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MORPHOLOGY OF COLLAGEN AND ANTI-CANCER DRUGS ASSEMBLIES ON MICA

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ABSTRACT. Self-assemblies of type I collagen (COL) from bovine Achilles tendon and an anti-cancer drug, such as 5-fluorouracil (FLU) or doxorubicin (DOX), on mica substrate are investigated by atomic force microscopy (AFM). The AFM technique allows visualisation of these assemblies and determination of their morphology and surface roughness. The AFM images show different morphology of self assemblies made of COL, COL-FLU and COL-DOX. The data suggest that the anti-cancer drugs guide to the formation of collagen self assemblies with a notable level of stability, reflecting a high level of nanometer scale order within the adsorbed layers on mica surface. These systems might be well appropriate as biological surfaces for biomedical, drug delivery and sensing applications.

Keywords: *collagen, fluorouracil, doxorubicin, mica surface, self-assemblies, morphology, atomic force microscopy*

INTRODUCTION

The formation of protein self-assemblies from aqueous solutions onto various surfaces is of increasing importance [1-18] with a wide range of applications, including implant biocompatibility, cell adhesion and growth, [10, 11] and biomaterials design [9,10]. Such applications require a controlled morphology of the self-assembled dried layers of biomolecules at various surfaces, as in the case of biosensor devices, for which the distribution of proteins can influence the signal transduction [14] and the cellular response [14, 15]. Several factors drive the nanometer scale organization of protein layers, such as distribution of charged groups in the protein interfacial layer [6, 7, 26] and the characteristics of the substrate surface [6, 7, 21-26].

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Type I collagen represents one of the most common form of structural proteins in vertebrates, comprising up to 90% of the skeletons of the mammals. In addition to bones, it is also widespread all over the body, in skin, tendons, ligaments, cornea, intervertebral disks, dentine, arteries and granulation tissues [1,2]. The stability of collagen and its importance for biological processes [26-31] make the collagen an ideal compound for the investigation of protein adsorption onto solid surfaces.

The structure of adsorbed collagen layers on solid substrates [21-27] is essential if the collagen surfaces are to be used as sensing or healing surfaces or as anchorage of biomolecules for drug delivery systems [28-31]. On the other hand, the self-assembly formation of type I collagen is important for biomedical research, the collagen nanostructures being generally involved in many human diseases, including cancer, osteoporosis, atherosclerosis. However, the nanostructure formation of collagen layers is not yet completely understood.

The type I collagen molecule comprises three polypeptide chains (α-chains) which form a unique triple-helical structure composed of two α_1 and one α_2 chains. Chemically, each chain is constructed from repeating amino acid sequences of glycine–X-Y, where X and Y positions may be occupied by any amino acid. Frequently, proline is in the X position and hydroxyproline is in the Y position [2,23]. The type I collagen molecule contains a triple helix of 1.5 nm in diameter flanked by short nonhelical telopeptides. The telopeptides count up for 2% of the collagen molecule and are essential for self-assembly formation [3].

The previous studies concerning the organization of collagen onto solid surfaces [21-27] showed that the collagen presents different morphologies according to the sample history. For example, homogeneous layers of collagen molecules are observed after adsorption or layer by layer deposition on solid substrates (e.g., glass) from diluted acidic collagen aqueous solutions [22]. In contrast, fibrillar structures were obtained depending on the collagen concentrations and the time elapsed for assembly formation in aqueous solutions kept at high temperature of 37 ^{o}C [21]. It was also indicated that the morphology of collagen films can be changed upon drying process, [5,24] or depending on the solid surface characteristics, [21,24-26] like roughness, hydrophilicity as well as the charged surface.

The purpose of this work is to characterize the influence of experimental conditions on the self-assemblies formation of the type I collagen from bovine Achilles tendon (COL) in adsorbed layers on mica, in order to get a deeper insight into the various collagen nanostructures.

The self-assemblies of collagen are examined using three parameters able to affect the nanostructure formation, namely, the chosen period of time for self assembly process in collagen solutions kept at high temperature,

the adsorption time on the mica substrate, and the presence of two anti-cancer drugs in collagen solutions. Among the anti-cancer drugs, we have used 5-fluorouracil (FLU) and doxorubicin (DOX). The self assemblies of COL, COL and FLU and COL and DOX were obtained by adsorption from bulk solutions and drying on mica surface. The nanometer scale organization of collagen both in the absence and in the presence of anti-cancer drugs is followed by atomic force microscopy (AFM).

AFM was specifically chosen to explore the nanostructure of these layers since it is a nondestructive experimental technique under the actual working conditions. In addition, during the last decade, it was recognized that AFM observations [2,5-7,15,18,21,22,26] allow a better understanding of biomolecule layer organization. The AFM has received considerably attention due to its potentiality to analyze in situ a broad range of biological objects. It can be therefore expected that the AFM serves to gain deeper insights into the morphology of collagen layers and into the collagen auto-associative properties. An advantage of AFM observations, particularly when AFM is used in the tapping mode (alternative contact), is offered by topographic and phase images, which are obtained simultaneously. The latter ones present better contrast and resolution than topographic images, and moreover, they furnish further systematic information upon the different phases present in collagen layers. In the following, it is worthwhile to note that the term layer is used interchangeable with film adsorbed on mica surface.

Previously, we studied the nanostructured organization of collagen and anti-cancer compounds on glass surface by using AFM [21,22]. Glass substrates are negatively charged and present a roughness, indicated by root mean square (RMS) values, of about 7 Å obtained by AFM, while the mica surface is atomically flat and more negatively charged than glass. It is therefore of a major interest to investigate these systems on mica support, in order to evidence the effect of a different substrate on the morphology of various collagen nanostructures. The resulted multifunctional bionanostructures are formed by molecular self-assembly of these biocompounds on mica surface during their adsorption from bulk solutions.

RESULTS AND DISCUSSION

A systematic study using atomic force microscopy for collagen self-assemblies adsorbed on mica surface was carried out, trying to evidence the morphology of these layers. We have also investigated the mixtures of type I collagen and an anti-cancer drug (e.g. 5-fluorouracil: FLU or doxorubicin: DOX) as described in experimental section.

The representative AFM images for the pure COL film assembled on mica from its aqueous saline solution are given for a scanned area of 1 μm x 1 μm (Fig. 1) and of 500 nm x 500 nm (Fig. 2).

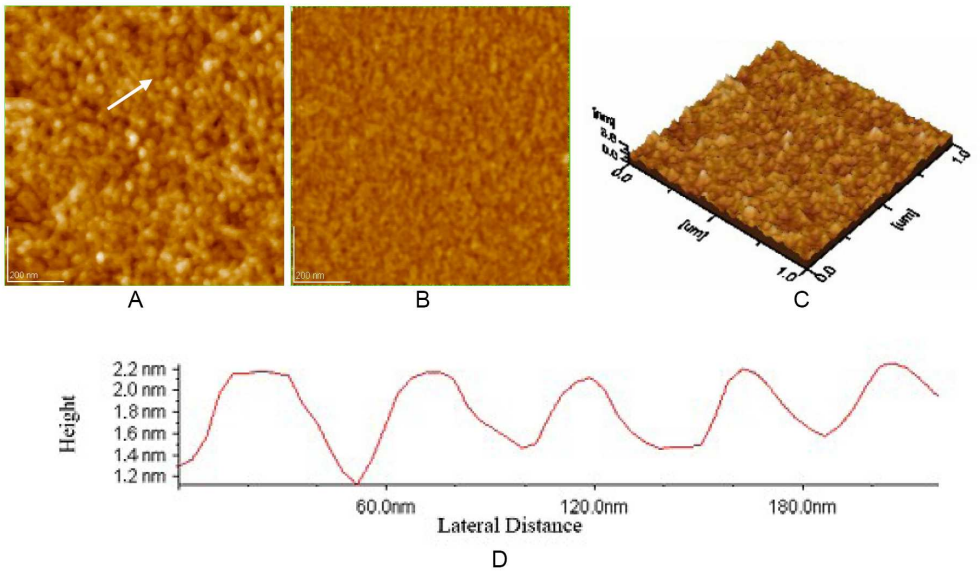

Figure 1. Collagen film on mica. A) 2D – topography; B) phase image; C) 3D-topography; D) profile of the cross section along the arrow in Fig. 1A. Scanned area: 1 μm x 1 μm.

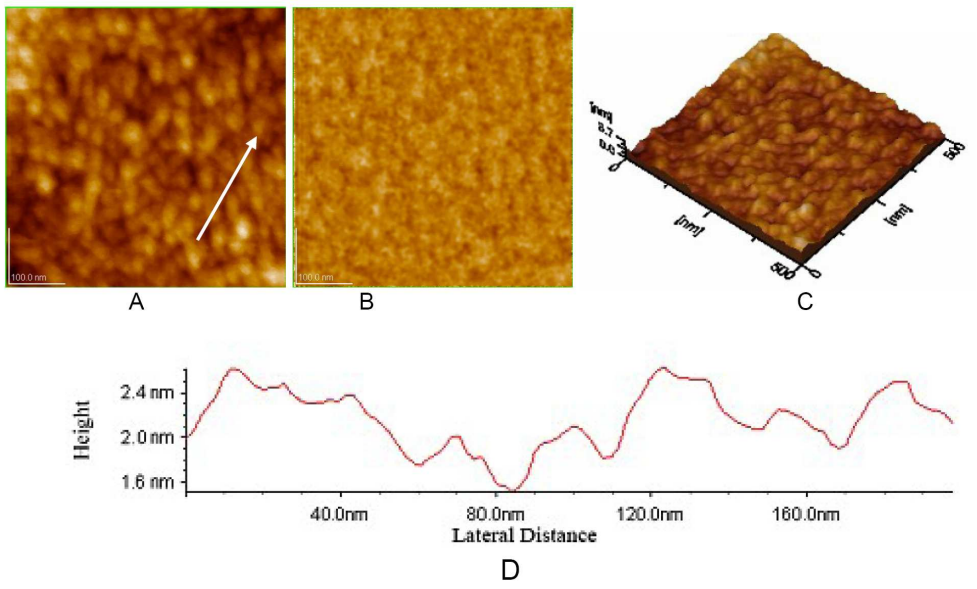

Figure 2. Collagen film on mica. A) 2D – topography; B) phase image; C) 3D-topography; D) profile of the cross section along the arrow in Fig. 2A. Scanned area: 0.5 μm x 0.5 μm.

From these images the self-organisation of collagen molecules can be observed, both in the topographic (Figs. 1A and 2A) and in the phase images (Figs. 1B and 2B), as well as in the three-dimensional (3D) topographies (Figs. 1C and 2C). The phase images present a very good contrast and high resolution. The AFM images indicate the association of collagen molecules in a globular structure. The height of the collagen film on mica, estimated from the cross section profiles (Figs. 1D and 2D), is in the range 2 to 2.2 nm. The thickness of the collagen film on mica is comparable with that corresponding to the collagen film on glass (comprised between 1.4 nm and 1.6 nm [22]) or with the 1.5 nm value reported for the diameter of the collagen molecule [1,2,17].

The surface roughness of the COL film on mica, measured by the RMS values on the film surface (0.4 nm, Figs. 1A and 2A, Table 1) and on the cross section profile (0.2 nm, Figs. 1D and 2D, Table 1) is much lower then for the similar films on glass (e.g., RMS of 1.0-1.8 nm on film area and RMS of 0.4-0.6 nm on cross profile) [22]. These observations indicate the formation of nearly homogeneous layers of collagen molecules by their adsorption on the mica surface. Obviously, a flattening of the collagen films is observed, caused at least in part by the strong interaction of films with the mica surface. Therefore the physical and chemical properties of the hydrophilic surface (e.g., mica or glass) affect the nanostructure of collagen self-assemblies. Accordingly, the mica surface structure mediates the interactions between the solid surface and the collagen molecules adsorbed onto that surface.

Table 1. RMS values for collagen, anti-cancer drugs, and collagen mixtures with an anti-cancer drug as adsorbed layers on mica surface, obtained from AFM observations. RMS roughness measurements are given for the scanned areas or on the cross section profiles of these layers

Fig.	Sample	RMS on area, nm	RMS on profile, nm
1	Collagen, scanned area: 1 μm x 1 μm	0.4	0.2
2	Collagen: 0.5 μm x 0.5 μm	0.4	0.2
-	5-Fluorouracil: 1 μm x 1 μm	0.7	0.5
-	Doxorubicin: 1 μm x 1 μm	0.8	0.4
-	Collagen and 5-fluorouracil: 1 μm x 1 μm	0.3	0.2
3	Collagen and 5-fluorouracil: 0.5 μm x 0.5 μm	0.3	0.3
-	Collagen and doxorubicin: 1 μm x 1 μm	0.9	0.6
4	Collagen and doxorubicin: 0.5 μm x 0.5 μm	1.1	0.4

Because mica is a hydrophilic, negatively charged substrate, while collagen is positively charged in acid aqueous solutions (pH about 3), it is apparent that electrostatic interactions might contribute, at least in part, to a relatively high stability of these assembled layers. These observations made on type I collagen from bovine Achilles tendon are quite similar to those made on dried samples of type I collagen from calf skin on hydrophilic substrate, such as plasma oxidized polystyrene [3] in suitable experimental conditions. On the other hand, random drying patterns of COL assemblies were also reported due to the drying process of COL solutions on mica [34].

Due to its structure, [1,23,29] collagen is not expected to undergo conformational changes [24] upon adsorption, except in the telopeptide zones. The driving force for adsorption is expected to be a gain of entropy due to the release of water molecules, [24] along with the role of electrostatic interactions that can not be ruled out under the working conditions.

The AFM images for the mixed films of collagen and an anti-cancer drug assembled on mica surface are given in Figs. 3 and 4.

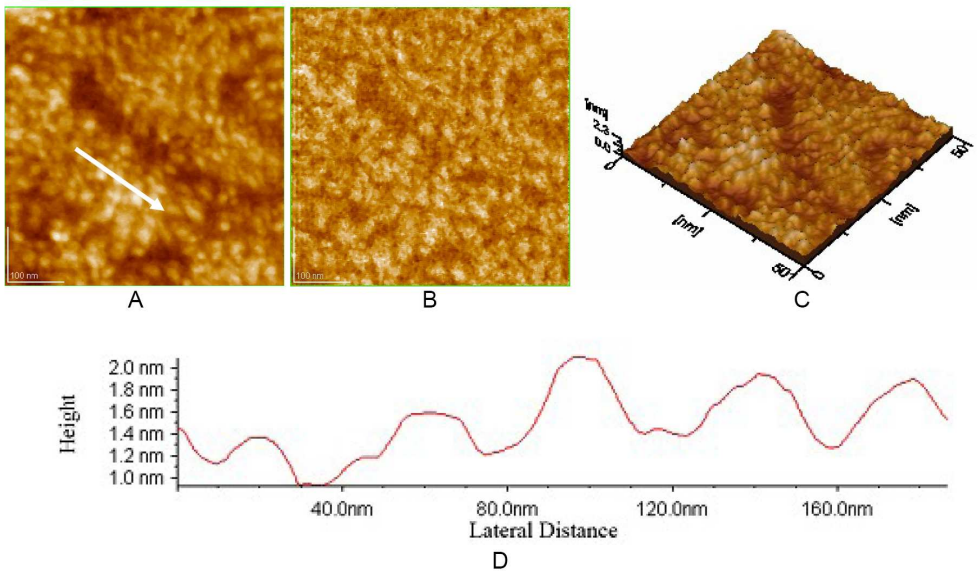

Figure 3. Collagen with 5-fluorouracil film on mica. A) 2D – topography; B) phase image; C) 3D-topography; D) profile of the cross section along the arrow in Fig. 7A. Scanned area: 0.5 μm x 0.5 μm.

For the mixed COL and FLU films, AFM images are given for a scanned area of 0.5 μm x 0.5 μm (Fig. 3). In Fig. 3 (A, B, C) a nanostructured mixed COL and FLU assembly is observed. The structure of COL and FLU film is rendered by rows arranged approximately parallel. Film thickness is about

2 nm (Fig. 3D). The RMS roughness of mixed COL and FLU films is of 0.3 nm for scanned areas (Fig. 3A) and 0.3 nm for profile given in Fig. 3D. It is to be noted that the roughness of mixed COL and FLU films shows almost the same RMS values as for pure COL films (Table 1). These findings indicate that FLU seems to penetrate and be entrapped within the COL matrix in a substantial agreement with data reported for the mixed hydrogel [28] made of collagen and poly(hydroxyethyl methacrylate) containing FLU. The FLU appears to stabilize the COL film and generate stable 2D- and 3D-nanostructures with a low roughness in comparison with its corresponding films on glass [22].

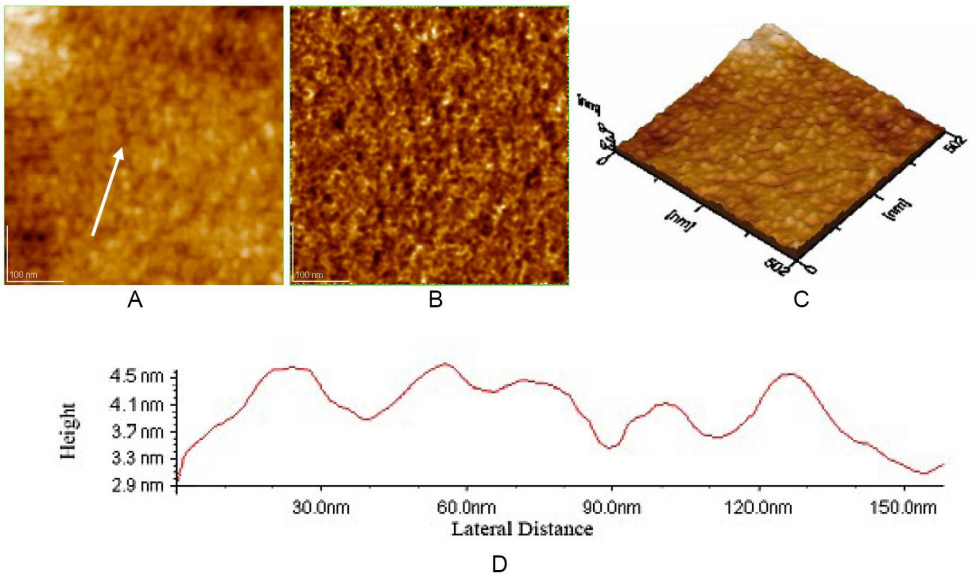

Figure 4. Collagen with doxorubicin film on mica. A) 2D – topography; B) phase image; C) 3D-topography; D) profile of the cross section along the arrow in Fig. 9A. Scanned area: 0.5 μm x 0.5 μm.

The AFM images for the COL and DOX films assembled on mica from mixed aqueous solutions are given for a scanned area of 0.5 μm x 0.5 μm in Fig. 4. The mixed COL and DOX films present a homogeneous nanostructure (Figs. 4A-4C), similar those formed on glass [22], but morphologically on mica surface these formations are much smaller. The film thickness is about 4.6 nm (Fig. 4D) very close to the values corresponding for pure DOX films on mica [35]. Because the DOX molecule is large, its binding to COL molecules leads to thicker mixed COL and DOX films when compared with pure COL layers (Figs 1D and 2D). Moreover, the RMS roughness (Table 1) is close to that of pure DOX film on mica [35]. For example, RMS value for mixed COL and

DOX film is of about 1.1 nm for scanned area of 0.5 μm x 0.5 μm rather close to RMS values for pure DOX film (of 0.8 nm) [35]. For cross section profiles, RMS roughness is about 0.4 nm (Fig. 4D) and coincides with 0.4 nm for pure DOX films [35]. The roughness of COL and DOX films is also increased in comparison with pure COL layers on mica (Table 1). However, the RMS roughness for COL and DOX films on mica is smaller than its corresponding value for the mixed COL and DOX layers on glass [22].

From this investigation, we suggest that the anti-cancer drugs as FLU or DOX can be incorporated into collagen layers by specific lateral interactions between components, such as hydrogen bonding, or simple by entrapment. The binding between collagen and anti-cancer drug might take place through molecular recognition between the less ordered zone of collagen, named telopeptides, and anti-cancer drug leading to more ordered mixed self assemblies. The mixed COL and FLU or COL and DOX layers present a good adhesion on mica surface, which in turn gives a high stability of these nanostructured self assemblies.

The morphology of the collagen assemblies is also modified depending on the characteristics of the solid surface, such as roughness and surface charge. As mentioned above, mica surface is atomically flat and has a more negative charge than glass. On the hydrophilic mica support, smooth collagen layers are formed, presenting a globular structure (Figs. 1 and 2). În the presence of anti-cancer drugs, the structure of collagen on mica presents characteristic features particularly for COL and FLU (Fig. 3). As compared with the corresponding layers on glass [22], it is clear that the interaction of these layers with the mica surface is stronger and generally the self assemblies are better organized showing low roughness in all these cases.

The advanced assembly of collagen both in absence and in presence of anti-cancer drugs are stable and may be used to cover the implants needed in nanomedicine, due to their biocompatibility with natural structures.

CONCLUSIONS

The nanostructured self assembly of type I collagen, in the absence and in the presence of two anti-cancer drugs (FLU or DOX) was investigated by adsorption from bulk solutions on mica surface by AFM, operated in tapping mode. The AFM observations enabled the examination of morphology, thickness and surface roughness of these layers at the nanometer scale and confirmed that these biomolecules form supramolecular associations on mica surface.

This work shows that the interaction of these self assemblies of COL, COL : FLU or COL : DOX with mica surface is strong and generally the layers are better organized than those adsorbed on glass substrate. The difference in the nanostructured organization of these layers is attributed, at

least in part, to the electrostatic interaction with the mica surface, which is more negatively charged than glass. In addition, the specific interactions between these molecules within the self assembled layers could be explained by hydrogen bonds, but the simple entrapment of drug molecules into the collagen matrix can not be rulled out.

Our findings could offer a strong promise for nanometer scale engineering of collagen self-assembling systems focused on the design and production of novel biomaterials with applications in nanoscience and nanobiotechnology.

On the other hand, a direct incorporation of small molecules, such as anti-cancer drugs, into the collagen assemblies represents a step toward rational design of nanostructured materials for potential applications in industry, medicine and synthetic biology, for drug delivery systems and nanobiotechnology.

In our laboratories the efforts to develop new drug delivery systems continue, because no anti-cancer drug kills selectively the cancer cells, without toxicity to normal cells.

EXPERIMENTAL SECTION

Materials and solutions. Type I collagen (COL, from bovine Achilles tendon) was purchased from Sigma-Aldrich Chemical, Corp., Milwaukee, WI. It was dissolved in 0.167 M acetic acid solution at 4 ^{o}C and an aqueous acidic solution of collagen concentration of 0.5 mg/mL was obtained (pH ≈ 3). After sonication for 30 min, the collagen solution was filtered through a 0.45 μm Millipore filter to remove pre-aggregated collagen oligomers. From this initial collagen solution, two series of stock solutions were prepared, one in the absence and the other in the presence of an anti-cancer drug. The stock collagen solution was obtained starting from the initial collagen solution mixed at 37 ^{o}C with an equal volume of 0.3 M NaCl solution. Similarly, the stock mixed collagen solutions containing an anti-cancer drug were prepared, but the aqueous saline solution contained also 0.1 mM anti-cancer drug.

The used anti-cancer drugs are doxorubicin hydrochloride (DOX, of purity >98% by TLC) and 5-fluorouracil (FLU, minimum 99% by TLC), both purchased from Sigma-Aldrich Chemical. The aqueous solutions of DOX (in 0.3 M NaCl) and FLU in ethanol and water mixture (1:1 v/v, containing 0.3 M NaCl), of the initial concentration in anti-cancer drug of about 0.1 mM, were obtained. Ultra pure deionised water [32,33] was used (pH 5.6) in all experiments. In the resulted final solutions of collagen or of collagen and anti-cancer drugs, the collagen concentration of about 250 μg/mL was obtained.

The final collagen solutions both in the absence and in the presence of anti-cancer drugs were allowed to stand at high temperature of 37 ^{o}C, for 1, 10 h or even 48 h, to let the auto-association of collagen in solutions with the view of the formation of supramolecular assemblies. For comparison

with our previous work, [22] we will present our results for 48 h elapsed time at high temperature for assembly formation in bulk aqueous solutions. After the heating period, the final solutions of collagen were further used to prepare self assemblies adsorbed on mica surface at room temperature. By using the above experimental strategy, the aggregation of collagen in bulk solution might be induced by increasing the ionic strength and the temperature of the initial cold collagen solution, in substantial agreement with findings on type I collagen from calf skin [2,5,15].

Mica substrate and self assembled layers. The used hydrophilic substrates are mica plates of 1 cm x 1 cm surface area. The mica surface was freshly cleaved before adsorption of collagen with or without anti-cancer drugs. At room temperature, the final solution (about 2 mL), both in the absence or in the presence of anti-cancer drugs, was added to each horizontal mica substrate. The samples were incubated at room temperature, for the chosen periods of time. For the sake of comparison with our previous studies, [22] for all samples presented here, the adsorption time lasted 30 min. This chosen period of time enabled the collagen, as well as the collagen with anti-cancer drug, to adsorb and assemble on mica surface.

After the adsorption time, the samples were rinsed with deionised water. For each sample, there were made two identical preparations, because two different washing modes were applied to the samples. A washing procedure was used by adding 3 mL of deionised water, directly to the sample being in contact with the bulk solution, stirring gently, pumping 3 mL of solution, adding 3 mL of water, and repeating these last steps five times, in order to eliminate the salt and other solution ingredients adsorbed on the sample.

For the other series of preparations, after the adsorption time, the samples were gently taken out from bulk solutions and another washing procedure was applied by adding water (about 20 mL) on slightly tilted substrates, with adsorbed layers on them.

Afterwards, all rinsed samples were dried slowly in air, being dust protected, and used for AFM examination. No significant differences were observed for the two different washing procedures indicating that the adsorbed layers have good adhesion and are rather stable on mica surface.

Atomic force microscopy (AFM). The AFM investigations were executed on collagen samples, without and with anti-cancer drugs, using a commercial AFM JEOL 4210, operating in tapping (noted *ac*) mode. Standard cantilevers, non-contact conical shaped of silicon nitride, coated with aluminium were used. The tip was on a cantilever with a resonant frequency in the range of 200 - 330 kHz and with a spring constant between 17.5 and 50 N/m. AFM observations were repeated on different areas from 20 μm x 20 μm to 0.5 μm x 0.5 μm of the same sample.

The images were obtained from at least ten macroscopically separated areas on each sample. All AFM experiments were carried out under ambient laboratory conditions (about 20 ^{o}C) as previously reported [6,7,21,22]. All images were processed using the standard procedures for AFM.

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