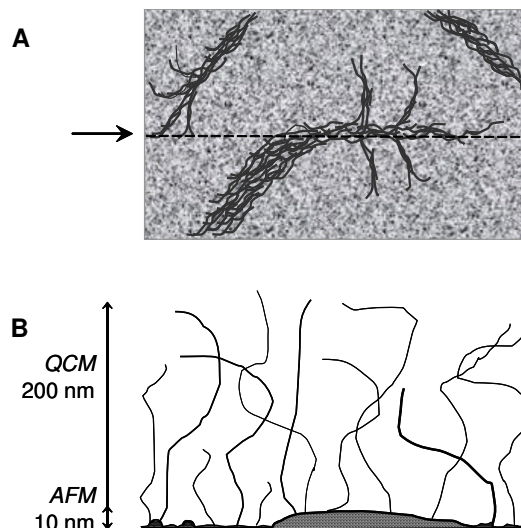


Figure II.1.9: Schematic representation of the collagen adlayer on PS and PSox substrates. A: top view; B: cross-section taken at the position indicated by the arrow. The thickness probed by each technique is indicated.

It also explains the evolution of AFM images in contact mode obtained upon increasing the loading force [42]. The development of a fibril network on PS [19] and PET [21] is responsible for a higher resistance to AFM probe damage. At this stage, it is not clear whether AFM probe bridging upon contact, which is observed on hydrophobic substrates, is due to the fibrils lying along the sample or to bundles protruding into the solution.

The difference between PS or other hydrophobic surfaces [6], on the one hand, and PSox or other hydrophilic surfaces [21], on the other hand, lies in the extent of fibril formation, which is also favored by increasing the collagen concentration and the adsorption time.

The radiochemical measurements (Table II.1.1) help to understand the difference between PS and PSox regarding collagen adsorption. While the amount adsorbed on PS is higher than on PSox, the collagen molecules adsorbed on PS are more readily involved in an exchange with molecules present in the solution. This indicates that they are more mobile and explains why they form fibrils more readily. This is consistent with the higher resistance to AFM probe damage observed on PSox compared to PS at short adsorption time and, or

low collagen concentration in the solution [20]. On the other hand adsorption is slower [19] and is characterized by a lower apparent affinity [18] on PSox compared to PS. All these observations can be tentatively rationalized if it is considered that collagen molecules are bound to PS through a limited number of contact points, either because of preferential affinity with hydrophobic segments of the molecules or because of crowding between molecules which approach the surface and stick immediately.

II.1.5 Conclusion

The interpretation of the QCM-D data is based on fitting physical quantities computed according to a model with the experimental data. This work does not provide arguments regarding the relevance of the Voigt-based viscoelastic model which is frequently used in the literature. However, it must be emphasized that the significance of the results obtained should be evaluated by examining the sensitivity of the fitted quantities (change of frequency, change of energy dissipation) to the fitting parameters (thickness, viscosity, shear modulus).

QCM-D reveals a thick (up to 200 nm) collagen adsorbed layer containing more than 96 % water. As the collagen concentration or adsorption time increases, both the adlayer thickness and viscosity increase. It develops faster and to a larger extent on PS compared to PSox, in agreement with a higher adsorbed amount measured by radiochemical measurements.

In a complementary fashion, AFM reveals the presence of a mat of collagen in which fibrils are formed. The density and size of fibrils increase with time and collagen concentration. Fibril formation occurs more readily on PS compared to PSox. This is attributed to a greater mobility of collagen adsorbed on PS, presumably due to a lower number of bridging points.

The combination of AFM, QCM-D and radioassays thus shows that the collagen adlayer consists of (i) a thin mat in which fibrils develop depending on substrate, concentration and time, (ii) a thicker overlayer (~ 200 nm) containing protruding molecules or bundles which are in very low concentration but modify noticeably the local viscosity and play a major role in the interaction with an approaching surface.

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Chapter II.2

Factors and mechanisms determining the formation of fibrillar collagen structures in adsorbed layers

Summary

The adsorption of collagen (type I from calf skin) was studied, comparing different collagen sources and using substrates which differ according to surface hydrophobicity (polystyrene, either native, with OH substitution of each repeat unit, with COOH substitution of a small fraction of repeat units, or surface modified by oxygen plasma discharge). The atomic force microscopy observation of the adsorbed layers showed that aggregation in the solution acts in competitions with the formation of fibrils in the adsorbed phase; more aggregated solutions behave like less concentrated solutions regarding adsorption. The fibrils formed in the adsorbed phase are much smaller than the fibrils formed in the suspension, and, in contrast with the latter, do not show regular band pattern. It is confirmed that fibrils formation occurs more readily on more hydrophobic surfaces, which is tentatively attributed to a greater mobility of individual molecules adsorbed on more hydrophobic substrates. This interpretation is supported by previously published radiochemical measurements. However, the comparison of strongly different adsorption procedures (progressive on the one hand; quick and massive on the other hand) did not provide any additional clue.

II.2.1 Introduction

Collagen is the major fibrillar protein in the extracellular matrix and in connective tissues. It is also a dominant component of skin, bone, cartilage, tendon and ligament [1]. Cells may co-express at least 27 distinct collagen types [2]. Fibrillar forms are the most abundant and represent over 90 % of the total collagen [3]. Structurally, type I collagen consists of three chains, which are twisted with each other to form a semi-rigid helical structure, the so-called collagen monomer. Nonhelical parts (telopeptides) are found at the extremities of each monomer. Chemically, each chain is constructed from repeated amino acid sequences glycine-X-Y, where X and Y positions may be occupied by any amino acid; frequently proline is in X position and hydroproline in Y. A characteristic of type I collagen is that, among the three polypeptide chains, two ($\alpha 1$) are identical and one ($\alpha 2$) is different. In calf skin collagen, the $\alpha 1(I)$ -chains are made of 1056 residues each, and the $\alpha 2(I)$ -chain of 1038 [4].

Collagen self-assembly is observed *in vivo* as well as *in vitro* and is an entropy-driven process. The major driving forces for fibril formation may be: water-mediated intra- and interbridges between the polypeptide helices [5], hydrophobic interactions between adjacent molecules [6] as well as electrostatic interactions [7]. An important role in collagen fibril formation is also attributed to the presence of the telopeptides [8]. Their participation seems to be catalytic rather than constitutive, as they would essentially facilitate achieving the appropriate packing into fibers [9]. Fibrillogenesis starts with assembly of collagen monomers, which associate into pentamers and form characteristic band patterns with a periodicity of 67 nm (D band pattern) along the fibril length. Further linear (end-to-end) and lateral association (entwining of the structures) [10], gives rise to microfibrils which can further assemble into large fibrillar structures [11]. Collagen is an adhesive protein, *i.e.* may be specifically recognized by cell surface receptors called integrins, and trigger cell adhesion and spreading [12]. The organization of collagen in adsorbed phases strongly influences these cell responses [13,14]. On the other hand, the occurrence of the 67 nm band pattern along the collagen fibers was shown to direct cell mobility [15].

Protein adsorption from aqueous solutions onto solid substrates may involve different interactions: redistribution of charged groups in the interfacial film,

dehydration of the protein and the substrate, and rearrangement of the protein molecule upon contact with the solid [16]. Owing to the semi-rigid character of the collagen molecule, its adsorption to solid surfaces cannot be primarily driven by conformational changes. The electrostatic protein-substrate interactions also seem to be minor [17]. The driving force for collagen adsorption is thus expected to be the entropy gain due to dehydration *i.e.* release of water molecules when hydrophobic patches on the protein exterior contacts hydrophobic substrate areas [18].

In our laboratory, different approaches were undertaken in order to better understand, control and utilize collagen adsorption on solid surfaces. The influence of several factors was investigated: (i) surface chemical composition, hydrophobicity and topography [19], (ii) state of collagen solutions, namely degree of aggregation [20] and of thermal denaturation [21], (iii) adsorption conditions, namely solution concentration [22] and duration of adsorption [23], (iv) competition of collagen with a polymer (Pluronic F68) [24] and other proteins (albumin) [25].

It was demonstrated that fibril formation takes place in the adsorbed phase. Regarding the influence of the surface hydrophobicity, a higher surface affinity was observed for polystyrene (PS) compared to oxidized polystyrene (PSox) [23, 26]. In contrast, fibril formation and exchange between adsorbed molecules and molecules in the solution occurred more readily on PS compared to PSox [22]. A combination of atomic force microscopy (AFM) and quartz crystal microbalance with dissipation monitoring (QCM-D) measurements has shown that the collagen adlayer consists of two parts: a dense and thin sheet in which fibrils are formed (directly observed by AFM) and an overlying thick layer (up to 200 nm) containing protruding molecules or bundles which are in very low concentration but modify noticeably the local viscosity. The thickness and viscosity of the semi-liquid adlayer both increase with adsorption time and collagen concentration. Fibril formation near the surface also increases with time and collagen concentration and occurs more readily on PS compared to PSox [22].

The aim of this work was to investigate further the factors influencing the nanoscale organization of collagen in adsorbed phases. The influence of the source of type I collagen was examined with a particular attention to the interference of collagen aggregation in solution. The influence of substrate hydrophobicity was investigated by using polymers with defined polar functions

in known concentration (OH, COOH), in comparison with an apolar polymer (polystyrene, PS) and with a complex modified polar surface (plasma treated polystyrene, PSox). In order to gain a better insight into the mechanisms responsible for the influence of substrate surface hydrophobicity, adsorption isotherms were obtained, using procedures which differed strongly according to the way of increasing the collagen concentration (sudden or progressive) to which the substrate was exposed.

II.2.2 Experimental

II.2.2.1 Polymer substrates

The polymers used are polystyrene (PS), and analogous polymers characterized by a substitution of all repeat units with hydroxyl groups (PSOH) and substitution of a small fraction of repeat units with carboxyl groups (random PS-co-PSCOOH; 8 % mole fraction substituted) on the aromatic ring, respectively. A homopolymer, PSCOOH was also tested. However, its dissolution in the buffer solution prevented adsorption studies. Table II.2.1 summarizes the name, formula, molar mass, density and source of the polymers. Polymer solutions were prepared using magnetic rod agitation for 1 h, as specified in Table 1. They were spin-coated on two types of glass samples: 12 mm diameter coverslips (VWR, Belgium) and 22 mm $\times$ 22 mm coverslips (VWR, Belgium). The parameters of spin-coating were: rotational acceleration = 20,000 rpm/s, speed = 6,000 rpm for round samples and 1,500 rpm for square samples, and time = 40 s. The round samples were spin-coated on one side (30 μ l) with the solutions of lower concentration and used for adsorption studies. The square samples were cleaned by immersion for one night in sulfochromic mixture followed by extensive rinsing with MilliQ water (Milli-Q Plus system, Millipore, Molsheim, France). Subsequently, they were directly dried on the spin-coating holder (speed = 6,000 rpm). In order to ensure a good polymer-glass adhesion, a thin film of an adhesion promoter (hexamethyldisilazan; HMDS; 80 μ l on each side) [27] was spin-coated prior to the polymer solution. The square samples were spin-coated twice on both sides (130 μ l each time) with the solutions of higher polymer concentration. They were used in the wetting measurements, which were performed after drying for at least 2 days in a dessicator. Analysis

Table II.2.1: Main characteristics of the polymers and copolymer used as the substrates.

	PS	PSOH	PS-co-PSCOOH
Name	polystyrene	poly(4-vinylphenol)	poly(styrene-co-4-vinyl benzoic acid)
Formula	$[-CH_2-CH-(C_6H_5)-]_n$	$[-CH_2-CH-(C_6H_4OH)-]_n$	$[-CH_2-CH-(C_6H_5)-]_n \times 0.92$ -co- $[-CH_2-CH-(C_6H_4COOH)-]_n \times 0.08$
Molar mass (g/mol)	230 000	20 000	84 400
Density (g/cm ³)	1.04	1.16	1.34
Spin-coating -solvent	toluene Vel, Louvain, BE	tetrahydrofuran Vel, Louvain, BE	dioxan Sigma-Aldrich, BE
-concentrations (% w/w)	20 and 10	20 and 10	10 and 5
Source	Sigma-Aldrich, BE 43,010-2	Sigma-Aldrich, BE 43,622-4	Polymer Source, Canada P1543-SVA _{ran}
O/C molar ratio -expected	0	0.125	0.020
-measured by XPS (SD)	0.002(0.001)	0.112(0.004)	0.017(0.001)

by XPS did not reveal the presence of the primer at the polymer surface (not shown). Plasma-oxidized polystyrene (PSox) was obtained by plasma discharge in oxygen. Therefore, PS samples were exposed to a plasma discharge for 1 min at a power of 150 W, under an oxygen pressure of 75 Pa (plasma cleaner 300 from TePla, GmbH, Feldkirchen, Germany).

II.2.2.2 Adsorption of collagen

Type I collagen from calf skin was purchased as solutions from three different companies. The first solution (3 mg/ml at pH = 3.0; Roche Diagnostic, Germany), called RD collagen, contained 95 % of type I collagen and 5 % of type III collagen. The second (4 mg/ml at pH = 3.0; AutogenBioclear, UK), called AB collagen, consisted of 90 % of type I collagen and 10 % of type III. For the third (10.5 mg/ml at pH = 3.7; USB Corporation, OH USA), called USB collagen, it was only specified that it contained the antiseptic agent Thimerosal (sodium ethyl mercurithiosalicylate) as a preservative. To obtain the desired concentrations, the collagen stock solutions were diluted at 4 °C in phosphate

buffered saline (PBS) (pH ~ 7.2 ; 137 mM NaCl, 6.44 mM KH_2PO_4 , 2.7 mM KCl, 8.0 mM Na_2HPO_4 ; Vel, Leuven, Belgium) and then warmed up at 37°C for 15 min just before proceeding to the adsorption.

For AFM and XPS measurements, 4 ml of the pre-warmed collagen solution was poured into the wells of a tissue culture plate (Falcon, NJ, USA) where the polymer substrates had been placed previously. The samples were left incubating at 37°C for the desired period of time and at the desired collagen concentration. After adsorption, the samples were rinsed 7 times without being withdrawn from the solutions. This was done by removing 2 ml of collagen solution and then adding 2 ml of deionised MilliQ water. Finally, the samples were dried by flushing with a stream of nitrogen, stored in a dessicator, and analyzed within 2 days.

Furthermore, RD collagen was adsorbed on PS at 37°C in two different ways as presented in Figure II.2.1. In the first procedure, called ‘one step’ (‘os’) method, five PS samples were immersed in a collagen solution of a given concentration for 24 h, each sample in a distinct well of a tissue culture plate. In the second procedure, called ‘step by step’ (‘sbs’) method, the concentration of the collagen solution was progressively increased. Therefore, all samples were incubated at a collagen concentration of $0.1\text{ }\mu\text{g/ml}$ for 24 h. One sample was then withdrawn and the other samples were incubated at a collagen concentration of $0.4\text{ }\mu\text{g/ml}$ for the next 24 h. One sample was then withdrawn and the procedure was repeated, increasing the collagen concentration at each step (7, 40 and $100\text{ }\mu\text{g/ml}$). Each sample was rinsed with water (MilliQ), as described before, and dried under a nitrogen flux.

II.2.2.3 Direct observation of collagen fibers

The aggregates formed in a $40\text{ }\mu\text{g/ml}$ solution of USB collagen aged for 24 h at 37°C were collected with a pipette (about 1.5 ml collected from 5 ml of solution). The suspension was delivered onto a slightly tilted gold surface, which enabled the collagen aggregates to anchor. Gentle water (MilliQ) rinsing was performed immediately in order to eliminate the PBS salts.

The gold substrates were obtained by titanium and subsequent gold deposition on silicon wafers (electron beam thermal evaporation; Siltronix, France; detailed description of gold substrates preparation described elsewhere [19]).

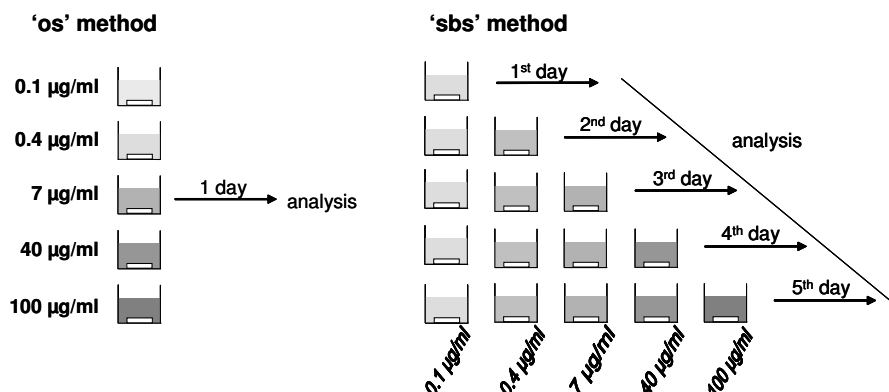


Figure II.2.1: Schematic representation of the two collagen adsorption procedures ‘one step’ (‘os’; left) and ‘step by step’ (‘sbs’; right). The collagen concentrations were 0.1, 0.4, 7, 40 and 100 µg/ml. The duration of each adsorption step was 24 h. The shade of the grey color indicates the increasing collagen solution concentration

A metal substrate was initially chosen in order to examine the collagen structures by scanning electron microscopy (SEM). However, SEM provided a poor resolution compared to AFM and thus, SEM images will not be presented. In comparison with polymers spin-coated on glass slides, gold substrates were much rougher (root-mean-square roughness $R_{r.m.s.}$ around 2.5 nm for gold and around 0.4 nm for polymers as computed from $5\text{ }\mu\text{m} \times 5\text{ }\mu\text{m}$ AFM contact mode images).

II.2.2.4 Measurements of wetting properties

Wetting of the polymers with water ($\text{pH} \sim 5.6$) was examined in dynamic conditions using the Wilhelmy plate method, with a DCA 322 equipment from Cahn Instruments (Cerrios, USA). All measurements were performed at room temperature using glass coated on both sides with the polymers. Seven successive immersion-emersion cycles (advancing-receding) were recorded as a function of the position (X) of the three-phases contact line [28]. For PS, PSOH and PS-co-PSCOOH samples, the rate of sample displacement was 20 µm/s. Cycles 4, 5 and 6 were run with a stop of 10 min, 30 min and 2 h, respectively, at maximum immersion. For PSox samples, the rate of sample displacement was 50 µm/s. A stop of 10 min, 30 min and 2 h was performed prior to cycles 4,

5 and 6, respectively (*i.e.* at maximum emersion). Each determination was performed twice.

In addition, water contact angles were measured for all polymers at ambient temperature using the sessile drop method. The water (MilliQ) droplet volume was 0.3 μl . Each determination was performed by averaging the results obtained on 10 droplets.

II.2.2.5 Atomic force microscopy (AFM)

AFM examination was performed at room temperature, on dry, collagen-conditioned samples, using a Nanoscope III MultiMode AFM (Digital Instrument, Santa Barbara, CA) in contact (CM) and tapping (TM) mode. The samples were fixed to the AFM pucks using a double-sided tape.

In CM, microlever D (spring constant of 0.03 N/m) of commercial silicon nitride (Park Scientific Instruments, Sunny Vale, CA) probes was used in all examinations. Topographic images (height) of $5\text{ }\mu\text{m} \times 5\text{ }\mu\text{m}$ were obtained by scanning the surface at a minimum loading force with a scan rate in the range of 2-4 Hz.

In TM, commercial etched silicon probes (Digital Instruments) were used; the spring constant ranged between 20 and 100 N/m and the resonant frequency between 300 and 362 kHz. The height images (constructed by the change in sample height needed to keep the cantilever vibrational amplitude constant) were recorded in light tapping conditions (A_s/A_0 in the range of 0.8-0.9 where A_s = setpoint amplitude and A_0 = free oscillation amplitude). Amplitude images were also recorded (constructed using the error signal from the feedback loop). The scan rate for $5\text{ }\mu\text{m} \times 5\text{ }\mu\text{m}$ images was in the range of 0.3-0.5 Hz.

The presented images were flattened using a third-order polynomial, with the image analysis software provided with the instrument. The cross-sections were taken in the middle of the non-flattened height images, unless specified otherwise.

II.2.2.6 X-ray photoelectron spectroscopy (XPS)

The XPS analyses were performed on a Kratos Axis Ultra spectrometer (Kratos Analytical, Manchester, UK) equipped with a monochromatized aluminum X-ray source (10 mA, 15 kV). The pressure in the analysis chamber was around

10^{-6} Pa. The angle between the normal to the sample and the lens axis was 0° . The hybrid lens magnification mode was used with the slope aperture resulting in an analyzed area of $700\ \mu\text{m} \times 300\ \mu\text{m}$. The pass energy was set at 40 eV for scanning individual peaks. In these conditions, the full width at half maximum (FWHM) of the $\text{Ag}_{3d5/2}$ was about 1.0 eV. Charge stabilization was achieved by using the Kratos Axis charge balance neutralization device. The spectra were recorded in the following order: survey spectrum, C_{1s} , O_{1s} , N_{1s} , Si_{2p} and C_{1s} again to check the charge stability and the absence of degradation of the sample during the analyses. Data treatment was performed with the Casa XPS program (Casa Software Ltd., UK). The binding energies were determined with respect to the C_{1s} peak component of carbon only bound to carbon and/or hydrogen, fixed at 284.8 eV. Molar concentration ratios were calculated using peak areas normalized on the basis of acquisition parameters and of the sensitivity factors and transmission function provided by the manufacturer [29].

II.2.2.7 UV-visible spectrophotometry

The UV-visible absorption spectra (DU 800 UV/visible spectrophotometer from Beckman Coulter, CA, USA) were recorded using a cell with silica windows and a light path of 10 cm to ensure higher sensitivity (homemade adaptation of the cell holder). The spectrum in absorbance was recorded from 200 to 800 nm with a scanning speed of 120 nm/min. All measurements were conducted at room temperature.

II.2.3 Results

II.2.3.1 Bare substrates characterization

The O/C molar ratios expected from stoichiometry and determined by XPS are given in Table II.2.1. The determined values were close to the expected once and are as follow: 0.002, 0.112 and 0.017 for PS, PSOH and PS-co-PSCOOH, respectively. The O/C ratio of PSox was 0.25. Typical C_{1s} and O_{1s} XPS peaks obtained on PS, PSOH, PS-co-PSCOOH and PSox are presented in Figure II.2.2. For PS and PSox they are similar to those presented

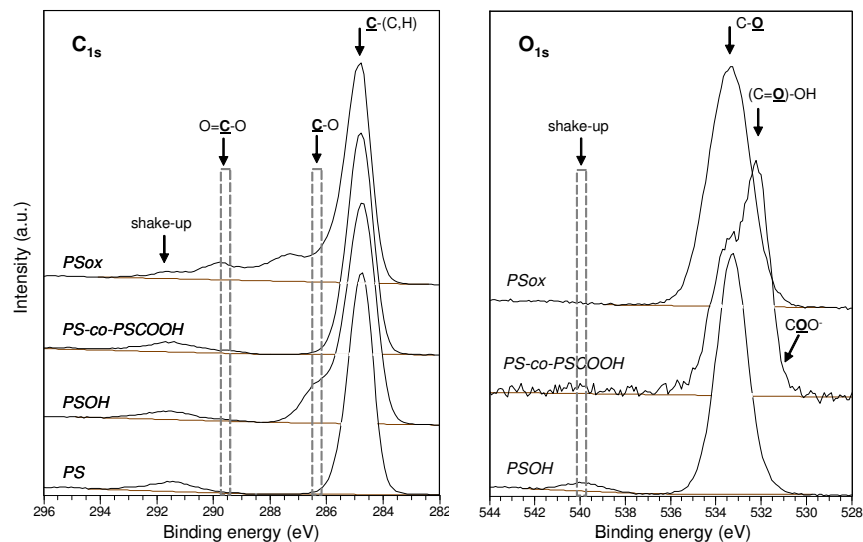


Figure II.2.2: Representative XPS C_{1s} and O_{1s} spectra recorded on PS, PSOH, PS-co-PSCOOH and PSox. The O/C molar concentration ratios for PSOH, PS-co-PSCOOH and PSox are also presented. The position of components characteristic for typical chemical functions is indicated by arrows. The ordinate scale was adjusted to obtain the maximum at the same height.

in the literature [23, 30]. For all polymers, the main C_{1s} peak situated at 284.8 eV is due to carbon atoms only bound to carbon and hydrogen [$\underline{C}$ -(C,H)] and a shake-up peak at about 291.4 eV is due to $\pi \rightarrow \pi^*$ transitions of the aromatic ring. For PSOH, a component due to alcohol functions [$\underline{C}$ -O] is observed near 286.3 eV, while for PS-co-PSCOOH, a small contribution of carboxyl functions [$O=\underline{C}$ -OH] is visible at about 289.5 eV. On PSox, a variety of functions with oxidized carbon (alcohol, carbonyl, carboxyl, carboxylate) is detected as described before [30]. The O_{1s} peak of PSOH presents a main component at about 533.3 eV [$C-\underline{O}$] and a shake-up at about 539.8 eV. PS-co-PSCOOH presents the two expected O_{1s} oxygen components: [$C-\underline{O}$] near 533.3 eV; [$(C=\underline{O})$ -OH] near 532.1 eV. However, they do not have the same intensity, presumably due to a partial deprotonation, the COO^- component being expected near 531.1 eV [31]. For PSox, the O_{1s} peak is broader due to the presence of oxygen in different functions, and shows no shake-up.

The wetting properties of PS, PSOH, PS-co-PSCOOH and PSox are pre-

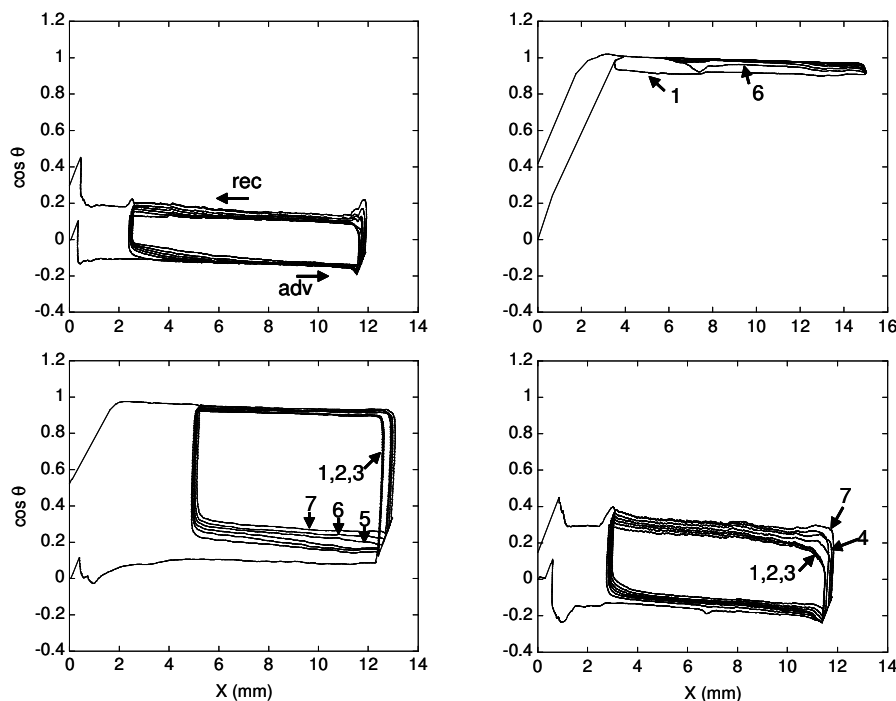


Figure II.2.3: Variation of cosine of the water contact angle (θ) as a function of the three-phases contact line position X during repeated immersion-emersion cycles in water ($\text{pH} \sim 5.6$) for PS, PSox, PSOH and PS-co-PSCOOH polymers. Cycles 1, 2 and 3 were run without any stop. For PS, PSOH and PS-co-PSCOOH, a stop of 10, 30 and 120 min was made at maximum immersion for cycles 4, 5 and 6 respectively. For PSox, a stop of 10, 30 and 120 min was made before starting cycles 4, 5 and 6 (maximum emersion). Cycle 7 was recorded immediately after cycle 6. Certain cycles are indicated by arrows.

sented in Figure II.2.3. The shape of the hysteresis loop remains almost constant upon repeating wetting cycles on PS and PS-co-PSCOOH, with advancing contact angles of $92\text{--}96^\circ$ for PS and $93\text{--}97^\circ$ for PS-co-PSCOOH, respectively. A slight shift ($84\text{--}71^\circ$) of the advancing contact angle upon repeating the wetting cycles is found on PSOH. The receding contact angle measured in the first cycle is 82° for PS, 75° for PS-co-PSCOOH and 23° for PSOH. Maintaining these materials in water for prolonged times (10 min, 30 min and 2 h during cycles 4, 5 and 6, respectively) does not influence markedly the hysteresis loop, indicating the absence of perturbation by swelling in water. PSox presents

a hydrophilic surface, as reported before for samples oxidized using another plasma reactor [30]. The hysteresis loop is very small (θ_{adv} of the first cycle equal to 23° ; other values of θ_{adv} and all values of θ_{rec} below 15°) and is not influenced by stops allowing the sample to dry before starting cycles 4, 5 and 6, indicating that there is no significant surface reorganization upon drying [30]. It was checked that the surface tension of water (Platinum ring method) was not affected by contact with the sample during wetting measurements.

Static contact angles were about 90° , 82° , 89° and 20° (average SD did not exceed 3°), respectively for PS, PSOH, PS-co-PSCOOH and PSox.

II.2.3.2 Influence of the collagen source on adsorption and aggregation

The rate of collagen aggregation upon ageing at 37°C was monitored by recording absorption spectra and measuring the absorbance at 400 nm [20]. The results presented in Table II.2.2 show that the rate of aggregation is similar for RD and AB and much larger for USB.

AFM TM images recorded after 10 min and 24 h adsorption of collagen from different sources ($40\text{ }\mu\text{g/ml}$) on PS and PSox are presented in Figure II.2.4. On PS, the RD and AB collagens formed fibrils already after 10 min adsorption with a fibril thickness of about 5 nm. After much longer adsorption (24 h), the collagen features were not significantly different. After 10 min adsorption, USB collagen formed a layer in which holes with a depth of 1 to 2.5 nm were observed (cross-section not shown); no fibrils were observed at this stage. After 24 h adsorption, large collagen fibers were observed. The cross-section taken through a single fiber revealed a periodicity with a distance between two valleys in the range of 62 to 68 nm. The background around the fibers appeared as rather smooth with small aggregates, as shown in Figure II.2.4 (top, right).

On PSox, RD and AB collagen showed small and thin filamentous aggregates after 10 min adsorption. After 24 h, more distinct fibrillar features appeared in the case of RD collagen. After 10 min adsorption on PSox, the USB collagen formed a uniform layer with small globular features. After 24 h, large fibers appeared as observed on PS. The cross-sections revealed again a periodicity along the large collagen fibers, with a distance between two valleys in the range of 63 to 64 nm. The height of the fibers was about 100 nm. The

Table II.2.2: Absorbance values measured at 400 nm for RD, AB, and USB type I collagen from calf skin; the solutions (40 and 150 $\mu\text{g}/\text{ml}$) were aged for 15 min, 30 min, 6 h, 1 day, 2 days and 7 days.

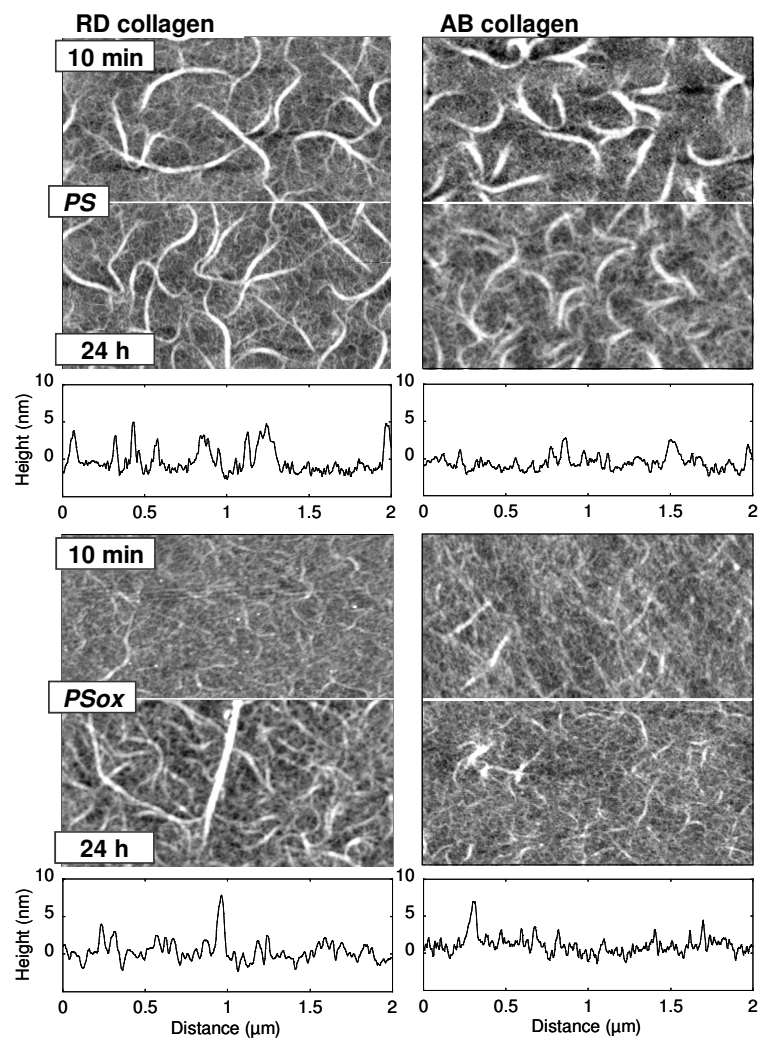
concentration ($\mu\text{g}/\text{ml}$)	aging time	absorbance at 400 nm		
		RD ^a	AB	USB
40	0.5 hour	-	0.01	0.01
	6 hours	-	0	0.01
	1 day	-	0.01	0.12
150	0.25 hour	0.01	-	0.10
	0.5 hour	-	0.01	0.31
	6 hours	-	0.01	b
	1 day	-	0.10	-
	2 days	0.06	-	-
	7 days	0.13	-	-

^a From Dupont-Gillain et al. [20]; measured at pH 7.5.

^b The measurements were not reproducible; large, elongated collagen aggregates were observed in solution.

background around the fibers (Figure II.2.4; bottom, right) was similar to that observed on PS and showed a similarity with the adsorbed layer formed on PSox after 10 min of contact with the substrate.

Representative AFM TM amplitude images of USB collagen fibers observed after adsorption on PS (40 $\mu\text{g}/\text{ml}$ collagen solution, 24 h contact at 37 °C) or collected from a 40 $\mu\text{g}/\text{ml}$ solution aged for 24 h at 37 °C), and deposited on a gold substrate are presented in Figure II.2.5. The fibers observed in adsorption experiments appeared as single twisted strands with a regular pattern or as more complex aggregates where the same strands were additionally entangled around each other. The periodicity observed on single strands (from 1 $\mu\text{m} \times 1 \mu\text{m}$ topographic image; top right) was between 62 and 74 nm (distance from valley to valley), while the height (valley to peak) was in the range of 3 to 6 nm. For collagen fibers deposited on gold, a similar periodicity was observed for single strands. In the case where several strands were involved in the fiber, a periodicity related to twisting between strands was sometimes observed. In the case of the fiber shown in the bottom right image of Figure II.2.5, the periodicity was between 280 and 230 nm (distance from valley to valley), while the height was about 13 nm (distance from valley to peak).



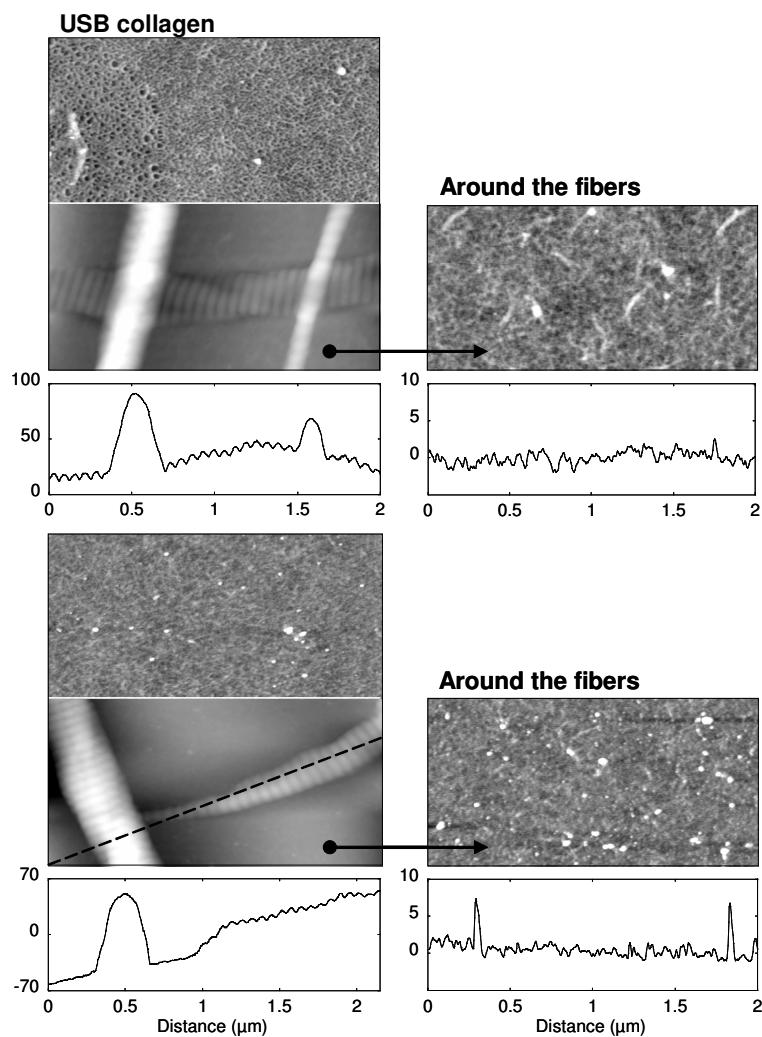


Figure II.2.4: Representative AFM tapping mode topographic images ($2\ \mu\text{m} \times 1\ \mu\text{m}$; z-range 7 nm except for USB collagen fibers, z-range 60 nm) recorded in air after adsorption of RD, AB and USB collagen ($40\ \mu\text{g}/\text{ml}$) on PS and PSox surfaces for 10 min (top half image) and 24 h (bottom half image). The cross-sections were taken in the middle of the non-flattened images or as indicated by the dashed line on the PSox-USB-24 h image. Right hand side of the figure: detailed images of the background around the fibers.

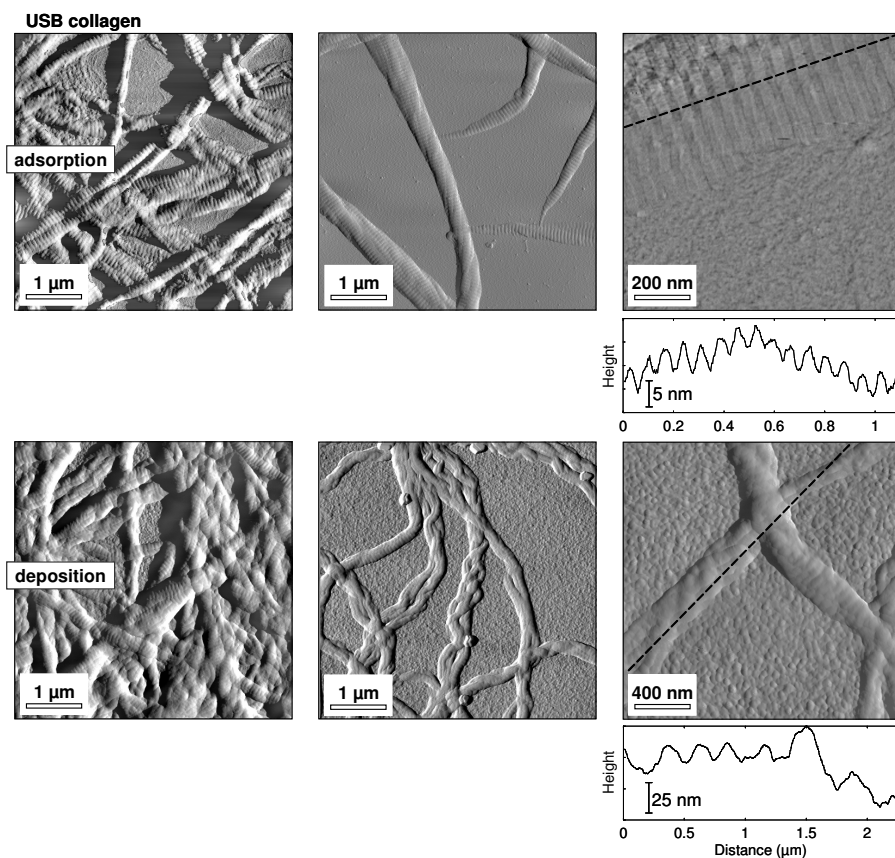


Figure II.2.5: Representative AFM tapping mode amplitude images (scale bars indicated on each image) recorded in air, either after 24 h of USB collagen adsorption on PS (top) or on fibers of USB collagen (from solution aged for 24 h at 37°C) deposited onto a gold substrate (bottom). The concentration of the collagen solution was 40 $\mu\text{g}/\text{ml}$. The cross-sections were taken from corresponding height images at the location indicated (dashed line) on the amplitude images.

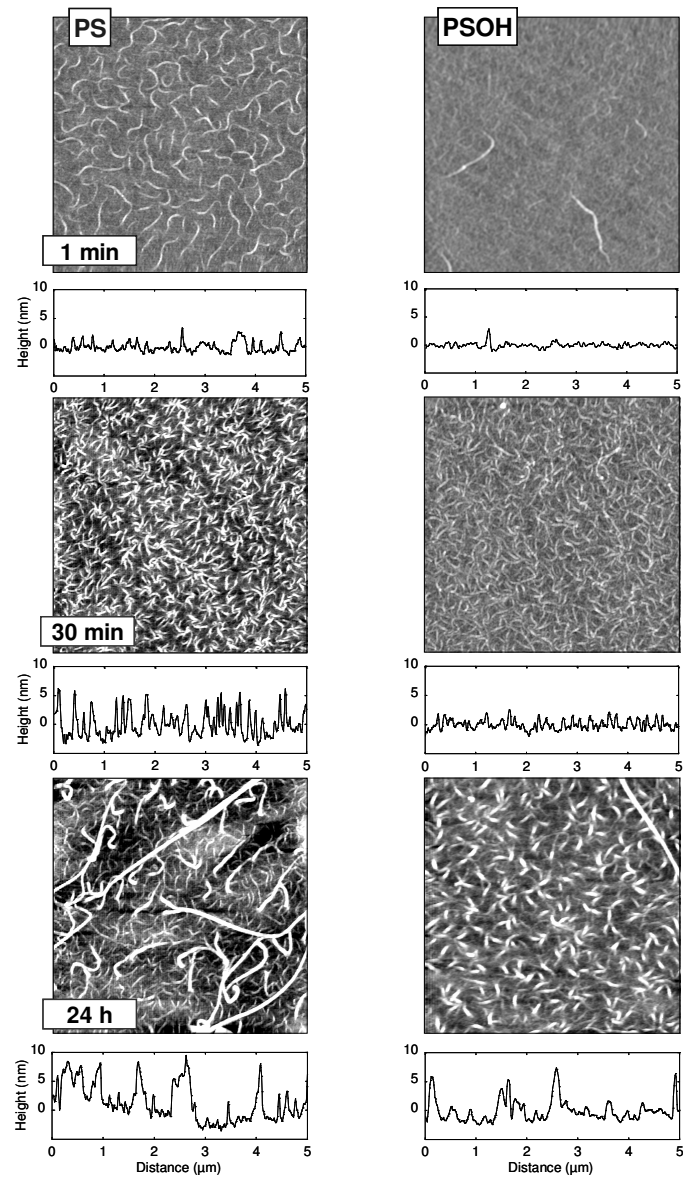
II.2.3.3 Influence of the substrate on adsorbed collagen organization

Representative AFM CM images of RD collagen layers on PS, PSOH, PS-co-PSCOOH and PSox surfaces, after 1 min, 30 min and 24 h adsorption from a 40 $\mu\text{g}/\text{ml}$ solution are presented in Figure II.2.6. After 1 min adsorption, distinct collagen features were observed on PS, while only occasional structures appeared on the oxygen-containing substrates. After 30 min adsorption, elongated features were observed on all substrates, their height increasing in the order $\text{PSox} < \text{PSOH} < \text{PS-co-PSCOOH} \approx \text{PS}$. The distribution of the aggregates was uniform and the density was comparable on PS and PSOH and seemed to be denser compared to PS-co-PSCOOH. On PSox, the density of the relief features was very low. After 24 h adsorption, bigger collagen aggregates appeared on all substrates. Occasionally, large fibers were observed on PS, PSOH and PS-co-PSCOOH. The collagen structures distributed on the whole surface seemed to be longer and thinner on PS and PS-co-PSCOOH in comparison with PSOH. The PSox surface became also covered by a uniform fibrillar layer after 24 h. The cross-section revealed that the thickness of the collagen fibrils distributed uniformly over the different polymers was in the range of 1 to 5 nm.

The C_{1s} peak recorded by XPS on pure collagen (thick layer obtained by evaporation of a drop of the stock collagen solution) is presented in Figure II.2.7 with the component assignment. The C_{1s} peaks recorded after adsorption of collagen on PS, PSOH, PS-co-PSCOOH and PSox after 24 h from 40 $\mu\text{g}/\text{ml}$ solutions are also presented in Figure II.2.7. They were fitted with a linear combination of the C_{1s} peaks of pure collagen and of bare substrates (given in Figure II.2.2) as shown in Figure II.2.7. It appears that the proportion of the carbon peak due to collagen is 55, 67, 57 and 54 % for PS, PSOH, PS-co-PSCOOH and PSox, respectively. The N/C molar concentration ratios after 24 h of adsorption were about 0.20 for PS and PSOH, 0.17 for PS-co-PSCOOH and 0.16 for PSox.

II.2.3.4 Influence of the adsorption procedure

Representative AFM topographic images recorded in air, of RD collagen layers adsorbed on PS according to the ‘one step’ (‘os’) and ‘step by step’ (‘sbs’) (Fig-



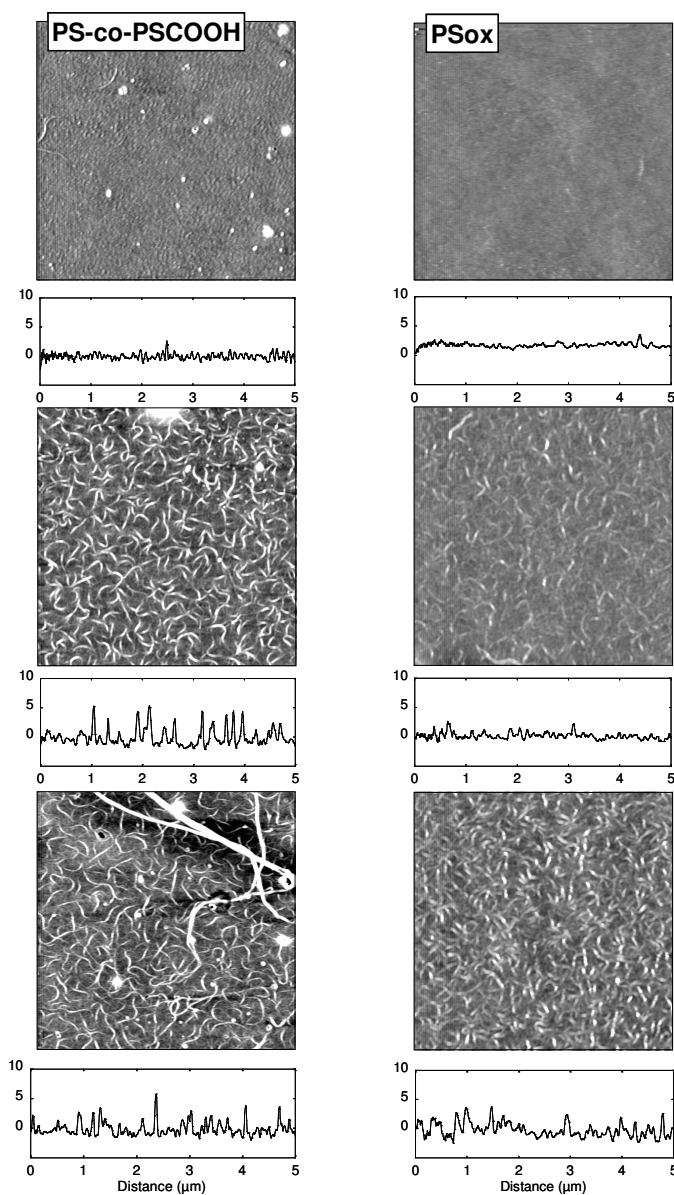


Figure II.2.6: Representative AFM contact mode topographic images ($5\ \mu\text{m} \times 5\ \mu\text{m}$; z-range 7 nm) recorded in air after 1, 30 min and 24 h of adsorption from $40\ \mu\text{g}/\text{ml}$ RD collagen solutions on PS, PSCOOH, PS-co-PSCOOH and PSox surfaces. The cross-sections were taken in the middle of the non-flattened images.

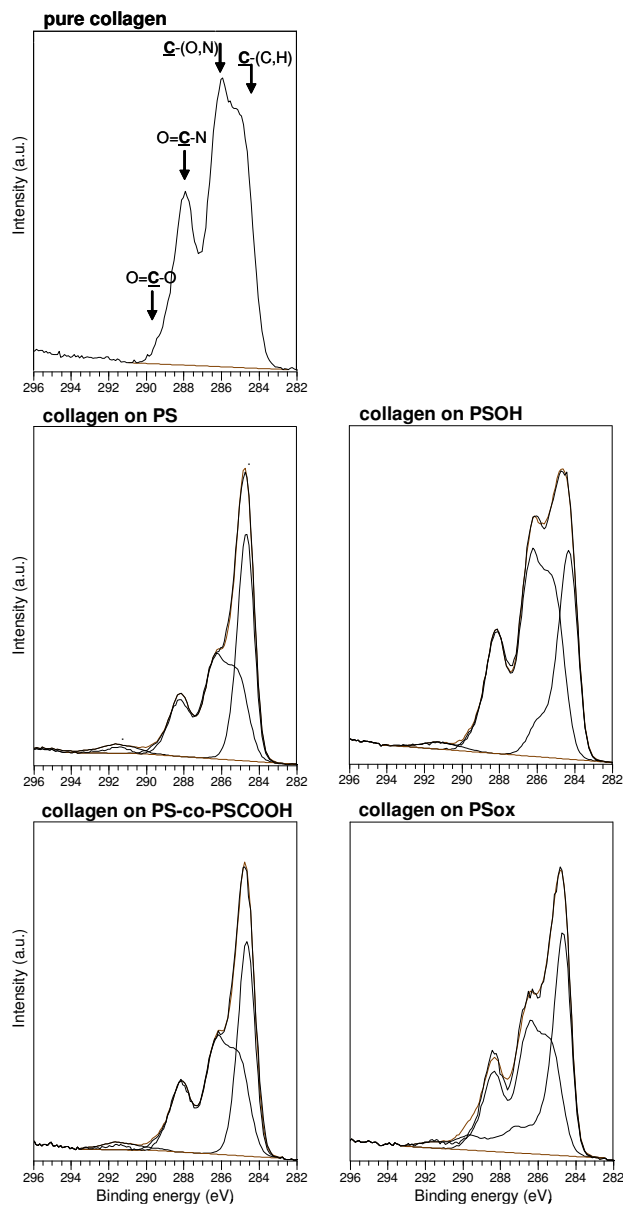


Figure II.2.7: XPS C_{1s} peaks recorded on pure RD collagen (thick layer) and after 24 h of adsorption from 40 $\mu\text{g/ml}$ RD collagen solution on PS, PSOH, PS-co-PSCOOH and PSox substrates. The peak decomposition was obtained by fitting the latter as a linear combination of the C_{1s} peak of pure collagen and the C_{1s} peak of the corresponding bare substrates (from Figure II.2.2).

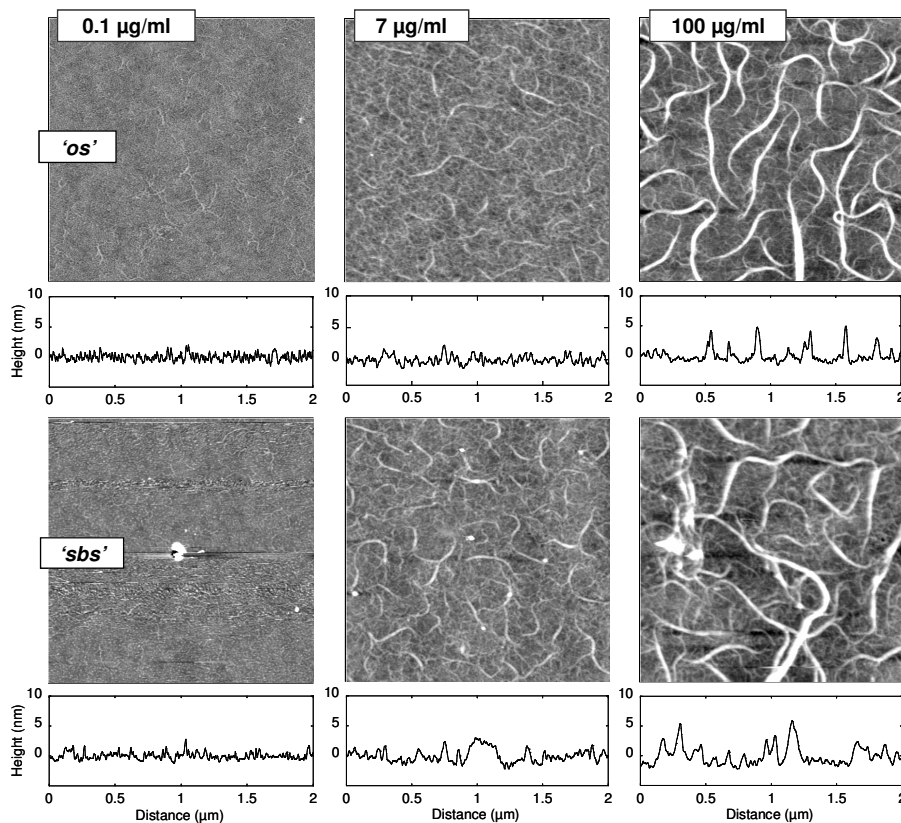


Figure II.2.8: Representative AFM tapping mode topographic images ($2\ \mu\text{m} \times 2\ \mu\text{m}$; z-range 7 nm) recorded in air on PS substrates after 24 h of RD collagen adsorption from solutions at a final concentration of 0.1, 7 and 100 $\mu\text{g/ml}$. The adsorption procedure was either ‘one step’ (top) or ‘step by step’ (bottom). The cross-sections were taken in the middle of the non-flattened images.

ure II.2.1) procedures are presented in Figure II.2.8. The images recorded when using solutions at 0.4 and 40 $\mu\text{g/ml}$ showed morphologies intermediate between those obtained with 0.1, 7 and 100 $\mu\text{g/ml}$ and are not presented. For the low concentrations (0.1 and 0.4 $\mu\text{g/ml}$), the images differed from that obtained on the bare substrate (not shown) by the occurrence of small filamentous features, also revealed by the cross-sections. With increasing concentrations (7 to 40 or 100 $\mu\text{g/ml}$), broader and longer fibrillar structures appeared, the surface coverage and height of which increased (height about 3.5 and 7 nm at 7 and

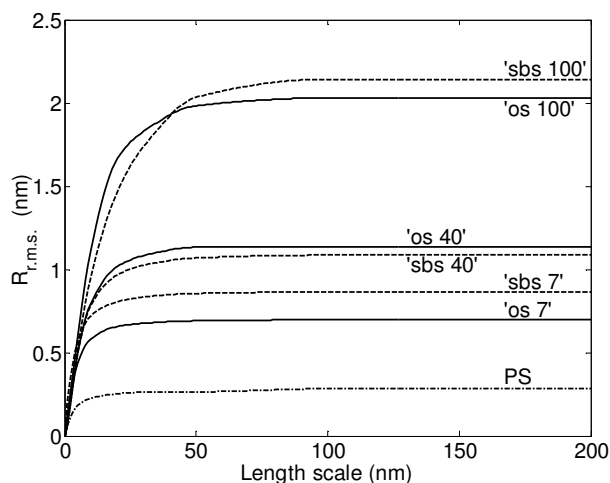


Figure II.2.9: Power Spectral Density (PSD) analysis of AFM tapping mode topographic ($5\ \mu\text{m} \times 5\ \mu\text{m}$) images recorded in air, presented as the variation of the surface roughness ($R_{r.m.s.}$) as a function of the length scale, for bare and collagen-conditioned substrates ('one step', full line; 'step by step', broken line). The presented curves are the average of analyses performed on three images from two independent sets of data.

100 $\mu\text{g}/\text{ml}$, respectively). The AFM images of the collagen layers showed a similar topography for both adsorption methods.

Figure II.2.9 presents the results of power spectral density analysis performed on AFM topographic images obtained after adsorption according to the two procedures ('os' *vs* 'sbs'), providing surface roughness ($R_{r.m.s.}$) as a function of the spatial wavelength. This is obtained by a fast Fourier transform [32]. In all cases, the $R_{r.m.s.}$ increased with length scale and reached a plateau for a length scale below 50 nm. This is much smaller than the image size ($5\ \mu\text{m} \times 5\ \mu\text{m}$) and is thus representative of the adsorbed layer topography. The length scale at which the plateau was reached and the $R_{r.m.s.}$ value at the plateau increased slightly from 7 to 40 $\mu\text{g}/\text{ml}$ and was much higher at 100 $\mu\text{g}/\text{ml}$. No significant difference was observed between the 'os' procedure and the 'sbs' procedure.

Each sample was also analyzed by XPS and showed the expected increase of the N/C concentration ratio. No significant difference was observed according to the adsorption procedure. At 100 $\mu\text{g}/\text{ml}$, the N/C ratio reached the value

of 0.15 and 0.17 for the ‘os’ procedure and the ‘sbs’ procedure, respectively.

II.2.4 Discussion

II.2.4.1 Adsorption vs aggregation in the solution

The three specimens of type I collagen investigated here differ remarkably regarding aggregation in the aqueous phase and adsorption. All three were purchased as solutions in acetic acid. To our knowledge, the extraction process for RD and USB collagen was adapted from Miller and Rhodes [33], and Gallop and Seifter [34], respectively. The role of dilute acids is to hydrolyze and to solubilize aggregates. However, collagen extracted from the same animal species may contain varying proportions of the monomers with respect to aggregates made by covalent cross-linking, depending on the extraction and purification procedure, as well as on tissue age and organ from which it is extracted. Pure monomer solutions are very difficult to obtain [4]. Another important factor is the proportion of monomer collagen with intact nonhelical ends. The lack of telopeptides significantly slows down collagen aggregation in vitro [35,36]. The content of type III collagen, about 5 % in RD, about 10 % in AB, unknown in USB, may also influence aggregation. The in vitro self-assembly study of triple-helical domains coming from two specimens of collagen indicated that the fibril diameter varied directly with the I/III molar ratio [37].

The organization of adsorbed collagen was similar for RD and AB, whether on PS or PSox (Figure II.2.4). The fibrillar structures were more pronounced on PS than on PSox at short adsorption time and developed with time on PSox. A drastically different behavior, both on PS and PSox, was observed in the case of USB collagen. After 10 min (Figure II.2.4), the PS surface was covered by a collagen layer, with small holes. This recalled the layer organization of type I collagen extracted from kangaroo, and also adsorbed on PS [38]; however, in the latter case, the hollow structures seemed to be larger and deeper. After 24 h of USB collagen adsorption, the substrate was covered by a layer which evolved only slightly as a function of adsorption time and did not show the formation of fibrils in the adsorbed phase. The large fibers observed were clearly formed in the aqueous phase, as revealed by the increase of optical density, and settled onto the substrate. As already reported for RD collagen samples which were

aggregated by a long conditioning at $\text{pH} \sim 5.8$ [20], aggregation occurring in the solution competes with adsorption. More aggregated solutions, which are depleted in free monomers, behave like less concentrated solutions, *i.e.* lead to a lower adsorbed amount and less fibril formation at the interface.

The large fibers formed in the aqueous phase present a striking regular band pattern with a periodicity of the order of 62 to 74 nm. This band pattern was first observed by electron microscopy [39] and was more recently visualized by AFM [40]. The observation of the necks with a well defined periodicity ($D \sim 69$ nm), marked by a 3 to 6 nm lower height may be attributed to the combination of an ordered lateral association of molecules due to acid-base interactions and to the gap between molecules aligned end-to-end, according to the model proposed by Hodge [41].

The collagen assemblages produced in the adsorbed phase differ markedly from those produced in the solution. The former are only small fibrils along which no periodicity is observed. As a matter of fact the fibril diameter is of the order of the height difference associated with the necks observed on the large fibers. The small size of the fibrils produced in the adsorbed phase may be attributed to the reduced mobility of adsorbed collagen in comparison with molecules in solution.

II.2.4.2 Influence of the substrate surface properties on the organization of the adsorbed collagen layers

The data reported here confirm previous information that fibrils formation in the adsorbed phase takes place more readily on PS than on PSox [23,26]. The examination of PSOH and PS-co-PSCOOH permits a deeper analysis of the relationship with wetting properties, using materials with a defined nature and concentration of polar functions (Table II.2.1). The wetting behavior of PS-co-PSCOOH is not appreciably different from that of PS. This is attributed to the low concentration of polar groups. PSox presents a hydrophilic surface at neutral pH as reported before [30]. PSOH shows an intermediate wetting behavior, with a high advancing and a low receding water contact angles. The occurrence of a large wetting hysteresis may be due to several phenomena, among which a reorientation of polar groups which tend to get into contact with water but are buried in the polymer to reduce the interfacial energy in contact

with air [42]. It is striking to observe that the tendency of collagen to form fibrils in the adsorbed phase (Figure II.2.6) varies in the order PSox < PSOH < PS-co-PSCOOH $\approx$ PS, *i.e.* in the same order as the overall hydrophobicity. The homopolymer PSCOOH would be an interesting substrate to compare with PSOH and PSox as its wetting curves are similar to those of PSOH; however it dissolves in the buffer used to prepare collagen solutions. This problem could be solved by cross-linking PSCOOH under UV light in air, keeping in mind that this may form new oxygen-containing functions [43, 44].

The differences between the organizations observed by AFM on the different substrates do not involve relief features which are sufficiently contrasted to be revealed by XPS. If the adsorbed layer is modeled as a continuous layer with constant thickness, the ratio of the contributions of collagen (I^{coll}) and the substrate (I^{sub}) to the C_{1s} peak may be computed by:

$$\frac{I_C^{coll}}{I_C^{sub}} = \frac{\lambda_C^{coll} C_C^{coll} [1 - \exp(-t/\lambda_C^{coll} \cos\theta)]}{\lambda_C^{sub} C_C^{sub} \exp(-t/\lambda_C^{coll} \cos\theta)} \quad (\text{II.2.1})$$

where λ_C^{coll} and λ_C^{sub} are the inelastic mean free path of C_{1s} photoelectrons in the collagen adsorbed layer and in the substrate ($\lambda_C^{coll} = 3.5$ nm; $\lambda_C^{sub} = 3.8$ nm for PS, considered to be the same for PSOH [45]); C_C^{coll} and C_C^{sub} are the concentrations of carbon in collagen and in the substrates ($C_C^{coll} = 58.9$ mmol/cm³ as calculated from the collagen type I calf skin sequence [4], considering a density of 1.4 g/cm³; $C_C^{sub} = 79.7$ mmol/cm³ for PS and 77.2 mmol/cm³ for PSOH); θ is the angle of photoelectron collection (0°); t is the cumulated thickness (neglecting voids) of the adsorbed layer. Fitting the experimental data ($I^{coll}/I^{sub} = 1.22$ for PS and 2.0 for PSOH) with this equation leads to an apparent collagen thickness of 3.6 nm on PS and 4.8 nm on PSOH. This difference is not considered to be significant owing to the limited precision of experimental data and approximations involved in the model. The deduced thickness may be compared with values of about 3 nm obtained for continuous collagen layers formed by adsorption on CH₃-terminated self-assembled alkanethiol from a 30 μ g/ml solution [19].

II.2.4.3 Mechanisms controlling collagen fibril formation in the adsorbed phase

The results presented here confirm that (i) fibril formation in the adsorbed state is favored by increasing adsorption time and collagen concentration, the occurrence of aggregation in the solution leading to the same effects as decreasing the concentration; (ii) fibrils formation occurs more readily if the substrate surface is more hydrophobic. On the other hand, the shape of adsorption isotherms indicated a higher affinity of collagen for PS compared to PSox [23, 26]. However, exchange between adsorbed collagen and collagen in the solution occurred more readily on PS compared to PSox [22].

A tentative explanation for the paradox between higher surface affinity, on the one hand, and increased mobility and organization, on the other hand, could be that the sticking efficacy of the collagen-surface collision is higher on PS than on less hydrophobic substrates. As a consequence, the PS would be quickly occupied by a high density of molecules retained by a small number of contact points, while on less hydrophobic substrates more space and, or time would be left for the molecules to adsorb in lying position, thereby engaging more segments in close contact with the surface. According to this tentative explanation, the adsorption of collagen on PS following the ‘sbs’ procedure could favor a stronger attachment of collagen to the surface compared to the ‘os’ procedure and could impede the formation of fibrils in the adsorbed phase. Figure II.2.8 and II.2.9 demonstrate that this is not observed: the supply of collagen molecules to the interface in a slow and progressive way (‘sbs’ procedure) leads to the same surface layer organization as the supply provided by a quick and massive procedure (‘os’ procedure).

Two aspects may then be considered further: (i) the non appropriate selection of PS as the model substrate to perform ‘sbs’ procedure and (ii) the nature of the collagen-substrate interactions on hydrophobic and hydrophilic substrates. Gurdak et al. [22] observed that radiolabeled RD collagen prepared in PBS solutions and brought in contact with adsorbed non-labeled collagen, revealed a much higher exchange with PS as the substrate compared to PSox (about 17-26 % on PS, close to zero on PSox). Deyme et al. [46] reported that, in the case of rat tail tendon collagen adsorption/desorption at pH about 2.7 on polyethylene (PE; water contact angle 95 °) and poly(maleic

acid) grafted polyethylene (grafted PE; water contact angle 74°), the amount desorbed by rinsing with buffer was higher from PE compared to grafted PE. When using mica (water contact angle close to 0°) as a substrate in adsorption/desorption measurements, less collagen was desorbed from mica surface compared to grafted PE [47]. This confirms that the adsorption of collagen molecules is more labile on hydrophobic surfaces. The reorganization within the adsorbed phase on PS could be a process too quick to be differentiated by the ‘os’ and ‘sbs’ adsorption procedures. Accordingly, it would have been preferable to compare ‘os’ and ‘sbs’ procedures using PSox rather than PS. The reason why collagen exchanges and reorganizes less readily on a less hydrophobic surface is not understood. A possible clue might be provided by a direct study of collagen mobility at the surface, as a function of the adsorbed amount, using fluorescence recovery after photobleaching [48] or single particle tracking [49] techniques.

II.2.5 Conclusion

The three type I collagen specimens investigated in this work show that fibril formation in the adsorbed phase is strongly influenced by aggregation in the solution. With respect to adsorption, more aggregated solutions behave like less concentrated and lead to less fibril formation at the interface. The morphology of the collagen assemblages also differs significantly. In the case of interfacial assemblages, small fibrils without regular band pattern are observed, while aggregates formed in solution appear as big fibers with well characterized periodicity.

Fibril formation in the adsorbed phase increases when the adsorption time is longer and when the solution concentration is higher. The substrate surface hydrophobicity is also a determining factor which favors collagen molecules organization in the adsorbed phase. As a result of previous investigations, it is known that collagen molecules have a higher apparent surface affinity, on the one hand, and an increased mobility, on the other hand, on hydrophobic compared to hydrophilic surfaces. The possible explanation of this paradox by the quick formation of a dense layer of collagen molecules forming a small number of contact points with a hydrophobic surface could not be demonstrated

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by a comparison of adsorption procedures leading to a progressive adsorption, and to a quick and massive adsorption, respectively. Further investigations where collagen mobility at the surface is directly probed may give more insight into the influence of surface hydrophobicity on collagen molecules assembly in the adsorbed phase.

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Chapter II.3

Influence of denaturation on the nanoscale organization of adsorbed collagen layers

Summary

Adsorption (at 37 °C) of type I collagen in native and heat-denatured (30 min at 40 and 90 °C) forms, on polystyrene was studied using quartz crystal microbalance with energy dissipation monitoring (QCM-D), atomic force microscopy (AFM) in tapping mode and X-ray photoelectron spectroscopy (XPS). The significance of the parameters deduced from QCM-D data was examined by comparing different approaches. The adsorbed layer of native collagen has a complex organization consisting of a thin mat of molecules near the surface, in which fibrils develop depending on concentration and time, and of a thicker overlayer containing protruding molecules or bundles which modified noticeably the local viscosity. As a result of drastic denaturation, the ability of collagen to assemble into fibrils in the adsorbed phase is lost and the protrusion of molecules into the aqueous phase is much less pronounced. The adsorbed layer of denatured collagen appears essentially as a monolayer of flattened coils. At low concentration, this is easily displaced upon drying, leading to particular dewetting figures; at high concentration, aggregates add to the first layer. Moderate denaturation leads to an adsorbed phase which shows properties intermediate between those observed with native and extensively denatured collagen, regarding both the ability to form fibrillar structures and the adlayer thickness and viscosity.

II.3.1 Introduction

Collagen is the major structural protein of the extracellular matrix. The type I collagen molecule (length ~ 300 nm, diameter ~ 1.5 nm) is a right-handed helix composed of three polypeptide chains with non helical ends (telopeptides). Each polypeptide chain is a left-handed helix and consists of repeating sequences of amino acids, $(G-X-Y)_n$, with glycine (G) found every third residue. About 20 % of the X and Y positions are occupied by proline and hydroxyproline [1–3]. The lateral and end-to-end (via N- and C-terminated telopeptides) self-association of molecules gives rise to the formation of highly ordered structures such as microfibrils and fibres.

The collagen triple-helix is stabilized, on the one hand, via direct inter-chain hydrogen bonds, and on the other hand, via inter- and intrachain water-mediated hydrogen bonds. The presence of hydroxyproline residues is crucial in strengthening collagen molecules, since water-mediated hydrogen bonds are formed between two hydroxyproline residues [2, 4, 5]. As a consequence, the packing of collagen molecules is largely determined by their hydration shell.

Collagen can be denatured upon raising the temperature. The denaturation process is initiated in so-called thermally labile domains where the concentration of hydroxyproline is low [6, 7]. The effect of collagen heating may be reversible or irreversible. The former is reached by moderate heating, for which only local unfolding within the protein takes place. The latter occurs under severe heating, for which the breaking of longer sequences of hydrogen bonds takes place [8]. The temperature above which denaturation occurs depends on the collagen specimen. For instance, type I human collagen denatures above 41.5°C , whereas collagen from the skin of an ice fish denatures at 6°C [9].

Heat denaturation of collagen is an important process not only in solution but also at interfaces. Volloch et al. [10] reported that the growth of aged cells on denatured collagen matrices attenuated the aging-related processes; moreover it appeared to reduce the rate of aging of young cells. Elliott et al. [11] showed with smooth muscle cells that thin films of temperature-denatured collagen promoted cell proliferation in contrast with native collagen films. Growen et al. [12] also reported that multifunctional immune cells as macrophages adhered poorly to fibrillar collagen. Much better adhesion was observed when the protein matrix was replaced by monomeric or heat-denatured forms of collagen.

The kinetics of the helix-coil transition of collagen upon denaturation has been extensively studied using different techniques such as differential scanning calorimetry [13], optical polarimetry [14], light and neutron scattering [15], and rheology [16] to determine thermodynamic parameters, reversion rate as well as helix conformational properties.

The quartz crystal microbalance (QCM) has been successfully applied to measure protein adsorption kinetics [17], antibody - antigen binding [18] as well as cell adhesion [19]. The merit of QCM lies in its high mass sensitivity (in the ng/cm^2 range) and its simplicity, as well as in the possibility to perform real-time measurements. The simultaneous measurement of energy dissipation (QCM with dissipation monitoring; QCM-D) enables to evaluate the viscosity and elasticity of the adsorbed layer [20–23].

The aim of this work is to investigate the influence of preheating on the nanoscale organization and mechanical properties of collagen layers adsorbed on a polystyrene substrate. To provide a complementary imaging, chemical and physical analysis, we have employed QCM-D, atomic force microscopy (AFM) and X-ray photoelectron spectroscopy (XPS).

II.3.2 Materials and methods

II.3.2.1 Substrata

Polystyrene (PS) (Sigma-Aldrich, Bornem, Belgium, ref. 43,010-2) was spin-coated (rotational velocity = 20,000 rpm/s, speed = 6,000 rpm, time = 30 s, volume = 40 μl) on one side of 15-mm diameter glass coverslips from a 10 % (w/w) solution in toluene (Vel, Leuven, Belgium). For QCM-D examinations, one side of the gold sensors (14-mm diameter; provided by Q-Sense, Göteborg, Sweden) was spin-coated with 30 μl of PS solution. After spin-coating, the samples were stored in a desiccator and used within a few days. The QCM-D sensors were reused; therefore they were cleaned by sonication in a mixture of toluene : water (1:1) for a few minutes, then rinsed with pure toluene and finally dried gently under a nitrogen flow.

II.3.2.2 Collagen adsorption procedure

Type I collagen from calf skin (Roche Diagnostic, Mannheim, Germany) was purchased as a solution (3 mg/ml at pH = 3.0). Denaturation was produced by heating a portion of the stock solution at 40 (*den40*) or 90 °C (*den90*) for 30 min in a water bath. The denatured collagen solutions were then immediately cooled and stored at 4 °C. Collagen solutions at 7, 40 and 1000 µg/ml (native and denatured) were prepared by dilution at 4 °C in phosphate buffered saline (PBS) (pH ~ 7.2; 137 mM NaCl, 6.44 mM KH₂PO₄, 2.7 mM KCl, 8.0 mM Na₂HPO₄ (Vel, Leuven, Belgium)) and then warmed at 37 °C for 15 min just before proceeding to the adsorption.

For AFM and XPS measurements, 4 ml of the collagen solutions were poured into the wells of a tissue culture plate (Falcon, NJ, USA) where the polymer substrates had been placed earlier. The samples were left incubating at 37 °C for the following periods of time: 1, 10, 30 min, 2, 6 and 24 h for native collagen and 1, 30 min and 24 h for denatured collagen. After the desired period of adsorption, the samples were rinsed without being withdrawn from the solutions. This was done by removing 2 ml of solution and then adding 2 ml of deionised water (Milli-Q system from Millipore, Molsheim, France), performing this operation 5 times. When the samples had to be dried, they were flushed with a stream of nitrogen to ensure quick drying, stored in a dessicator, and analyzed within 2 days.

II.3.2.3 Atomic force microscopy

In the case of AFM analysis under water, the glass slides were glued (epoxy bisphenol glue; Araldite, Sodiema, France) to the sample pucks of the AFM (Digital Instruments, Santa Barbara, CA) before collagen adsorption.

AFM topography (height) images were obtained at room temperature in tapping mode, under water or in air, using a Nanoscope III MultiMode AFM (Digital Instruments, Santa Barbara, CA). The images were recorded in light tapping conditions (A_s/A_0 in the range of 0.8-0.9 where A_s = setpoint amplitude and A_0 = free oscillation amplitude). The images were flattened using a third order polynomial.

Under water, NP-S oxidation-sharpened silicon nitride probes were used (Digital Instruments), with a force constant = 0.01-0.6 N/m and resonant fre-

quency of 5 - 50 kHz (working frequency always chosen close to 10 kHz). Images were acquired at a scanning rate of about 2-3 Hz. The samples were analyzed immediately after rinsing.

In air, tapping mode etched silicon probes (Digital Instruments) were used; the spring constant ranged between 20 and 100 N/m and the resonant frequency between 300 and 362 kHz. The scan frequency was about 2.5 Hz for $1\ \mu\text{m} \times 1\ \mu\text{m}$ images.

II.3.2.4 X-ray photoelectron spectroscopy

The XPS analyses were performed on a Kratos Axis Ultra spectrometer (Kratos Analytical, Manchester, UK) equipped with a monochromatized aluminum X-ray source (10 mA, 15 kV). The pressure in the analysis chamber was around 10^{-6} Pa. The angle between the normal to the sample and the lens axis was 0° . The hybrid lens magnification mode was used with the slope aperture resulting in an analyzed area of $700\ \mu\text{m} \times 300\ \mu\text{m}$. The pass energy was set at 40 eV. In these conditions, the full width at half maximum of the $\text{Ag}_{3d5/2}$ was about 1.0 eV. Charge stabilization was achieved by using the Kratos Axis charge balance neutralization device. The spectra were recorded in the following order: survey spectrum, C_{1s} , O_{1s} , N_{1s} , Si_{2p} and C_{1s} again to check the charge stability and the absence of degradation of the sample during the analyses. Data treatment was performed with Casa XPS program (Casa Software Ltd., UK). The binding energies were determined with respect to the C_{1s} peak component of carbon only bound to carbon and/or hydrogen, fixed at 284.8 eV. Molar concentration ratios were calculated using peak areas normalized according to the acquisition parameters, and the sensitivity factors and transmission function provided by the manufacturer [24].

II.3.2.5 Quartz crystal microbalance with dissipation monitoring

The QCM-D measurements were performed with a Q-Sense D300 System (Göteborg, Sweden) at a temperature of $25.0 \pm 0.1^\circ\text{C}$. The QCM-D sensors with gold electrodes were polished crystals with a fundamental frequency of about 5 MHz. One side of the electrode was coated with PS (for details see ‘substrata’). Before collagen adsorption, the crystal was brought into contact with

PBS to establish a baseline. The geometry of the cell design provided a fast replacement of PBS by the collagen solution, and the response of the QCM-D sensor was monitored as a function of time. All measurements were conducted for three harmonics (3, 5 and 7) at 15, 25 and 35 MHz; some of them were repeated independently. The contribution of the adlayer may be considered to be additive to the contribution of the polystyrene coating owing to the small thickness of the latter [25]. Data analysis was performed according to the Voigt-based model [26,27]. Best fitting values of the adlayer parameters were obtained using experimental values of Δf and ΔD at harmonics 3 and 5 (Q-Tools software, Q-Sense D300, Göteborg, Sweden). Details are given elsewhere concerning the QCM-D experimental setup [21,22], the Voigt model used in QCM-D modeling [28,29], the fitting procedure as well as the sensitivity of the results with respect to the parameters involved in the model [30].

II.3.3 Results

II.3.3.1 Morphology of collagen layers by AFM

Representative AFM images obtained under water, after 1, 30 min and 24 h of adsorption on PS surfaces from a collagen solution at 40 $\mu\text{g}/\text{ml}$, are shown in Figure II.3.1, for native collagen and collagen denatured at 40 and 90 °C. Different collagen layer organizations were observed depending on heat-denaturation. For native collagen, fibril formation was observed as described before [31–33]. Cross-sections revealed a fibril thickness up to 10 nm. For *den40* collagen, the density of fibrils was strongly reduced. For *den90* collagen, no fibrils were visible, and globular features appeared after 30 min and 24 h (diameter 20 to 30 nm, thickness of the order of 1 nm).

Representative AFM images recorded in air after 2 h adsorption of denatured collagen using different concentrations (7, 40 and 1000 $\mu\text{g}/\text{ml}$) are shown in Figure II.3.2. For collagen *den40* at 7 and 40 $\mu\text{g}/\text{ml}$ and collagen *den90* at 40 $\mu\text{g}/\text{ml}$, globular structures with a size similar to that described for collagen *den90* under water were observed. However, many of them (see *den40* at 7 $\mu\text{g}/\text{ml}$) presented a depression in the center. Larger holes were also observed, showing depths up to 4 nm, surrounded by a ring characterized by a thickness of 1 to 2 nm above the background. The depth of about 4 nm seemed

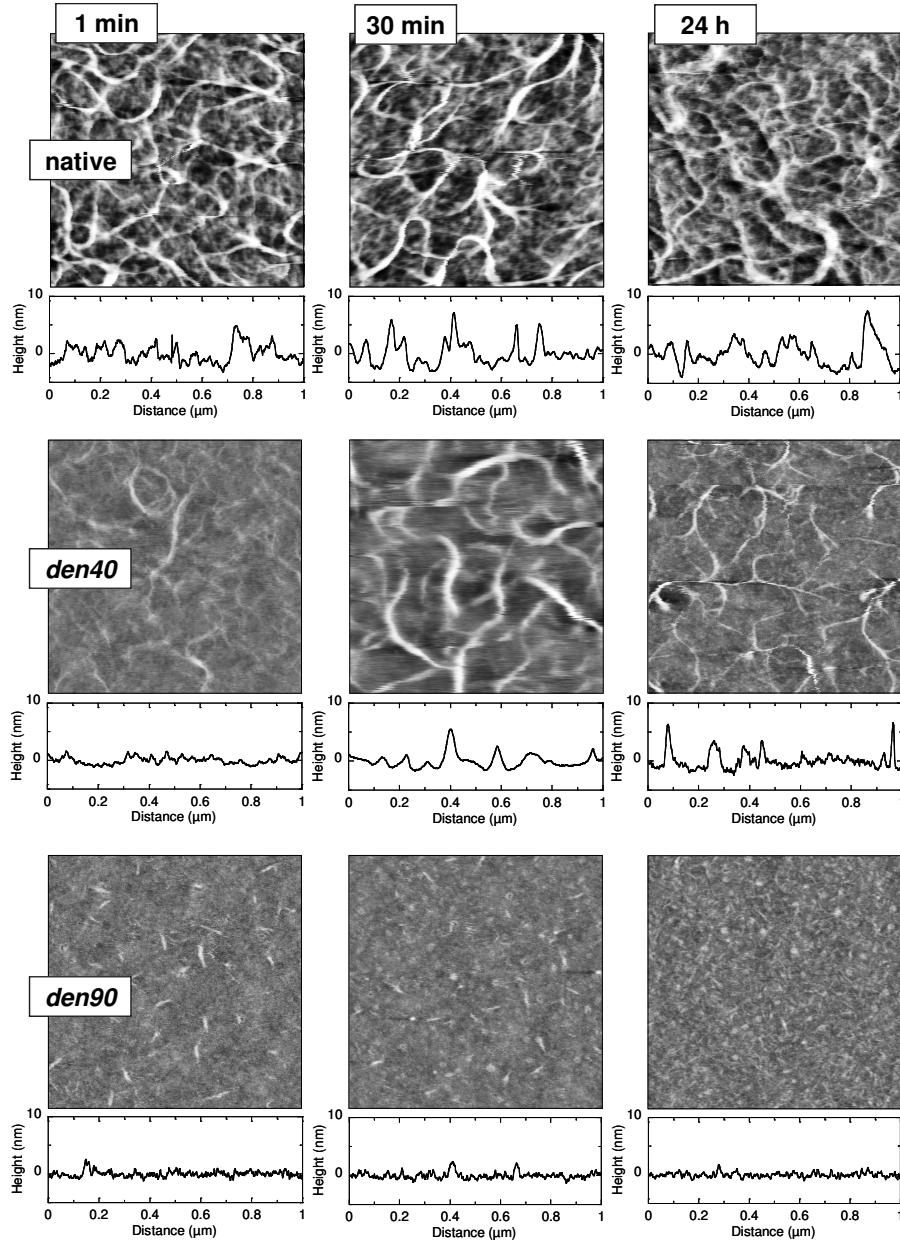


Figure II.3.1: AFM topographic images (1 $\mu\text{m} \times 1 \mu\text{m}$, z-range = 15 nm) recorded in tapping mode under water on PS substrates after 1 min, 30 min and 24 h of adsorption (from left to right) at a collagen concentration of 40 $\mu\text{g}/\text{ml}$. Top line, native collagen; central line, collagen preheated at 40 $^{\circ}\text{C}$; bottom line, collagen preheated at 90 $^{\circ}\text{C}$. The cross-sections were taken in the middle of the non-flattened images.

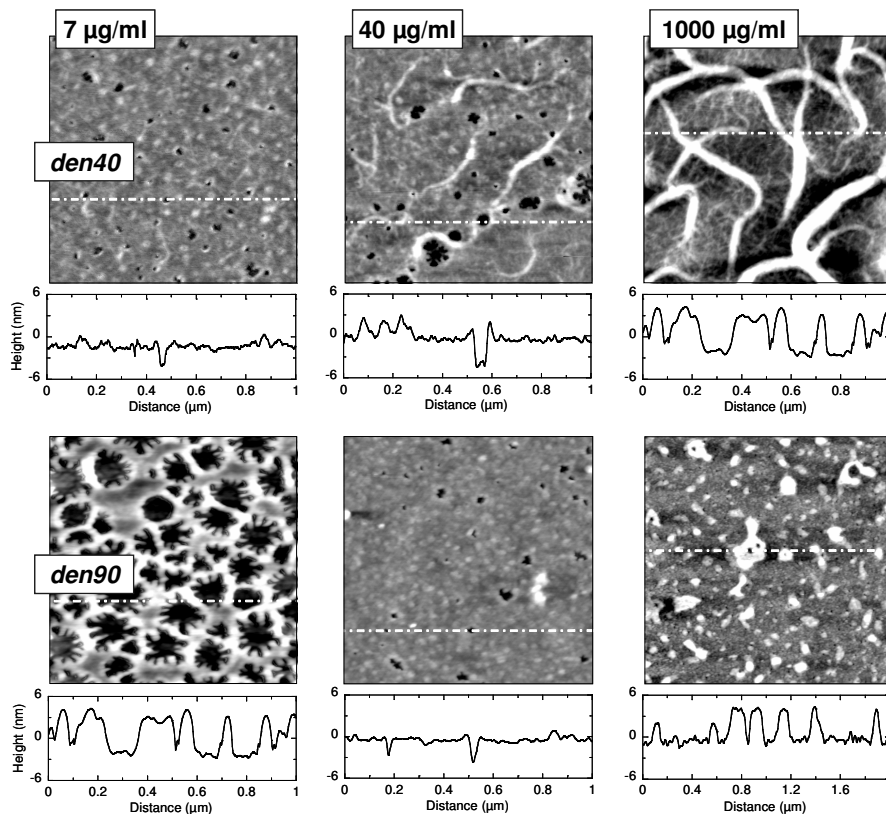


Figure II.3.2: AFM topographic images ($1\ \mu\text{m} \times 1\ \mu\text{m}$, $z\text{-range} = 10\ \text{nm}$, except for *den90*/1000 $\mu\text{g/ml}$, $2\ \mu\text{m} \times 2\ \mu\text{m}$, $z\text{-range} = 10\ \text{nm}$) recorded in tapping mode in air on PS substrates after 2 h of adsorption of collagen preheated at 40°C (*den40*) and 90°C (*den90*), using concentrations of 7, 40 and 1000 $\mu\text{g/ml}$. The cross-sections were taken from non-flattened images as indicated by the dotted lines.

to correspond to the average thickness of the adsorbed layer. The shape of the holes indicated that they were formed by a process in which smaller holes were growing and merging together. For the system *den90* at 7 $\mu\text{g/ml}$, this process leads to a layer in which star-shape holes about 6 nm deep represented about 45 % of the surface (as estimated using the Bearing function of the Nanoscope III Multimode software).

For *den40* at 40 $\mu\text{g/ml}$, fibrillar structures were visible, as observed under water (Figure II.3.1). For *den40* at 1000 $\mu\text{g/ml}$, the observed layer morphology

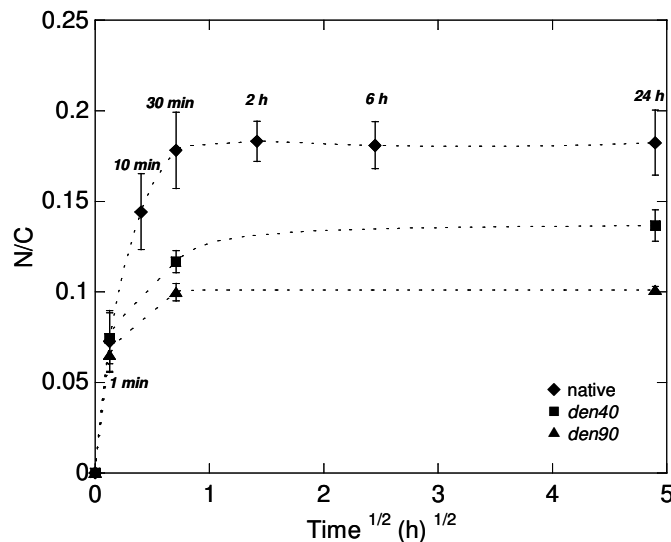


Figure II.3.3: N/C ratio determined by XPS on PS substrates after adsorption for increasing time at a collagen concentration of 40 $\mu\text{g}/\text{ml}$: native collagen (diamonds), collagen preheated at 40 $^{\circ}\text{C}$ (squares) and collagen preheated at 90 $^{\circ}\text{C}$ (triangles). The presented results are the average from four (native collagen) or three (preheated collagen) independent sets of data. The error bars show the standard deviation.

was dominated by small and large fibrillar structures recalling those found with native collagen. For *den90* at 1000 $\mu\text{g}/\text{ml}$, no fibrillar structures were observed but large (size of 40 to 200 nm) and flat aggregates (thickness of about 6 nm) were distributed on a smooth (roughness about 1 nm) adsorbed layer.

The images recorded in air after adsorption of collagen denatured at 60 $^{\circ}\text{C}$ (*den60*) at the concentrations of 7, 40 and 1000 $\mu\text{g}/\text{ml}$ showed similar layer morphology compared to collagen *den40*: globular structures with holes at 7 $\mu\text{g}/\text{ml}$, globular and fibrillar structures at 40 $\mu\text{g}/\text{ml}$ and finally well formed fibrils at 1000 $\mu\text{g}/\text{ml}$ (not shown).

II.3.3.2 Adsorption kinetics by XPS

The evolution of the N/C molar concentration ratio as a function of the adsorption time is presented in Figure II.3.3, for native and denatured collagen, at a concentration of 40 $\mu\text{g}/\text{ml}$. In all cases, the N/C ratio reached a plateau after

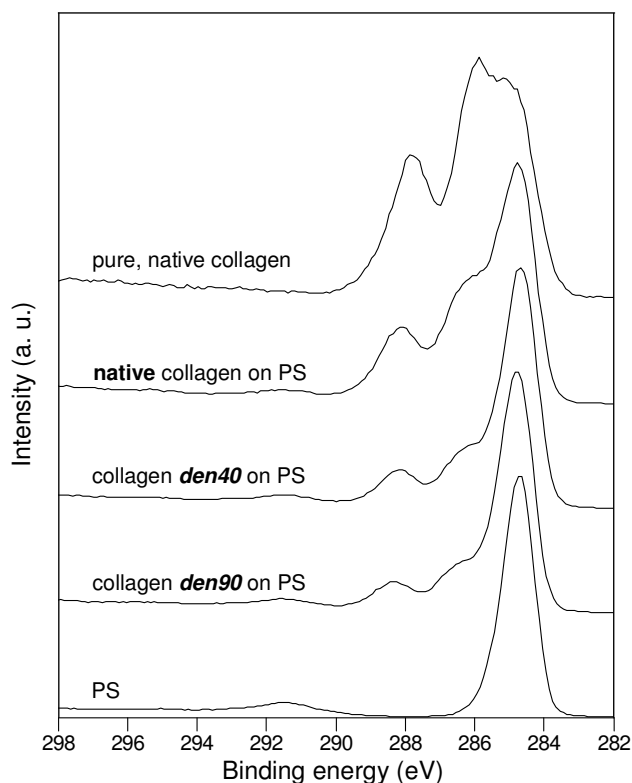


Figure II.3.4: C_{1s} spectra recorded on PS substrates after 24 h of adsorption of native or preheated collagen from 40 $\mu\text{g}/\text{ml}$ solutions. The spectra of native polystyrene (PS) and ‘pure’, native collagen (*i.e.* thick layer formed by drop deposition) are also shown for comparison. The ordinate scale was adjusted to obtain the maximum of the main peak component (284.8 eV) at the same height.

about 30 min of adsorption. For native collagen, the N/C ratio at the plateau was about 0.18. It was roughly half this value for collagen *den90* and had an intermediate value for collagen *den40*. The O/N ratios calculated at different adsorption times for the three types of collagen solutions were in the range of 1.10 to 1.16. As points of comparison, the N/C and O/N ratios for native collagen (thick film made by drop deposition of non diluted collagen on glass) were 0.26 and 1.01, respectively; they were 0.25 and 1.03 for collagen denatured

at 40 and 90 °C. The ratios computed from the amino acid composition of type I collagen from calf skin are $N/C = 0.32$, $O/N = 1.09$ [5].

Representative C_{1s} peaks recorded on PS prior to and after adsorption (40 $\mu\text{g}/\text{ml}$, 24 h) of native, *den40* and *den90* collagen are shown in Figure II.3.4. For comparison, a C_{1s} peak of pure, native collagen is also presented. For PS, the main component at 284.8 eV is due to carbon bound only to carbon and/or hydrogen $\underline{\text{C}}\text{-(C,H)}$ and the small peak at 291.7 eV is due to an energy loss attributed to $\pi \rightarrow \pi^*$ transitions of the aromatic rings (shake-up). After collagen adsorption, new components were detected, at 288.0 eV (amide, $\text{O}=\underline{\text{C}}\text{-N}$) and at about 286.3 eV (carbon singly bound to oxygen or nitrogen, $\underline{\text{C}}\text{-(O,N)}$), respectively. The contribution of the peptide link increased in the following order: *den90* < *den40* < native collagen, in agreement with N/C ratios.

II.3.3.3 QCM-D data

Typical records of frequency (Δf) and dissipation (ΔD) shifts as a function of adsorption time (40 $\mu\text{g}/\text{ml}$ solution) are shown in Figure II.3.5, for native collagen, collagen *den40* and collagen *den90*. The solid lines show the raw records whereas the symbols show fitting curves (see discussion section). The frequency and dissipation shifts decrease and increase respectively, as a result of native collagen adsorption. Less pronounced but still significant changes are recorded for collagen *den90*. The behavior of collagen *den40* is intermediate. In all cases, most of the f and D variations took place in less than 1 hour. Values of Δf and ΔD no longer changed appreciably after 3 h of adsorption of native collagen, whereas small variations were observed till 10 hours for collagen *den40* and *den90*.

The relation between ΔD and $\Delta f/3$ within a kinetic run is presented in Figure II.3.6 for the three collagen states at a concentration of 40 $\mu\text{g}/\text{ml}$, using harmonic 3. The arrows indicate adsorption times of 6 min (first arrow) and 60 min (second arrow), and the end of the plots corresponds to 10 h of adsorption. It appears from Figure II.3.6 that all adsorption runs followed the same trend, however the ranges Δf and ΔD were strongly decreased as a result of denaturation.

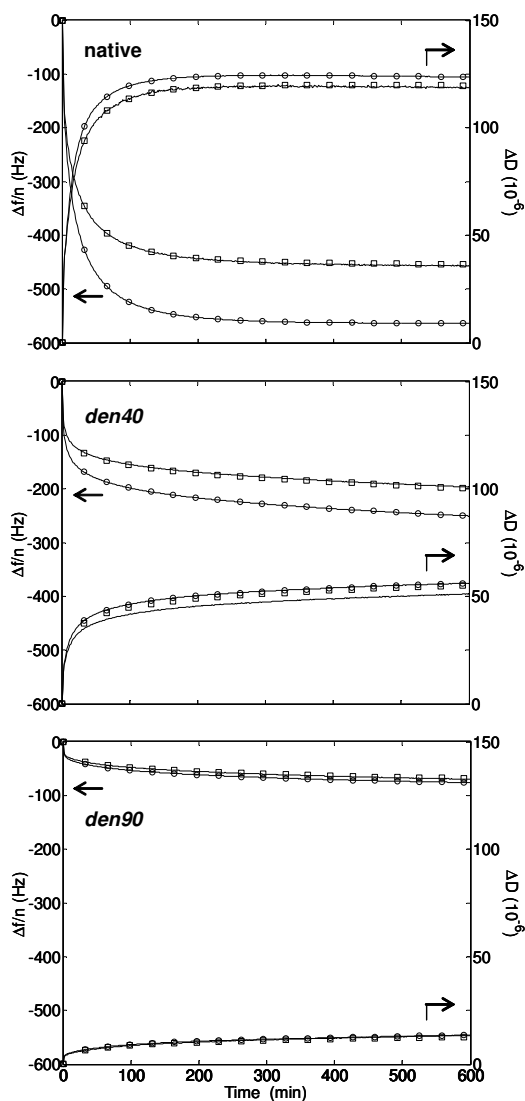


Figure II.3.5: Raw QCM-D (frequency shift, $\Delta f/n$ and dissipation shift, ΔD) records for collagen adsorption on PS (solid lines) and fitting based on the Voigt viscoelastic model (dots: circles, overtone $n = 3$; squares, overtone $n = 5$). Native, native collagen; *den40*, collagen preheated at 40 °C; *den90*, collagen preheated at 90 °C. The collagen concentration was 40 $\mu\text{g/ml}$.

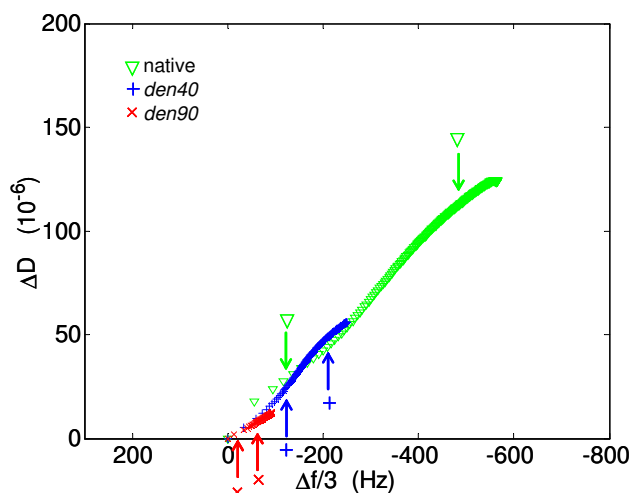


Figure II.3.6: ΔD vs $\Delta f/n$ plots using QCM-D data at the third overtone ($n = 3$). Adsorption on PS from 40 $\mu\text{g/ml}$ solutions of collagen: native (green downward triangles), denatured at 40 $^{\circ}\text{C}$ (blue crosses) and denatured at 90 $^{\circ}\text{C}$ (red x-marks). Each curve ends after 600 min of adsorption. The arrows indicate adsorption times of 6 minutes and 60 minutes.

II.3.4 Discussion

II.3.4.1 Collagen layer morphology as deduced from XPS and AFM

The AFM images obtained with native collagen adsorbed on PS substrate are in excellent agreement with data reported before (Figure II.3.1) [32, 34]. The fibril formation takes place in the adsorbed phase [35] and is dependent on the substrate surface properties, as well as on the details of the collagen solution preparation and of the adsorption procedure [32, 34]. It is favored by hydrophobicity of the substrate and also by increasing the adsorption time and/or the solution concentration. Fibril formation is thus the result of an aggregation process at different scales, starting from molecules lying along the surface, as indicated by root-like ends which seem to anchor big fibrils at the surface [11, 30].

If the adsorbed layer had a constant thickness, the N/C ratio of 0.18 (Figure II.3.3) would correspond to an effective thickness (thickness of densely

packed collagen) in the range of 3.7-4.7 nm (depending on the C, N, O concentrations considered for pure collagen: either those measured on a thick deposited film, or those calculated from the amino-acid composition of type I collagen [5]) [36].

As a result of extensive denaturation (Figure II.3.1 and II.3.2), fibrillar structures were no longer formed in the adsorbed phase. In contrast, the surface showed the presence of globular structures of about 20 to 30 nm diameter and 1 nm thickness. The holes observed after adsorption from 40 $\mu\text{g/ml}$ *den90* and *den40* collagen solutions followed by drying indicate that the layer adsorbed in these conditions has a thickness of about 4 nm (Figure II.3.2). Neglecting the holes and considering the layer to be smooth, the N/C ratios of 0.13 and 0.10 for *den40* and *den90* would correspond to a thickness of about 2.9 nm and 2.1 nm, respectively, on the basis of the XPS analysis of a thick collagen film [33, 36]. In the case where 10 % of the adsorbed layer is occupied by holes in which PS is exposed, these values become respectively about 3.6 nm and 2.5 nm. Heat-denatured collagen adsorbed from 7 and 40 $\mu\text{g/ml}$ solutions and subsequently dried thus resulted in the formation of discontinuous layers. With a higher concentration (330 $\mu\text{g/ml}$) of heat-denatured (boiling for 30 min) collagen placed in contact with self-assembled monolayers, Elliott et al. found a layer thickness of 3.6 nm [11]. The layer morphology described here for collagen *den90* at 1000 $\mu\text{g/ml}$ (Figure II.3.2) is consistent with that observed by these authors.

If we consider one of the three strands constituting the collagen molecules being about 1×10^5 g/mol, then a sphere of densely packed material (118 nm^3) would have a radius of 3 nm. A coil containing 90 vol. % of water would have a radius of 6.5 nm and a hydrated flattened coil is compatible with a particle of about $20 \times 20 \times 3 \text{ nm}^3$. The layer formed by denatured collagen may thus tentatively be viewed as a monolayer of flattened coils. At very high concentration of completely denatured collagen (*den90*, 1000 $\mu\text{g/ml}$), aggregates add to the first layer, constituting islands. This recalls the behavior of poly (N-isopropylacrylamide) adsorbed on glass, which readily formed a continuous layer at the surface, while single-chain coils, globules or aggregates were formed on top of this layer, depending on concentration and temperature [37]. Similarly, an amphiphilic diblock copolymer was found to readily form a layer of adsorbed individual molecules at a surface, on top of which micelles or micellar

aggregates were retained above the critical micelle concentration [38].

It appears that adsorbed denatured collagen is easily displaced by dewetting, leading in extreme cases to large star-shape holes in the adsorbed layer (Figure II.3.2, *den90*, 7 $\mu\text{g}/\text{ml}$). It seems that dewetting starts from the center of distinguishable particles which might be individual coils (Figure II.3.2; *den40*, 7 $\mu\text{g}/\text{ml}$; *den90*, 40 $\mu\text{g}/\text{ml}$). More detailed experiments would be needed to discuss this point further.

Preheating at 40 °C reduced drastically the amount of fibrils (Figure II.3.1 and II.3.2). However, a high density of fibrils, including large fibrils clearly taking roots in a background adsorbed layer, is found upon adsorption at a very high concentration. Partial denaturation as obtained after pretreatment at 40 °C may lead to a mixture of intact and fully denatured molecules or to molecules in which certain domains of the triple helix are unfolded. In the latter case, the structural disorganization may be partially reversible [39]. The formation of fibrils at a high concentration of partially denatured collagen (*den40*, 1000 $\mu\text{g}/\text{ml}$) may possibly be due to the displacement of preadsorbed coils by intact molecules; however in situ curing of partially disordered molecules may not be excluded.

II.3.4.2 Collagen adlayer characteristics as deduced from QCM-D

From the raw records of Δf and ΔD shifts upon collagen adsorption (Figure II.3.5; solid lines), it may be deduced that a thicker and, or more viscous adlayer is created by native compared to heat-denatured collagen and that major changes take place before 60 min adsorption time (second arrows in Figure II.3.6) for all collagens. Comparison with radioassays (the plateau of adsorbed amount is reached within 1 h [32]) and AFM, confirms that collagen adsorption mostly occurs within the first hour, while rearrangements may take place subsequently in the adlayer. The ΔD vs Δf plot shows a change of slope after about 6 min (first arrow of Figure II.3.6). In the case of adsorption of hemoglobin on self-assembled CH₃-terminated thiol monolayer, also monitored by QCM-D, Höök and co-workers [40] reported a change of ΔD vs Δf slope which was attributed to the transition between adsorption of rigidly attached molecules and formation of a second layer. However, this interpretation should

take the complex character of the QCM response into consideration.

Sauerbrey [41] has shown that the total change in mass (Δm) of a rigid, elastic and sufficiently thin adlayer on an oscillating crystal is directly proportional to the recorded frequency shift (Δf):

$$\Delta m = \left(\frac{C_{QCM}}{n} \right) \Delta f \quad (\text{II.3.1})$$

where C_{QCM} is a constant based on the physical properties of the quartz crystal ($= 17.7 \text{ ng/cm}^2\text{Hz}$ at $f = 5 \text{ MHz}$) and n is the overtone number. According to this equation and considering an adlayer density of 1050 kg/m^3 , the plateau reached by Δf with harmonics 3, 5 and 7 corresponds to thickness values presented in Table II.3.1. However, in the case of collagen adsorption (Figure II.3.5), significant dissipation shifts were recorded, indicating that the added mass acts as soft and viscoelastic adlayer. The assumptions at the basis of Sauerbrey equation are thus not met.

Figure II.3.6 indicates a correlation between ΔD and $\Delta f/n$ which is followed by all collagens, the denaturation influencing the extent of the changes resulting from adsorption. In order to extract useful information from the QCM-D data, Δf and ΔD have to be related to collagen layer properties, namely thickness h , viscosity η and shear modulus μ . This was achieved by applying the Voigt-based model [28, 29] (best fitting procedure) to the maximum Δf and ΔD values at two harmonics (3 and 5). Figure II.3.5 demonstrates a good agreement between the raw data (solid lines) and the results obtained by best fitting (symbols). Prior to fitting, the density of the collagen adlayer had to be chosen and maintained as a constant input. This parameter must be between that of water (1000 kg/m^3) and of dry protein (1400 kg/m^3) [5]. Previous work showed that the density only slightly affects the value of the best fitting parameters, thus a constant density of 1050 kg/m^3 was chosen in all computations [30]. The best fitting parameters deduced from the Δf and ΔD values measured after 10 h for native, *den40* and *den90* collagen are presented in Table II.3.1. The adlayer characteristics differ strongly depending on the collagen state: denaturation decreases the thickness, viscosity and shear modulus reached at the plateau.

The best fitting values of the parameters characterizing the collagen adlayer are not necessary the true values. Indeed from Δf - ΔD pairs measured at two

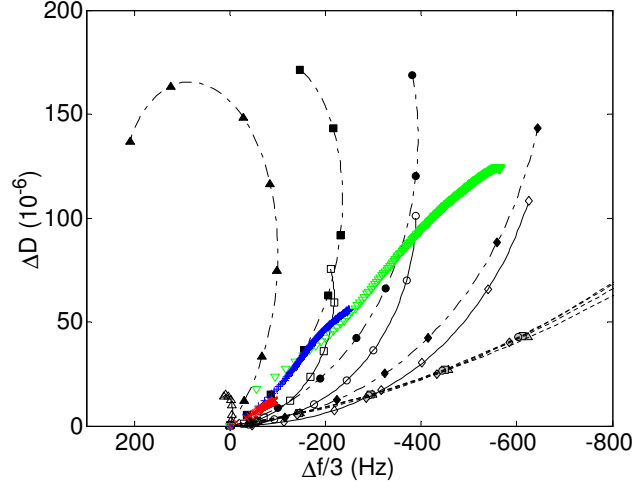


Figure II.3.7: ΔD vs Δf charts computed for a collagen adlayer, considering a density of 1050 kg/m^3 , a thickness of 0.1, 10, 25, 50, 75, 100, 150 and 200 nm (set of 8 dots in the increasing order on each spiral curve), a viscosity of $1 \times 10^{-3} \text{ Pa.s}$ (triangles), $2 \times 10^{-3} \text{ Pa.s}$ (squares), $3 \times 10^{-3} \text{ Pa.s}$ (circles), $5 \times 10^{-3} \text{ Pa.s}$ (diamonds) and a shear modulus of $1 \times 10^4 \text{ Pa}$ (open symbols, continuous curve), $1 \times 10^5 \text{ Pa}$ (black symbols, long and short strokes), $1 \times 10^6 \text{ Pa}$ (grey symbols, short strokes). In colors, plots of experimental data obtained by QCM-D for native collagen (green downward triangles), collagen denatured at 40°C (blue crosses) and collagen denatured at 90°C (red x-marks) adsorbed on PS from $40 \text{ } \mu\text{g/ml}$ solution during 10 h (reproduced from Figure II.3.6).

harmonics, three unknowns must be adjusted: thickness, viscosity and shear modulus. Munro et al. [42], studying adsorption of polyacrylamide (PA) on gold and silver surfaces, showed that the precision of the estimated film properties may be quite poor. For a film thickness determined independently (in the range of 6 to 45 nm) a large range of viscosity and shear modulus fitted the experimental data. Therefore, the sensitivity of ΔD and Δf to the parameters (h , η and μ) was examined as before [30]. Figure II.3.7 presents, for harmonic 3, a chart of dissipation shifts as a function of frequency shifts computed on the basis of the Voigt model (equations A5, A6 of reference [29]), with a thickness ranging from 0.1 to 200 nm, a viscosity varying from 1×10^{-3} to $5 \times 10^{-3} \text{ Pa.s}$ and a shear modulus between 10 and 1000 kPa. The experimental data (from Figure II.3.6) are presented in color. The same trends are given by harmonics 5

Thermal denaturation of collagen solution

Table II.3.1: Three ways to estimate the maximum thickness (h), viscosity (η) and shear modulus (μ) reached by the adlayer of collagen (native and denatured at 40 and 90 °C): (i) Sauerbrey relation, (ii) best fitting based on the Voigt model and (iii) chart shown in Figure I.20.

	native			den40			den90		
	h (nm)	η (10 ⁻³ Pa.s)	μ (kPa)	h (nm)	η (10 ⁻³ Pa.s)	μ (kPa)	h (nm)	η (10 ⁻³ Pa.s)	μ (kPa)
<i>Sauerbrey relation</i>									
$n=3$	95	-	-	42	-	-	13	-	-
$n=5$	77	-	-	33	-	-	12	-	-
$n=7$	65	-	-	28	-	-	11	-	-
<i>best fitting^a</i>									
$n=3$ and 5	165	4.3	130	140	2.1	7	56	1.4	8
<i>chart^b</i>									
$n=3$	220	4.2	10	135	2.2	10	47	1.6	10
	175	4.4	100	80	2.5	100	26	2.4	100

^a The best fitting values were obtained with the data of overtones 3 and 5.

^b The chart was generated for overtone 3; h and η were evaluated for set at 10 and 100 kPa. Collagen adsorption was performed on PS substrates from 40 μ g/ml solution and the considered adsorption duration was 10 h. In all computations, the collagen adlayer density was set at 1050 kg/m³.

and 7 (not shown). Figure II.3.7 shows that, for a given experimental pair Δf - ΔD , values of thickness, viscosity and shear modulus of the collagen adlayer are not unique. The curves based on a shear modulus of 10 kPa (white symbols) and 100 kPa (black symbols) are close to each other and may cross at high thickness. Thus, the values of shear modulus obtained from best fitting are imprecise. Table II.3.1 presents the values of the adlayer thickness and viscosity after 10 h of adsorption, evaluated using a shear modulus of 10 kPa and 100 kPa. The dependence of the assumed shear modulus is lower for the deduced viscosity, compared to the deduced thickness.

Table II.3.1 shows that, whatever the approach used to interpret the QCM-D data, the heat-denaturation leads to a strong decrease of the adlayer thickness at the end of the kinetic run. This is in agreement with the work of Silberberg and Klein [43], who also observed a significant decrease of the adsorbed layer thickness of calf skin collagen at pH 7.4, after heat-denaturation. By surface force measurements, where the force acting between two mica plates covered by collagen was measured, they found a collagen layer thickness of 212 nm and

53 nm for native and denatured collagen, respectively. By combining QCM-D and optical waveguide lightmode spectroscopy, Vörös [44] has shown that the amount of water taking part to the protein adsorbed layer is higher as the molar mass of the protein increases.

The viscosity of the adlayer obtained at the end of the kinetic run decreases correlatively with the thickness, as a result of denaturation. This may be related to the observation for *den90* that the thickness deduced from Sauerbrey equation is practically not influenced by the harmonic number (Table II.3.1), indicating that the adlayer is more solid-like. On the other hand, the relationship between ΔD and Δf over a whole kinetic run is not appreciably affected by denaturation (Figure II.3.6), indicating that the relationship between viscosity and thickness is not appreciably modified.

II.3.5 Conclusion

The adsorption of native collagen on polystyrene gives rise to a characteristic layer organization as revealed by in situ monitoring [30]: (i) AFM analysis reveals the presence of a dense and thin sheet in which fibrils are formed; (ii) QCM-D senses an overlying thick layer (up to about 200 nm), attributed to the protrusion of molecules or bundles which are in very low concentration but modify noticeably the local viscosity.

As a result of drastic denaturation by heating at 90 °C, (i) the ability of collagen to assemble and form fibrils in the adsorbed phase is lost, leading to the observation of a smooth adsorbed layer by AFM; (ii) the protrusion of molecules into the aqueous phase is much less pronounced, giving a more solid-like adlayer compared to native collagen, as revealed by QCM-D. The relative decrease of apparent thickness found by QCM-D is then higher than the relative decrease of the adsorbed amount deduced from XPS and AFM. Although denaturation reduces the thickness and the viscosity values of the adlayer obtained, the relation between thickness and viscosity is not appreciably influenced.

Moderate denaturation of the collagen solution (40 °C) leads to an adsorbed phase which shows properties intermediate between those observed with native and completely denatured collagen, regarding both the ability to form fibrillar structures, as observed by AFM, and the adlayer thickness and viscosity, as

deduced from QCM-D.

Upon fast drying, denatured collagen shows a greater tendency to be displaced, as a result of dewetting, compared to the native collagen. It seems that dewetting starts from the center of distinguishable particles which might be individual coils. It must be emphasized that the significance of the results obtained by QCM-D must be valuated by examining the sensibility of the fitted quantities (changes of the frequency and energy dissipation) to the fitting parameters (density, thickness, viscosity and shear modulus).

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Appendix II.A

Supramolecular assembly of type I collagen upon adsorption on simultaneously micro- and nanostructured PMMA surfaces

Summary

Type I collagen, an extracellular matrix protein, is widely used as a coating to control cell-material interactions. The aim of this study is to understand the effect of the topography of the substrate on the supramolecular organization of adsorbed type I collagen. Poly(methyl methacrylate) (PMMA) substrates were structured, using embossing, at the micro- and nanometer scale. The bottom of the microgrooves (depth: 70-80 nm; width at the level of the surface ~ 1.6 - $1.8 \mu\text{m}$; gentle edges) was modified with nanogrooves (depth ~ 5 nm; periodicity ~ 70 nm) running in the perpendicular direction. The periodicity of the nanogrooves recalled the banding pattern of collagen fibers, and was therefore expected to interfere with collagen assembly. The detailed study of atomic force microscopy (AFM) images obtained on collagen layers adsorbed on the structured PMMA surfaces did not reveal any influence of the fabricated topography on collagen organization. Collagen did not accumulate in the microgrooves, and the collagen assemblies did not align, neither in the microgrooves nor according to the nanopattern. The collagen layers were not perturbed by the surface structure and simply followed the fabricated contours.

II.A.1 Introduction

Collagen type I molecules are semi-flexible rods of about 300 nm in length and about 1.5 nm in diameter [1]. They are formed from three peptide chains entwined into a helix. The unusual characteristics of collagen molecules are at the origin of their self-assembling properties. The association of five collagen molecules gives rise to the formation of microfibrils, which in turn form more complex structures called fibrils. Native fibrils are characterized by a banding pattern with a periodicity of about 67 nm, known as the D-period [2, 3]. These fibrils are then entangled, together with other biomolecules, to form a part of the extracellular matrix. In *in vivo*, cells are thus in contact with an environment which is highly structured at different levels.

Collagen is widely used as a coating for *in vitro* cell culture applications. Indeed it contains regions which are specifically recognized by cell-surface receptors, or by other proteins which are in turn interacting with cell-surface receptors [2, 4]. However, recent studies have shown that beyond these recognition events, the supramolecular organization of adsorbed collagen layers has a strong effect on cell behavior [5–7]. These studies point to the important role played by the micro- and nanostructure of the extracellular matrix for the regulation of cell functions.

The design of surfaces presenting ordered collagen structures is thus a key to further understand the influence of such structures on cell behavior. Different approaches have been followed to produce artificially organized collagen layers. Collagen was ordered into concentric ring patterns by evaporating droplets of collagen solution (concentration higher than 5 mg/ml) on glass substrates [8]. Slow drying of adsorbed collagen layers on polystyrene led to the appearance of discontinuous structures [9]. Flat substrates, which were heterogeneous with respect to the surface hydrophobicity (tracks of methyl-terminated alkylsilanes drawn in an oligo(ethylene glycol)-terminated matrix), forced adsorbed collagen molecules to align along the hydrophobic tracks, owing to their width (30–90 nm) which is small compared to the length of collagen molecules [10]. Jiang and coworkers [11] aligned collagen microfibrils in a given direction on a mica substrate using adsorption under a hydrodynamic flow, and then they realigned collagen fibrils in the perpendicular direction via controlled manipulation by means of an atomic force microscope (AFM).

On the other hand, it has been established that the micro- and nanotopography of inert substrates may have a strong influence on cells [12, 13]. On microgrooves, cells are typically oriented and elongated in the groove direction, in a so-called contact-guidance process [14–16]. Nano-pillars have been shown to strongly reduce epitenon (tendon) cell adhesion [16]. Human fibroblasts cultured on nano-pits (75 and 120 nm in diameter and 100 nm in depth) experienced nanocontact guidance via cell filopodia and retraction fibers [17]. The mechanisms by which cells react to topography are not yet well known. Among several hypotheses, a possible explanation is that the substrate topography affects the organization of the adsorbed proteins involved in cell adhesion. Denis and colleagues [18] investigated the assembly of adsorbed collagen on smooth *vs* nanostructured surfaces. Protrusions of a height of ~ 15 nm separated by a distance of 50–150 nm were fabricated on a gold substrate by means of colloidal lithography. A homogeneous chemical surface composition was ensured by self-assembly of alkanethiols. On methyl-terminated substrates, the results showed that collagen assembly at the interface, which occurred on the smooth substrate, was prevented by the nanometer-scale protrusions.

The aim of the present work is to investigate the organization of collagen layers obtained by adsorption on topographically structured polymer substrates. Therefore, poly(methyl methacrylate) (PMMA) substrates were structured, using embossing, at the micro- and nanometer scale. While the microstructure consisted in grooves, the nanostructure, which was located in the bottom of the microgrooves, recalled the banding pattern of collagen. Adsorption of collagen from diluted solutions was carried out on smooth (control) and structured PMMA. Atomic force microscopy in tapping mode (TM AFM) was used to probe the surface topography of the PMMA substrates prior to and after collagen adsorption. Additionally, the surface chemical composition of the control substrates was determined by X-ray photoelectron spectroscopy (XPS).

II.A.2 Experimental

II.A.2.1 Surface structuration of PMMA

PMMA substrates were provided by N. Gadegaard (University of Glasgow). Their grooved motif was prepared in a three-stage process involving electron

beam lithography, nickel die fabrication and hot embossing. The procedure was similar to that described in details previously [19]. The aim was to fabricate a one dimensional periodic pattern with a repeat period similar to that of fibrillar collagen. It was hoped that this would encourage fibrils to form on such a template.

Briefly, resist-coated silicon substrates were exposed in a Leica HR100-EBPG5 beamwriter at 50 kV. A parallel array of microgrooves was drawn over 1 cm² area of the substrate. Each groove was designed to be flush with the surface but an over-exposure of the pattern formed a trough-like shape, with a depth of 80 nm and a width at the level of the surface of 300 nm. The distance between two adjacent grooves was 5 μ m. In each microgroove, parallel nanogrooves (depth of 5 nm) running perpendicular to the microgroove direction were fabricated with a periodicity of 70 nm.

Nickel dies were made directly from the developed patterned resists (thickness of about 300 μ m), using sputtering followed by electroplating.

Polymer replicas were made in PMMA by hot embossing. PMMA was melted on a hot plate and the nickel die pressed by a thumb into it. The PMMA samples were then transferred to a water bath at room temperature.

Smooth, control PMMA samples were cut outside of the groove-containing area.

II.A.2.2 Procedure of collagen adsorption

Type I collagen from calf skin (Roche Diagnostic, Germany) was purchased as a solution (3 mg/ml at pH = 3). To obtain the desired concentrations, the collagen stock solution was diluted in phosphate buffered saline (PBS) (pH $\sim$ 7.2; 137 mM NaCl, 6.44 mM KH₂PO₄, 2.7 mM KCl, 8.0 mM Na₂HPO₄, (Vel, Leuven, Belgium)). The collagen solutions were prepared at 4 °C; 4.5 ml of these solutions were directly placed in a tissue culture plate (Falcon, NJ, USA) and pre-warmed in a water bath at 37 °C for 15 min just before proceeding to the adsorption. After collagen solution pre-warming, polymer samples were immersed, and left for 2 h at 37 °C. After adsorption, each sample was rinsed, without being withdrawn from the solution, by 8 successive water rinsings (deionized Milli-Q water; Milli-Q system from Millipore, Molsheim, France). This was done by removing 2 ml of solution and then adding the same volume

of water. When the samples had to be dried, they were flushed with a stream of nitrogen and analyzed immediately.

II.A.2.3 Atomic force microscopy (AFM)

AFM examination was performed at room temperature, both in water on hydrated samples and in air on dried samples, using Nanoscope III and IV MultiMode AFMs (Digital Instruments, Santa Barbara, CA) in tapping mode (intermittent contact between the probe and the sample surface during scanning). Height images were recorded (the setpoint value is a given amplitude of oscillation), as well as amplitude images (the image is then built using the error signal from the feedback loop).

To perform the analysis under water, the PMMA samples were glued (epoxy bisphenol glue; Araldite, Sodiema, France) to the AFM metal pucks 2 days before adsorption. Commercial silicon nitride probes (Park Scientific Instruments, Sunny Vale, CA) were used (microlever B, spring constant of 0.24 N/m, resonant frequency of 23-37 kHz).

In air, commercial etched silicon probes, TESP (Veeco Instruments SAS) were used; the spring constant ranged between 20 and 80 N/m and the resonant frequency between 230 and 410 kHz; the working frequency was in the range of 270 to 310 kHz. The nominal radius of curvature of the tip was 10 nm.

The images, both recorded under water and in air, were acquired in light tapping conditions (A_s/A_0 in the range of 0.8-0.9 where A_s = setpoint amplitude and A_0 = free oscillation amplitude). The scan rate for $5\ \mu\text{m} \times 2.5\ \mu\text{m}$ images was in the range of 0.3-0.5 Hz. The height and amplitude images were recorded for two sample orientations with respect to the probe scanning direction, the microgrooves being positioned either parallel (scan angle of 0°) or perpendicular (scan angle of 90°) to the probe scanning direction. The presented images are either raw (non-flattened) or flattened using a zero or third order polynomial.

II.A.2.4 X-ray photoelectron spectroscopy (XPS)

The XPS analyses were performed on a Kratos Axis Ultra spectrometer (Kratos Analytical, Manchester, UK) equipped with a monochromatized aluminum X-ray source (10 mA, 15 kV). The pressure in the analysis chamber was around

10^{-6} Pa. The angle between the normal to the sample and the lens axis was 0° . The hybrid lens magnification mode was used with the slope aperture resulting in an analyzed area of $700\ \mu\text{m} \times 300\ \mu\text{m}$. The pass energy was set at 40 eV. In these conditions, the full width at half maximum of the $\text{Ag}_{3d5/2}$ peak was about 1.0 eV. Charge stabilization was achieved by using the Kratos Axis device. The spectra were recorded in the following order: survey spectrum, C_{1s} , O_{1s} , N_{1s} , Si_{2p} and C_{1s} again to check the charge stability and the absence of degradation of the sample during the analyses. Data treatment was performed with the Casa XPS program (Casa Software Ltd., UK). The binding energies were determined with respect to the C_{1s} peak component of carbon only bound to carbon and/or hydrogen, fixed at 284.8 eV. Molar concentration ratios were calculated using peak areas normalized on the basis of acquisition parameters and the sensitivity factors and transmission function provided by the manufacturer [20].

II.A.3 Results

II.A.3.1 Characterization of the smooth PMMA substrate prior to and after collagen adsorption

AFM images of a collagen layer adsorbed onto a smooth, control PMMA substrate from a solution at $100\ \mu\text{g}/\text{ml}$ are presented in Figure II.A.1. As a point of comparison, a bare, smooth PMMA substrate is also shown (Figure II.A.1a). The bare polymer showed a rather smooth surface topography (peak-to-valley roughness of the order of 5 nm). However, some deeper hollows (depth around 10-15 nm) were occasionally observed. After collagen adsorption, the polymer surface was clearly covered by the protein layer: large collagen fibrils formed a network (Figure II.A.1b) on a background layer (thickness of about 6-7 nm) presented small holes (Figure II.A.1c). Lower concentrations of the collagen solution (10 and $40\ \mu\text{g}/\text{ml}$; results not shown) led to a much lower coverage of the surface by much shorter collagen assemblies.

XPS measurements performed on smooth, control PMMA samples prior to and after collagen adsorption (solution at $100\ \mu\text{g}/\text{ml}$) showed the appearance of a nitrogen signal ($\text{N}/\text{C} = 0.19$) following adsorption. The O/C molar concentration ratio was not significantly affected by collagen adsorption ($\text{O}/\text{C} = 0.26$ and 0.28 before and after adsorption, respectively). The corresponding C_{1s} and

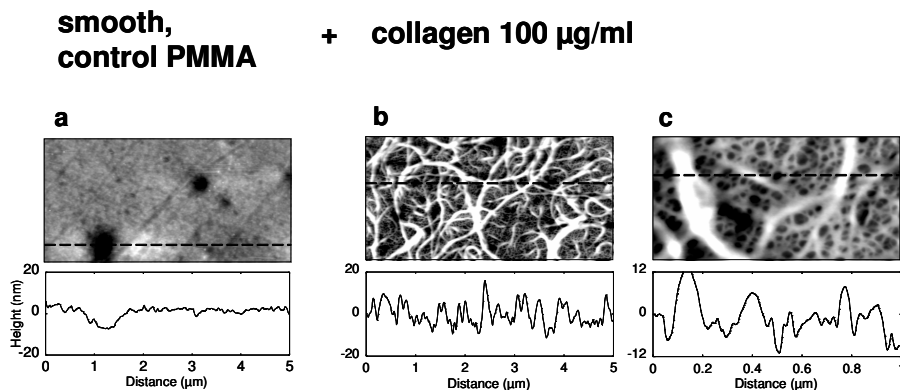


Figure II.A.1: AFM topographic images recorded in air of smooth, control PMMA before (a) and after (b,c) collagen adsorption from a solution at 100 $\mu\text{g/ml}$. Images size: 5 $\mu\text{m} \times 2.5 \mu\text{m}$; $z = 15 \text{ nm}$ (a,b) and 1 $\mu\text{m} \times 0.5 \mu\text{m}$; $z = 15 \text{ nm}$ (c). The images were flattened using a third-order polynomial. The cross-sections were taken on non-flattened images, as indicated.

O_{1s} peaks are shown in Figure II.A.2. The main component of the C_{1s} peak is due to carbon bound only to carbon and hydrogen ($\text{C}-(\text{C,H})$) at 284.8 eV (a,b). For the bare PMMA substrate (a), other components could be found within the C_{1s} peak: at 286.6 eV due to carbon singly bound to oxygen ($\text{C}-\text{O}$), and at 288.7 eV due to ester groups ($\text{O}=\text{C}-\text{O}-\text{C}$) [21]. After collagen adsorption (b), the C_{1s} peak is modified, owing to the contribution of components typical of the protein appeared: ($\text{C}-\text{N}$) at around 286.1-286.3 eV and amide groups ($\text{N}-\text{C}=\text{O}$) at 287.8 eV [22]. The O_{1s} peak recorded prior to collagen adsorption (a) showed the two components typical for PMMA, with oxygen doubly bound to carbon ($\text{C}=\text{O}$) at 531.8 eV and singly bound ($\text{C}-\text{O}$) at 533.3 eV. After collagen adsorption (b), the O_{1s} peak was modified, owing to a new component ($\text{O}=\text{C}-\text{N}$) near 531.3 eV.

II.A.3.2 Characterization of the grooved PMMA substrate

AFM topographic images recorded in air on bare, grooved PMMA surfaces are presented in Figure II.A.3. The top images (I,II) were obtained with a scanning direction nearly perpendicular to the microgrooves, while it was almost

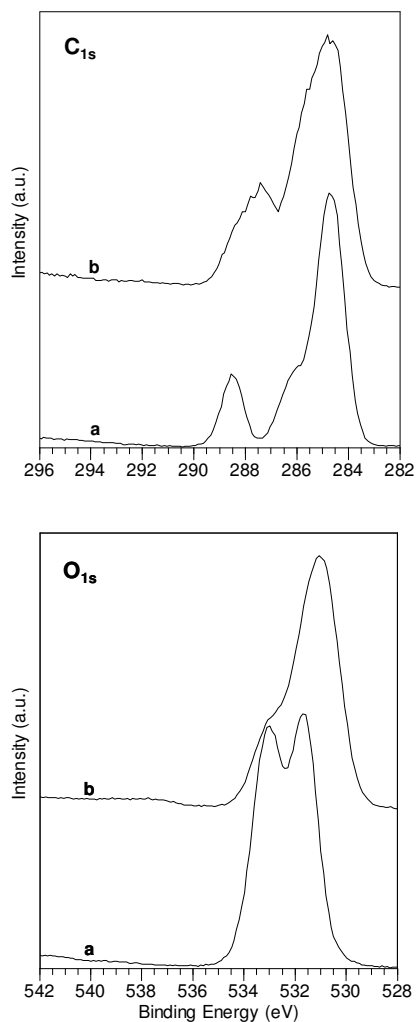


Figure II.A.2: C_{1s} and O_{1s} spectra recorded on control PMMA before (a) and after (b) collagen adsorption from a solution at $100 \mu\text{g/ml}$. The ordinate scale was adjusted to obtain the maximum of the peak at the same height.

parallel to the microgrooves for the bottom images (III,IV,V). Large size images ($100 \mu\text{m} \times 50 \mu\text{m}$) revealed that the shape and the dimensions of the microgrooves were repetitive and regular over a large area (section Ia). The depth of the grooves was around 70 to 80 nm, the distance between two ad-

grooved PMMA

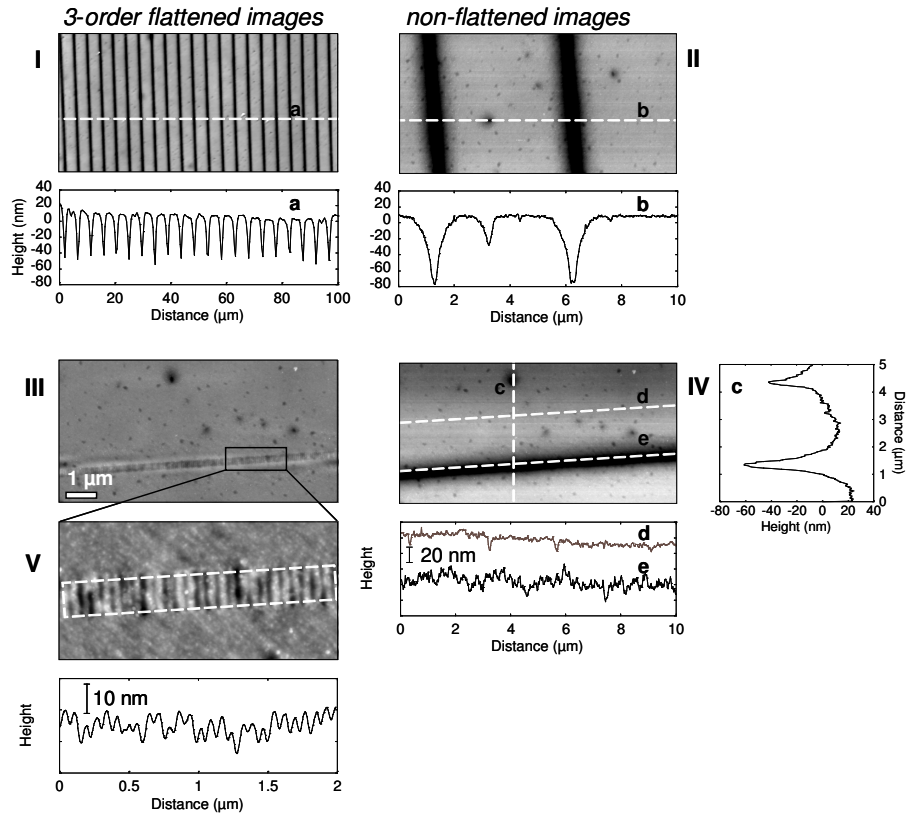


Figure II.A.3: AFM topographic images recorded in air on grooved PMMA substrate. Images size: $100\ \mu\text{m} \times 50\ \mu\text{m}$, $z = 60\ \text{nm}$ (I); $10\ \mu\text{m} \times 5\ \mu\text{m}$, $z = 60\ \text{nm}$ (II-IV); $2\ \mu\text{m} \times 1\ \mu\text{m}$, $z = 20\ \text{nm}$ (V). Images I and II were acquired by scanning perpendicular to the microgrooves direction, while images III, IV and V were acquired parallel to the microgrooves direction. Images I, III and V were flattened using 3-order polynomial, while images II and IV are raw. Images III and IV were the same and were flattened and non-flattened, image V shows the area indicated in image III at high resolution. The cross-sections were taken from the non-flattened images along the indicated dashed lines. The cross-section shown below image V is the average of all cross-sections contained in the area indicated by the dashed rectangle.

jacent groove valleys was around 5 μm , while the width of a microgroove at the level of the surface was about 1.6-1.8 μm (sections IIb, IVc). Additionally, the grooved PMMA samples were imaged in contact mode AFM (probe in contact with the surface while scanning), giving the same groove dimensions (not shown). Beside the microgrooves, the surface revealed some small defects being as deep as about 40 nm (section IIb). Topographic analysis of the same surface area, but with a scan direction parallel to the microgrooves, gave the same groove dimensions (section IVc). The roughness was higher inside the groove (section IVe) compared to outside the groove (section IVd). After flattening, high resolution image recorded with a scanning direction parallel to the microgrooves (image V) showed regular relief features, with a depth and periodicity, measured on averaged cross-sections (section V; measured on 10 nanogrooves) of about 5 nm and 76 nm, respectively.

II.A.3.3 Collagen layers adsorbed on grooved PMMA

AFM height images of different sizes (x-axis: 25, 10 and 5 μm) recorded in air on the bare, grooved PMMA substrate and on the same PMMA substrate covered with collagen layers adsorbed from solutions at 100, 300 and 600 $\mu\text{g/ml}$ are shown in Figure II.A.4, along with horizontal cross-sections. Increasing the collagen concentration led to a more dense surface coverage by the collagen fibrils (the surface defects were less and less visible). Collagen adsorption did not significantly change the depth of the microgrooves, which remained 70 to 80 nm deep whatever the concentration used. The surface roughness over the flat areas of the PMMA substrate increased with the collagen concentration, in accordance with the formation of bigger collagen structures at higher concentration.

Representative, AFM topographic images recorded in air and under water after adsorption of collagen from a 300 $\mu\text{g/ml}$ solution on grooved PMMA substrates are presented in Figure II.A.5. On the one hand, the high resolution of the images recorded in air revealed the double character of the adsorbed collagen layer: the uppermost layer consisted of big fibrillar structures, while the underneath layer was continuous. On the other hand, the images recorded in water revealed the collagen structures, which were not disturbed by drying and were probably the features typical of the uppermost layer. The compari-

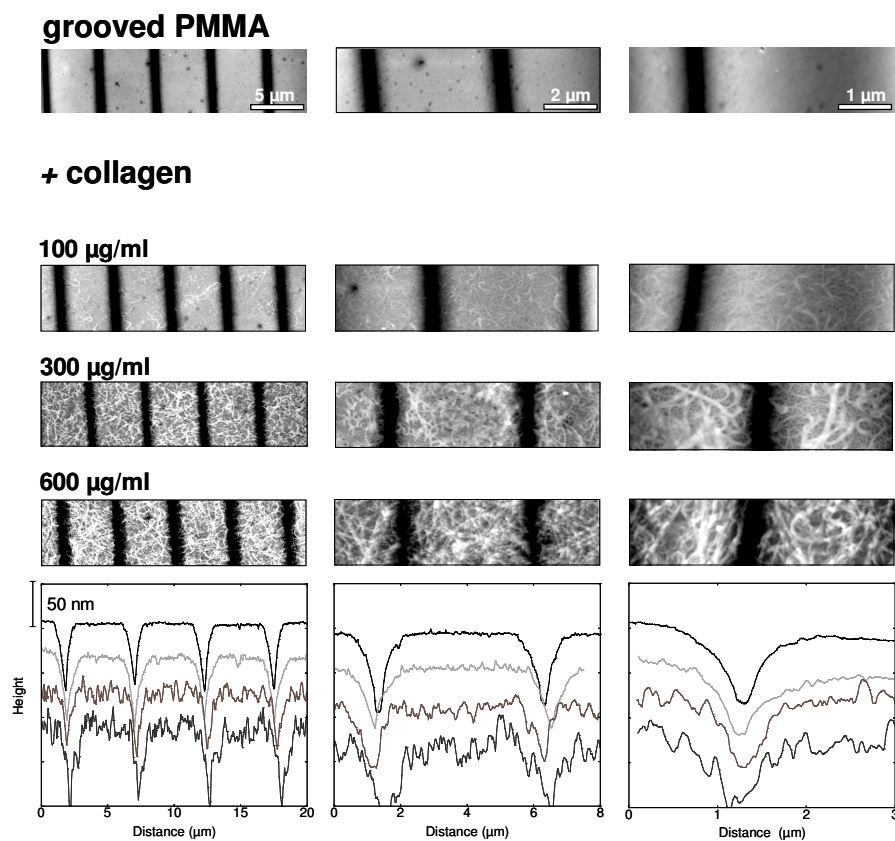


Figure II.A.4: AFM topographic images recorded in air on grooved PMMA substrates before (top) and after collagen adsorption from solutions at 100, 300 and 600 $\mu\text{g/ml}$. Image size: 25 $\mu\text{m} \times 6.25 \mu\text{m}$; $z = 40 \text{ nm}$ (left); 10 $\mu\text{m} \times 2.5 \mu\text{m}$; $z = 60 \text{ nm}$ (center); 5 $\mu\text{m} \times 1.25 \mu\text{m}$; $z = 60 \text{ nm}$ (right). All images were flattened using a third-order polynomial. The cross-sections were taken in the middle of the non-flattened images; from top to bottom the x-axis was shifted in order to align the grooves of different images.

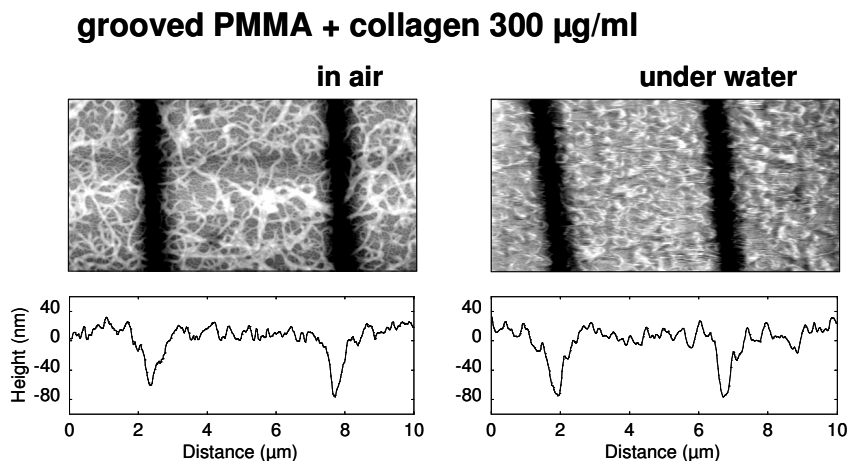


Figure II.A.5: AFM topographic images ($10\ \mu\text{m} \times 5\ \mu\text{m}$; $z = 60\ \text{nm}$) recorded in air and under water on grooved PMMA after collagen adsorption from a solution at $300\ \mu\text{g/ml}$. The images were flattened using a third-order polynomial. The cross-sections were taken in the middle of the non-flattened images.

son between dried *vs* hydrated collagen layers demonstrated a similar density of collagen assemblies. The microgrooves showed a comparable depth (70 to 80 nm) on both kinds of samples.

II.A.4 Discussion

AFM images show that the surface of control PMMA samples was rather smooth, except for some defects (Figure II.A.1a). The general shape of the C_{1s} and O_{1s} peaks obtained by XPS was in line with the literature [23]. The theoretical O/C ratio for pure PMMA is 0.4. However, due to the lack of accuracy of the sensitivity factors provided with the spectrometer used in this study, the oxygen content may be underestimated by about 15 %, which means that the expected O/C ratio for PMMA is about 0.34 [24]. The value measured here (0.26) is still lower. This points to the presence of an excess of carbon involved in $\underline{\text{C}}\text{-(C,H)}$ bonds, probably due to surface contamination occurring in the process of sample fabrication and storage.

The AFM and XPS results obtained for the smooth, control PMMA substrate after collagen adsorption are in good agreement with a previous re-

port [21]. After adsorption from a 100 $\mu\text{g/ml}$, collagen fibrils were clearly formed. At lower collagen concentrations, the onset of collagen assembly may be observed after 2 hours, but the size and the density of these assemblies are much lower [21]. At higher collagen concentrations (300 and 600 $\mu\text{g/ml}$), observations made on the flat PMMA areas between the microgrooves pointed to the increased thickness of the collagen fibrils formed at the surface (Figure II.A.4).

AFM topographic images of the grooved PMMA substrates showed that the microgrooves had a trough-like section. Their width at the upper level was large (about 1.8 μm) compared to their depth (70-80 nm). The width in the bottom of the microgrooves was 200-300 nm. The slope of the microgroove edges was thus very gentle. The microgrooves were repeated over the whole pattern area with a high regularity (Figure II.A.3). Comparing the collagen molecule dimensions (~ 300 nm in length and ~ 1.5 nm in diameter [2]) to the size of the microgrooves indicates that only one fourth of a standing molecule is enough to span the entire depth of a microgroove, while 47 molecules in lying position must be stacked to span the same depth. Inside each groove and perpendicular to their orientation, nanogrooves with a depth of about 5 nm and a periodicity of about 76 nm were found as expected. This periodicity is close to the one of the banding pattern of collagen fibers formed in a solution (67 nm). The AFM tip broadening effect could not influence the microgroove dimension in a drastic manner, since the latter are much wider than the curvature radius of the tip (~ 10 nm). On the other hand, the exact shape of the nanogrooves remains partly unknown. Their width, depth and periodicity are indeed of the same order than the AFM tip radius.

The central question raised in this paper is related to the influence of the micro- and nanotopography on collagen supramolecular organization at the surface. The cross-sections taken from AFM images obtained on the grooved PMMA substrate after collagen adsorption (Figure II.A.4 and II.A.5) show that the depth of the nanogrooves is comparable (70-80 nm) (i) prior to and after collagen adsorption, whatever the concentration used, and (ii) for dried and hydrated adsorbed layers. This indicates that, no collagen accumulation has occurred inside the microgrooves. Moreover, the images show that the organization of the adsorbed layer (*i.e.* molecular assembly as well as orientation of the molecules in the adsorbed phase) is not affected by the microgrooves.

Visualizing small details inside comparatively deep grooves is difficult on

AFM images, since the contrast due to the microgrooves depth masks these details. Raw images thus allow the collagen layer to be observed on the flat areas surrounding the microgrooves, but the microgrooves appear as entirely black regions. The details of the collagen organization inside the grooves may be visualized by varying the way of flattening the images as provided with the AFM software (in this case Nanoscope III Multimode). Briefly, the zero order flattening (Z_{zero}) removes the Z offset between scan lines by subtracting the average Z value of a given scan line from every point in this scan line, according to the equation:

$$Z_{zero}(X_i, Y) = Z(X_i, Y) - \frac{\sum_{i=1}^n Z(X_i, Y)}{n} \quad (\text{II.A.1})$$

where X_i is the row number of i -th point in the scan line, Y is the line number and n is the number of points in the scan line. The third-order flattening, beside the elimination of the Z offset between scan lines, removes also the tilt and the bow in each scan line, by fitting it with a third-order polynomial and subtracting the latter from the experimental Z values [25].

In order to better visualize the effects of the zero and third-order flattening, an image recorded after collagen adsorption on grooved PMMA from a 300 $\mu\text{g/ml}$ solution is presented in Figure II.A.6 as a 3D raw image, before flattening (image a) and after zero- (image c), and third-order (image e) flattening procedure. Additionally, the profiles which are subtracted from the raw image data upon flattening are also presented (profile b for zero-order and profile d for third-order flattening). In this case, where the microgroove was orientated along the scanning direction, flattening allows the almost complete removal of the microgroove from the image. The details of the collagen layer adsorbed within the grooves may then be seen.

AFM images acquired after collagen adsorption (300 $\mu\text{g/ml}$), with a scanning direction either parallel or perpendicular to the grooves, and either raw or flattened (zero- and third- order) are presented in Figure II.A.7, together with cross-sections. When the images are not flattened (images a and b and corresponding sections), they faithfully reflect the dimensions of the grooves, showing the groove depth to be unchanged whatever the scanning direction (the deepest point is observed at 60 nm on both images). On the cross-section b, in

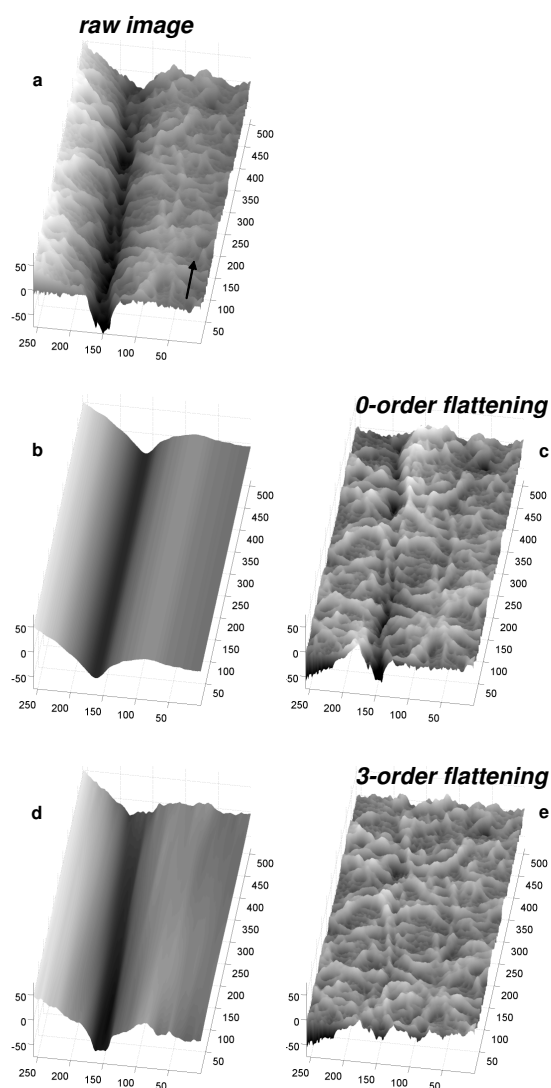


Figure II.A.6: 3D images illustrating flattening for an AFM topographic image ($10\ \mu\text{m} \times 5\ \mu\text{m}$; $z = 90\ \text{nm}$) recorded in air on grooved PMMA substrate after collagen adsorption from solution at $300\ \mu\text{g}/\text{ml}$ using the scanning direction indicated by the arrow: raw image (a), profile of the correction involved in zero-order flattening (b), result of zero-order flattening (c), profile of the correction involved in third-order flattening (d), result of third-order flattening (e). The images were built using the Matlab software (version 6.1).

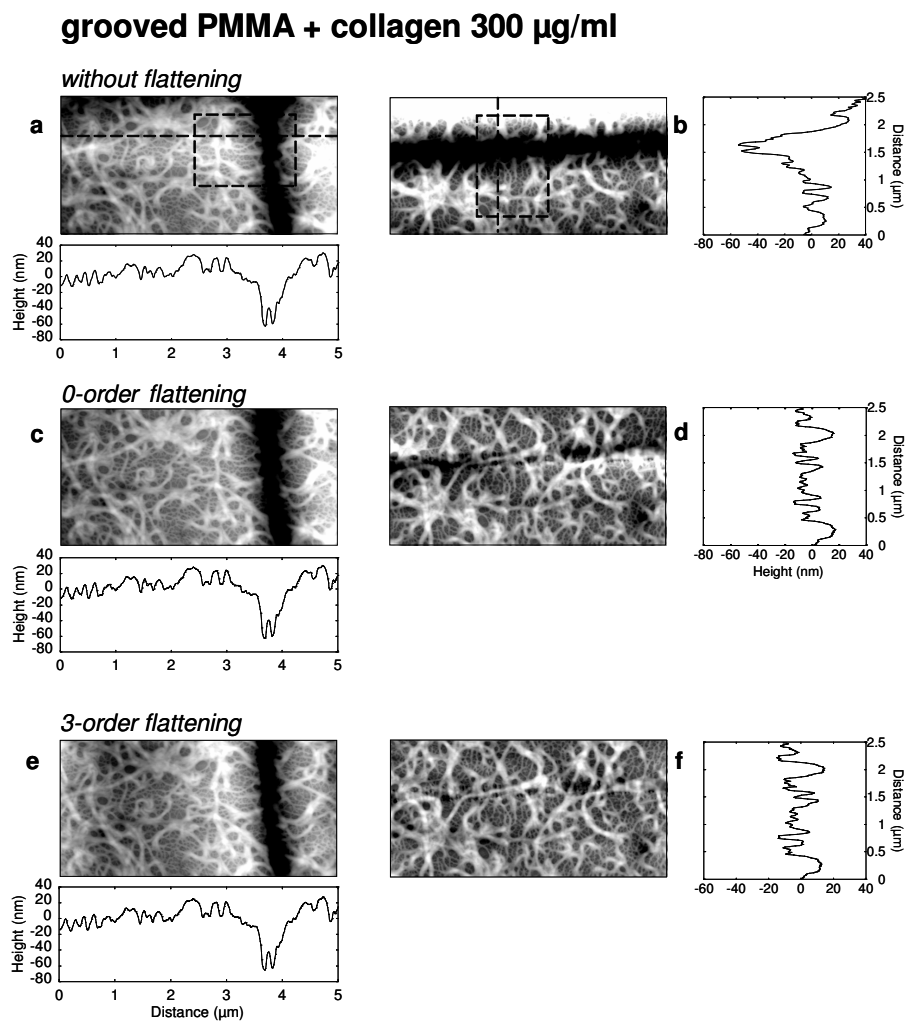


Figure II.A.7: AFM topographic images ($10\ \mu\text{m} \times 5\ \mu\text{m}$; $z = 60\ \text{nm}$) recorded in air on grooved PMMA substrate after collagen adsorption from $300\ \mu\text{g/ml}$ while the scanning direction perpendicular to the groove direction (left column) and parallel to the groove direction (right column): non-flattened (a,b), zero- (c,d), and third-order (e,f) flattened. The cross-sections were taken as indicated by the dotted lines (in a and b); the dashed rectangles (in a and b) represent the same area on the sample.

the middle of the microgroove, a small hill was observed (about 15 nm high), probably indicating the presence of a collagen fibril. As expected, flattening of the images acquired with a scan direction perpendicular to the microgrooves (images c and e) does not reveal the morphology of the layer adsorbed inside the groove. In contrast, the zero- and third-order flattening removed the groove (better for third- compared to zero-order) from the image when the AFM scanning orientation was parallel to the grooves direction (image d and f, respectively and corresponding sections), enabling the collagen layer organization inside the microgroove to be perfectly seen. The obtained images (d and f) strikingly show that the adsorbed layer organization is not affected by the microgroove. Clearly, some big fibrils are crossing the groove. The observation of many images obtained in this way never revealed any discontinuity in the adsorbed layer due to presence of the microgroove. Moreover, no alignment of fibrils in the direction of the microgroove could be observed. Finally, the nanogrooves present in the bottom of the microgroove could no longer be seen

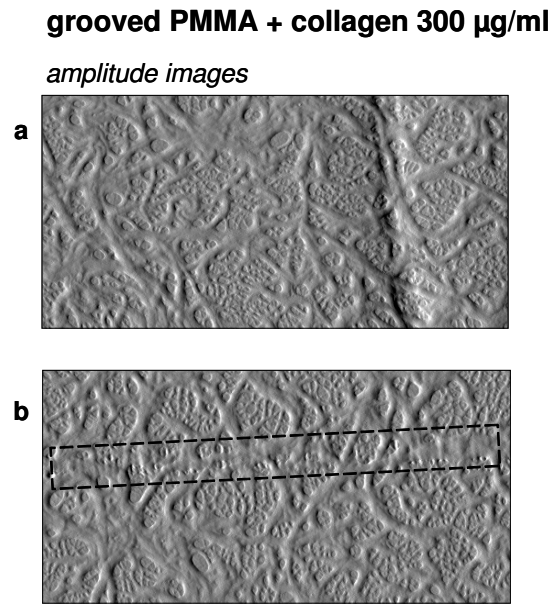


Figure II.A.8: AFM raw amplitude images originating from records presented on the left (a) and the right hand side (b) of Figure II.A.7. The rectangle (image b) indicates the position of the microgroove.

after collagen adsorption.

Another approach that can be used to remove the microgrooves from the images, in order to visualize the adsorbed layer within the groove, is to record amplitude images. In TM AFM, the feedback loop tends to maintain the amplitude of the probe oscillation constant. Amplitude images thus reflect the error signal of the feedback loop. If scanning is performed in a direction parallel to the microgrooves, the latter do not contribute to the error signal, which then essentially shows the topography of the adsorbed layer (note that the z scale is arbitrary since it depends on the parameters used to regulate the feedback). TM AFM amplitude images of a microgroove after collagen adsorption ($300\text{ }\mu\text{g/ml}$), acquired with a scanning direction either perpendicular or parallel to the groove, are shown in Figure II.A.8. When the microgroove is perpendicular to scanning, it can be observed on the amplitude image. However, the image acquired parallel to the microgrooves confirms that the collagen organization is not perturbed by the micro- nor by the nanogrooves. These latter appear slightly in the image, under the collagen layer.

II.A.5 Conclusion

PMMA structured at the micro- and nanoscale was obtained by hot embossing. The fabricated microgrooves (periodicity of about $5\text{ }\mu\text{m}$) had a trough-like section, with a depth of 70-80 nm, a width of 1.6-1.8 μm at the level of the surface and of 200-300 nm in the bottom of the grooves. Nanogrooves with a periodicity close to that of the banding pattern of collagen fibrils (*i.e.* 67 nm) were drawn inside each microgroove, in the perpendicular direction.

The influence of these micro- and nanogrooves on the supramolecular organization of adsorbed type I collagen was investigated using AFM in the tapping mode. On smooth, control PMMA, adsorbed collagen formed a network of entangled fibrils, lying on a thin background layer. On structured PMMA, image analysis was needed to visualize the morphology of the adsorbed collagen layers inside the microgrooves. The comparison of cross-sections of images acquired prior to and after adsorption as well as in the hydrated *vs* the dry state showed that collagen did not accumulate in the microgrooves, and indicated that the orientation of the collagen molecules or assemblies was similar in the grooves

compared to the flat surrounding areas. Flattening of images acquired with a scanning direction parallel to the microgrooves allowed the removal of the microgroove relief from the images, enabling the collagen layer to be observed within the grooves. The adsorbed collagen layer was not influenced by the microgrooves, no alignment of fibrils was observed, and no particular effect on the nanogrooves could be detected. These observations were confirmed by amplitude images.

It can thus be concluded that, in the adsorption conditions used here (collagen concentration from 100 to 600 $\mu\text{g/ml}$), the supramolecular organization of type I collagen is not influenced by the surface micro- and nanotopography. The adsorbed collagen layers, which present a high density of fibrillar assemblies, followed the contours of the microgrooves. More narrow grooves with sharper edges could be tested; these should interrupt the organization of the adsorbed layers and may force the alignment of assemblies. On the other hand, the nanogrooves did not interfere with the molecular assembly of collagen. This might be due to the absence of collagen accumulation within the microgrooves, or to the choice of the collagen concentration.

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