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NANOSTRUCTURE FORMATION OF COLLAGEN AND ANTI-CANCER DRUGS INVESTIGATED BY ATOMIC FORCE MICROSCOPY

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Atomic force microscopy (AFM) has been used to investigate several systems comprising a fibrous protein, namely type I collagen (COL) from bovine Achilles tendon, which co-assemble with an anti-cancer drug, such as 5-fluorouracil (FLU), doxorubicin (DOX) or lipoic acid (LA) to form nanostructured layers on mica substrate. The AFM technique allows visualisation of these nanostructures and determination of their surface roughness. The data show different morphology and good stability of self assemblies made of COL, FLU, DOX, LA, COL-FLU, COL-DOX or COL-LA. The anti-cancer drugs lead to the formation of collagen nanostructures with a remarkable level of nanometer scale order on mica, reflecting a high level of internal order within the adsorbed molecules network. Using anti-cancer drugs to self assemble with collagen molecules, more control over the collagen assembly formation is reached, consequently making these systems well suited as biological surfaces for biomedical, drug delivery and sensing applications.

INTRODUCTION

The characterization of protein nanostructure formation from aqueous solutions onto various surfaces is of increasing importance[1-18] in surface science, both from fundamental and practical point of view, with a wide range of applications, including implant biocompatibility, cell adhesion and growth,[10,11] and biomaterial design.[9,10] Such applications require a controlled morphology of the self-assembled dried layers of biomolecules at different 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] the structural rearrangements in the protein molecules,[19,20] the characteristics of the substrate surface,[6,7,21-26] and the spatial organization at the supramolecular scale.[1,9,21-26] From the various investigated parameters, the influence of the protein nature and of the solid substrate has received a considerable attention.[1,2,5-7,9-16,21-26]

Among proteins, 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.[1,2,29,30] The stability of collagen along with its significance for important biological processes[26-31] make it an ideal compound for the investigation of protein adsorption onto solid surfaces.

Further, the structure of adsorbed collagen layers on solid substrates[21-27] is crucial if the

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collagen surfaces are to be used as biomaterials, sensing or healing surfaces or as scaffolds for cells attachment[26,27] and anchorage of biomolecules for drug delivery systems.[28-31] Such layers have to exhibit biocompatibility and long-term stability. On the other hand, the self-assembly of type I collagen is important for biomedical research, the collagen nanostructured layers being generally involved in many human and animal diseases, including fibrosis, osteoporosis, cancer, 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.[1,23] Thus, 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 adsorption and 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 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 explore and further characterize the influence of experimental conditions on the nanostructure formation of the type I collagen from bovine Achilles tendon (COL) in adsorbed layers on mica substrate. In order to get a deeper insight into the various collagen nanostructures, both in the absence and in the presence of anti-cancer drugs 5-fluorouracil, doxorubicin and lipoic acid, the nanometer scale organization of collagen is followed by atomic force microscopy.

EXPERIMENTAL

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), 5-fluorouracil (FLU, minimum 99% by TLC), both from Sigma-Aldrich Chemical, and lipoic acid (LA, minimum 99%) obtained from Sinochem Jiangsu Corp., China. 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. The 0.1 mM solution of lipoic acid in ethanol (pro analysis, from Merck, Darmstadt, Germany) was also prepared. Ultra pure deionised water[32,33] was used (pH 5.6) in all the 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 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 results for 48 h elapsed time for assembly formation in bulk aqueous solutions. After the heating period, the final solutions of collagen were further used to prepare thin layers deposited or 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]

Solid substrate and adsorbed layers

The used hydrophilic substrate are mica plates of 1 cm x 1 cm surface area. Its surface was freshly cleaved before adsorption of anti-cancer drugs, or adsorption of collagen with or without anti-cancer drugs. The final solution (about 2 mL), both in the absence and in the presence of anti-cancer drugs, was added to each horizontal mica substrate at room temperature. The samples were incubated at room temperature for the chosen periods of time. Usually, the adsorbed amount can be monitored through the variation of the adsorption time.[6,7,24] For the sake of comparison with our previous studies,[22] for all samples presented here, the adsorption time lasted 30 min. This period of time enabled the anti-cancer drugs, the collagen, as well as the collagen with anti-cancer drug, to adsorb and assemble on mica surface. After adsorption, the samples were rinsed with deionised water. For each sample, two identical preparations were made, because

two different washing modes were applied to the samples. A washing procedure consists in adding 3 mL of deionised water directly to the sample 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 the samples were gently taken out from bulk solutions after the adsorption time, water was added (about 20 mL) on slightly tilted substrates with adsorbed layers on them. All rinsed samples were dried slowly in air, 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 anti-cancer drug samples and on collagen samples, without and with anti-cancer drugs, using a commercial AFM JEOL 4210, equipped with a 10 x 10 (x-y) µm scanner and operating in tapping (noted *ac*) mode. Standard cantilevers, non-contact conical shaped tips 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 x 20 μm^2 to 0.5 x 0.5 μm^2 of the same sample. The images were obtained from at least ten macroscopically separated areas on each sample. All images were processed using the standard procedures for AFM. All AFM experiments were carried out under ambient laboratory conditions (about 20 ^oC) as previously reported.[6,7, 21,22]

RESULTS AND DISCUSSION

A systematic study using atomic force microscopy for collagen and anti-cancer drugs adsorbed on mica surface was carried out, trying to evidence the bi- and three-dimensional organization of these biomolecules in adsorbed layers on mica.

We investigate the type I collagen, some anti-cancer drugs (5-fluorouracil, doxorubicin and lipoic acid) and the mixtures of collagen and an anti-cancer compound, as described in experimental section.

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

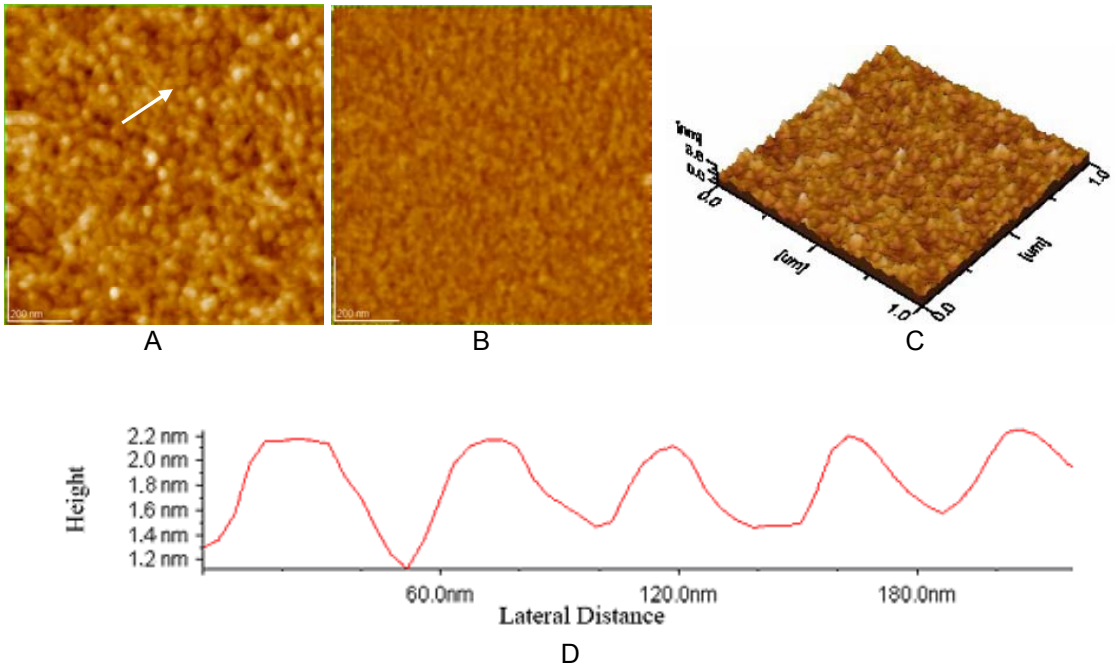


Fig. 1 – Collagen film on mica. A) 2D – topography; B) phase image; C) 3D-topography; D) profile of the cross section along the arrow in Fig. 1A. Scanned area: 1 µm x 1 µm.

From these images the self-organisation of collagen molecules can be observed, both in the topographic (Fig. 1A) and in the phase image (Fig. 1B), as well as in the three-dimensional (3D) topography (Fig. 1C). The phase image presents a very good contrast and high resolution. The AFM image in Fig. 1A indicates the association of collagen molecules in a globular structure with linear and branched rows. The height of the collagen film on mica, estimated from the cross section profiles (Fig. 1D), 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 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, Fig. 1A and Table 1) and on the cross section profile (0.2 nm, Fig. 1D and 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 emphasize 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 matrix layer. Accordingly, the substrate surface structure mediates the interactions between solid surface and the collagen molecules adsorbed onto that surface.

Table 1

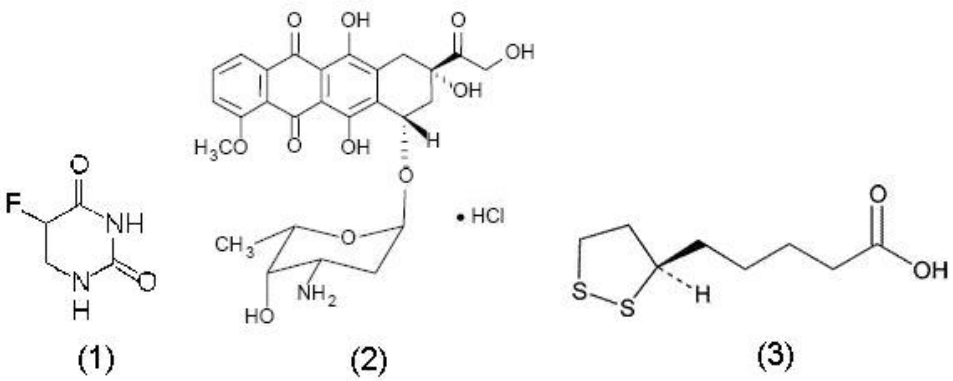
RMS values for collagen, anti-cancer drugs, and collagen mixtures with an anti-cancer drug as adsorbed layers on mica surface, obtained from AFM observations. RMS roughness measurements are given for the scanned areas or on the cross section profiles of these layers

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

Since mica is a hydrophilic substrate negatively charged, 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 adsorbed 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[3] in suitable experimental conditions. On the other hand, it was suggested that the drying effects might be involved in structuring phenomena of collagen layers[34] on mica.

Although we are not monitoring the adsorption mechanism, we still can note that 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 three anti-cancer drugs used and their formulas are given in Scheme 1.



Scheme 1 – Formulas of 5-fluorouracil (FLU, 1), doxorubicin·HCl (DOX, 2) and α-lipoic acid (LA, 3).

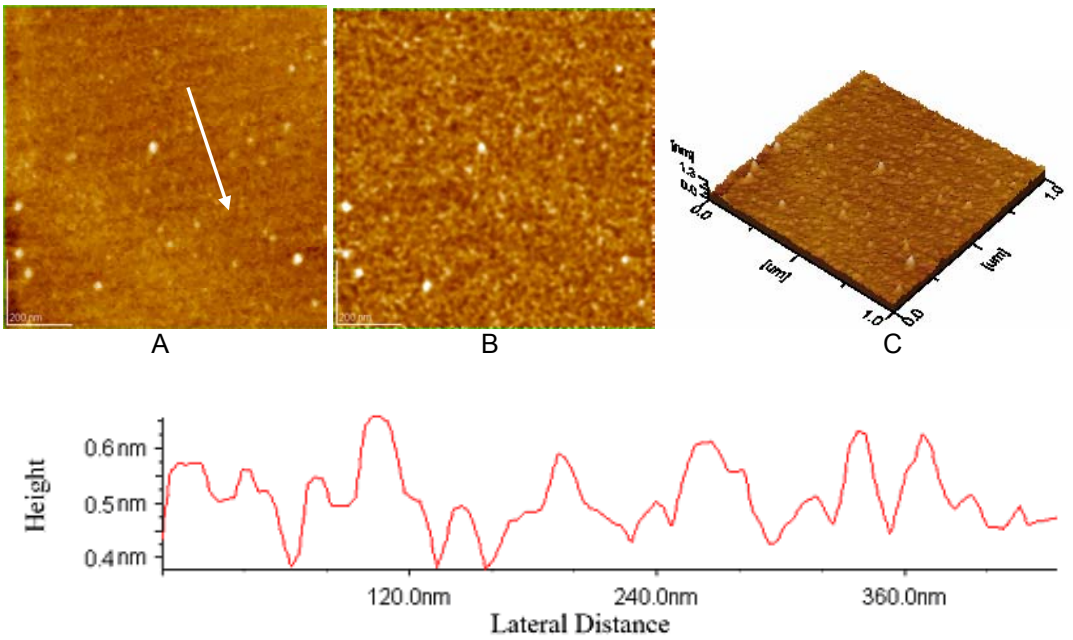


Fig. 2 – Fluorouracil film on mica: A) 2D–topography; B) phase image; C) 3D-topography; D) profile of the cross section along the arrow in Fig. 2A. Scanned area: 1 μm x 1 μm.

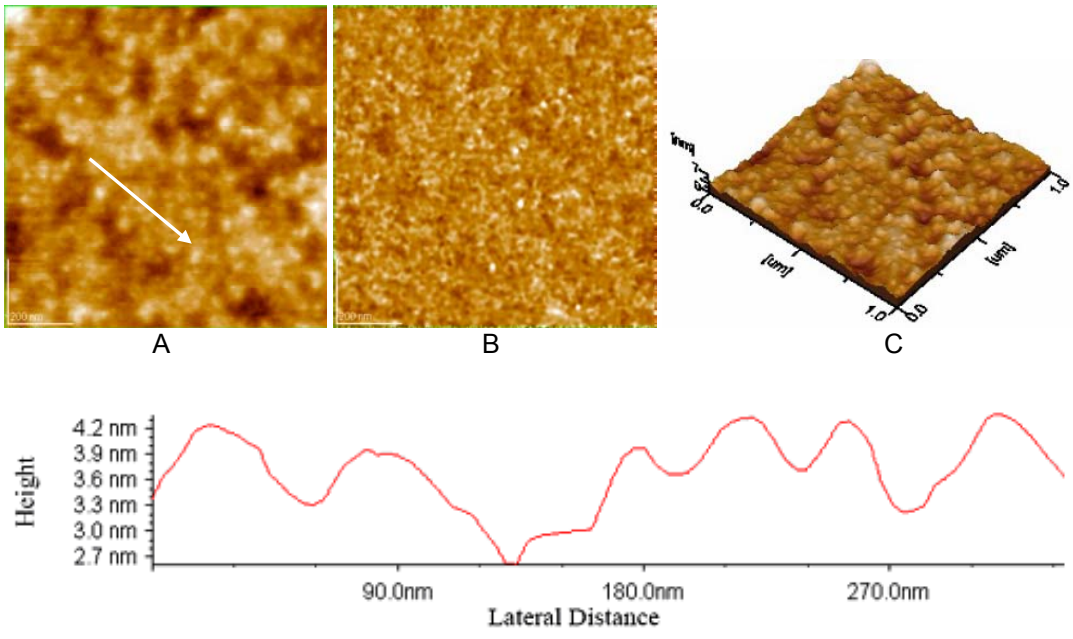


Fig. 3 – Doxorubicin film on mica: A) 2D–topography; B) phase image; C) 3D-topography; D) profile of the cross section along the arrow in Fig. 3A. Scanned area: 1 μm x 1 μm.

The representative AFM images of the pure anti-cancer drugs adsorbed on mica surface are given in Figs. 2-4.

The small, plane and rigid FLU molecules (Scheme 1, compound 1) form a film (Fig. 2) of only about 0.6 nm thick, which almost corresponds to the FLU molecule size if it is somehow horizontally oriented on the mica surface. The FLU film is smoother on mica than on glass substrate,[22] presenting RMS values of 0.7 nm on scanned area and of 0.5 nm on cross section profile (Table 1) as compared with RMS roughness measurements on glass of 2.5 nm on area and 1.5 nm on cross profile.[22]

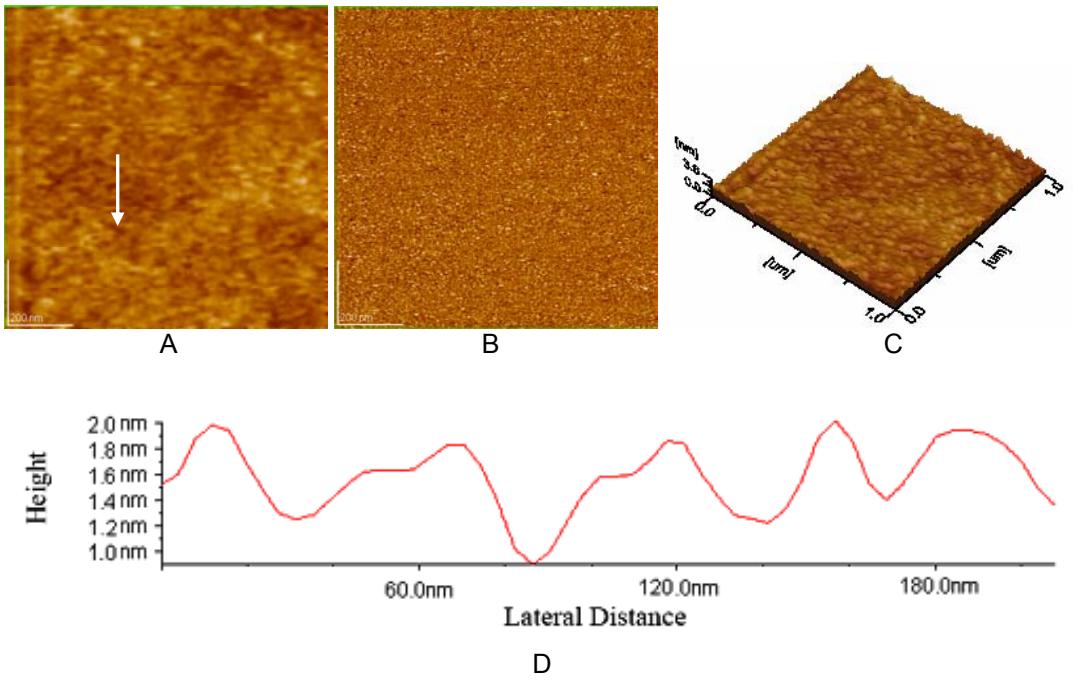


Fig. 4 – Lipoic acid film on mica: A) 2D – topography; B) phase image; C) 3D-topography; D) profile of the cross section along the arrow in Fig. 4A. Scanned area: 1 μm x 1 μm.

The DOX molecules (Scheme 1, compound 2) adsorbed on mica surface (Fig. 3) give a nanostructured adsorbed film (Figs. 3A, B and C) of about 4 nm thick (Fig. 3D), with DOX associations morphologically larger (Fig. 3A) than those observed for FLU film (Fig. 2A). In the case of DOX film, the RMS roughness (Table 1) is of 0.8 nm for a scanned area of 1 x 1 μm^2 and about 0.4 nm for the cross profile (Fig. 3D). The RMS values are much lower than those corresponding to the analogous DOX film on glass (RMS values of 3.8 nm for scanned area and 0.9 nm for cross profile analysis).[22]

The LA molecules (Scheme 1, compound 3) adsorbed on mica surface (Fig.4) give an adsorbed film (Figs. 4A, B and C) of about 2 nm thick (Fig. 4D). From morphological point of view, the LA pattern on mica (Fig. 4A) is similar with that of FLU film on mica (Fig. 2A). The LA film presents the lowest roughness among all the films of the anti-cancer drugs. The RMS roughness (Table 1) is of 0.3 nm on scanned area (Fig. 4A) and of 0.2 nm on cross section profile (Fig. 4D), while on glass the corresponding RMS values were higher, e.g., 1.4 nm on scanned area and 0.8 nm on cross section profile.[22] It seems that the films formed by pure anti-cancer drugs, except lipoic acid, present a somewhat higher roughness than pure collagen adsorbed films on mica.

The AFM images for the mixed films of collagen and an anti-cancer drugs adsorbed on mica surface are given in Figs. 5-7.

For the mixed COL and FLU films adsorbed on mica surface (see experimental part) images are given for the scanned area, of 1x1 μm^2 (Fig. 5). In Fig. 5 A-C a similar nanostructure formation of the mixed COL and FLU film is observed. The structure of COL and FLU film is rendered by rows arranged approximately parallel. Film thickness is between about 1.6 (Fig. 5D). The RMS roughness of mixed COL and FLU films is of 0.3 nm for scanned areas (Figs. 5A) and between 0.2 and 0.3 nm for cross section profile given in Fig. 5D and Table 1. It must be noted that the roughness of mixed COL and FLU films shows almost the same RMS values as the 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. Further, FLU molecules interact with collagen and seem to structure it into rows from 300 nm to 1 μm long (Fig. 5). Thus, 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]

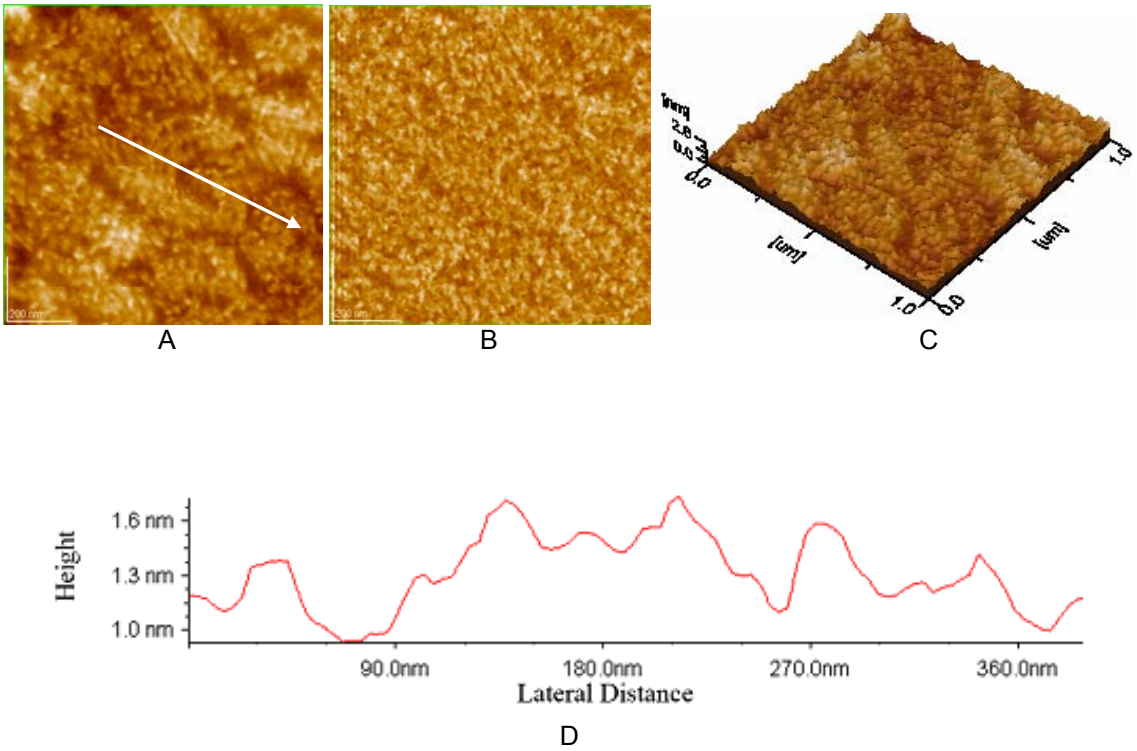


Fig. 5 – Collagen with 5-fluorouracil film on mica: A) 2D – topography; B) phase image; C) 3D-topography; D) profile of the cross section along the arrow in Fig. 5A. Scanned area: 1 μm x 1 μm.

The AFM images for the COL and DOX films adsorbed on mica from mixed aqueous solutions are given for the scanned area in Fig. 6. The mixed COL and DOX films present a homogeneous nanostructure constituted from concentric features (Figs. 6A-C), similar with those formed on glass[22], but morphologically these formations are much smaller on mica surface. The film thickness is between about 5 nm (Fig. 6D), close to the value (about 4.2 nm) corresponding for pure DOX films on mica (Fig. 3D). 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 (Fig 1D). Moreover, the RMS roughness (Table 1) is close to that of pure DOX film on mica. For example, RMS values for mixed COL and DOX films are of about 0.9 nm for scanned area of 1 x 1 μm^2 (Fig. 6A) and 1.1 nm for scanned area of 0.5 x 0.5 μm^2 (Table 1), close to RMS values for pure DOX film (of 0.8 nm, Table 1). For cross section profiles, RMS roughness ranges between 0.4 (Table 1) and 0.6 nm (Fig. 6D) for mixed COL and DOX films, and 0.4 nm for pure DOX film (Table 1). 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]

Fig. 7 shows the representative AFM images for mixed COL and LA films adsorbed on mica surface from their mixed bulk solutions. Their morphology (Figs. 7A-C) presents a homogeneous structure, rather different from that of the mixed COL and FLU films (Fig. 5) and the mixed COL and DOX ones (Fig. 6).

The LA molecule contains a ring with two sulphur atoms and a short hydrocarbon chain having a terminal carboxyl group (see Scheme 1). The completely different structure built up by the mixture of LA and COL molecules in comparison to COL and FLU or COL and DOX could be caused by some type of chemical bonding of LA to COL via its carboxylic group, leading to the LA penetration into the COL film, filling out the gaps between the COL molecules and building a well organized mixed COL and LA film on mica surface. An arrangement of this kind could explain the thickness of the mixed COL and LA film of about 1.6 nm (Fig. 7D), which is close to the thickness of both mixed COL and FLU films (about 1.7 nm, Fig. 5D) and to the pure COL film (about 2 nm, Fig. 1D).

It must be emphasised that the mixed COL and LA film shows a low RMS roughness (Table 1), 0.3 nm for scanned area (Fig. 7A) and 0.2 nm on cross section profile (Fig. 7D). Its roughness is identical with that for pure LA film or with that for mixed COL and FLU films on mica surface and very close to the roughness of pure COL films. As a general characteristic, the roughness of mixed COL and LA films is much lower on mica surface than its value for adsorbed layers on glass.[22]

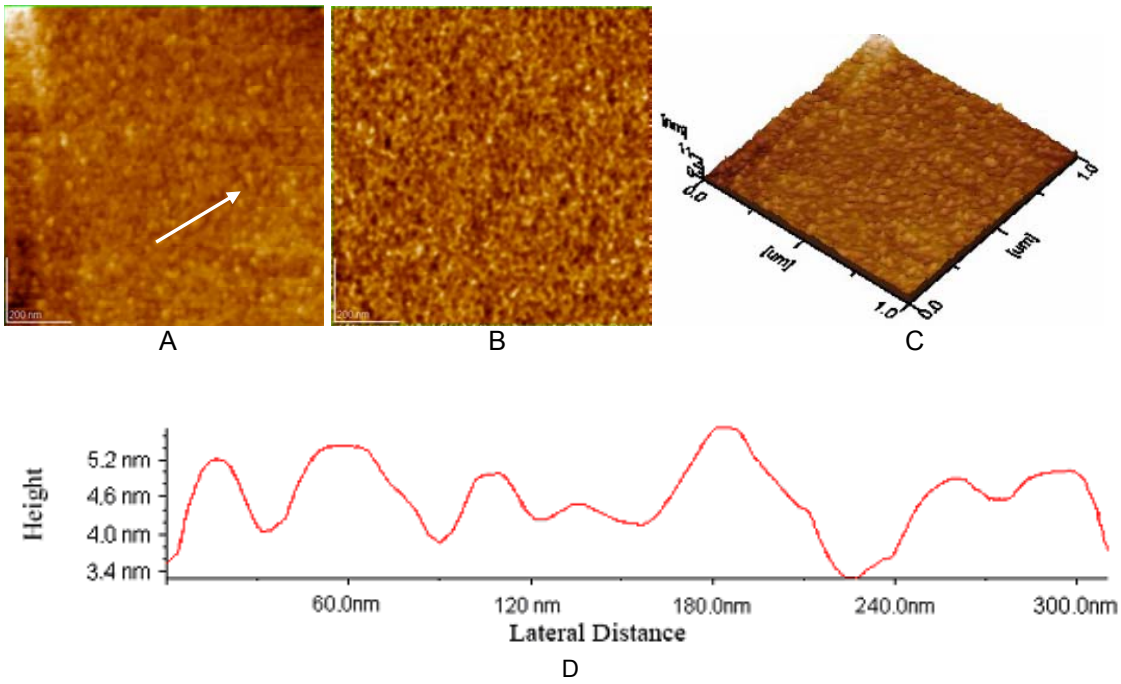


Fig. 6 – Collagen with doxorubicin film on mica: A) 2D – topography; B) phase image; C) 3D-topography; D) profile of the cross section along the arrow in Fig. 6A. Scanned area: 1 µm x 1 µm.

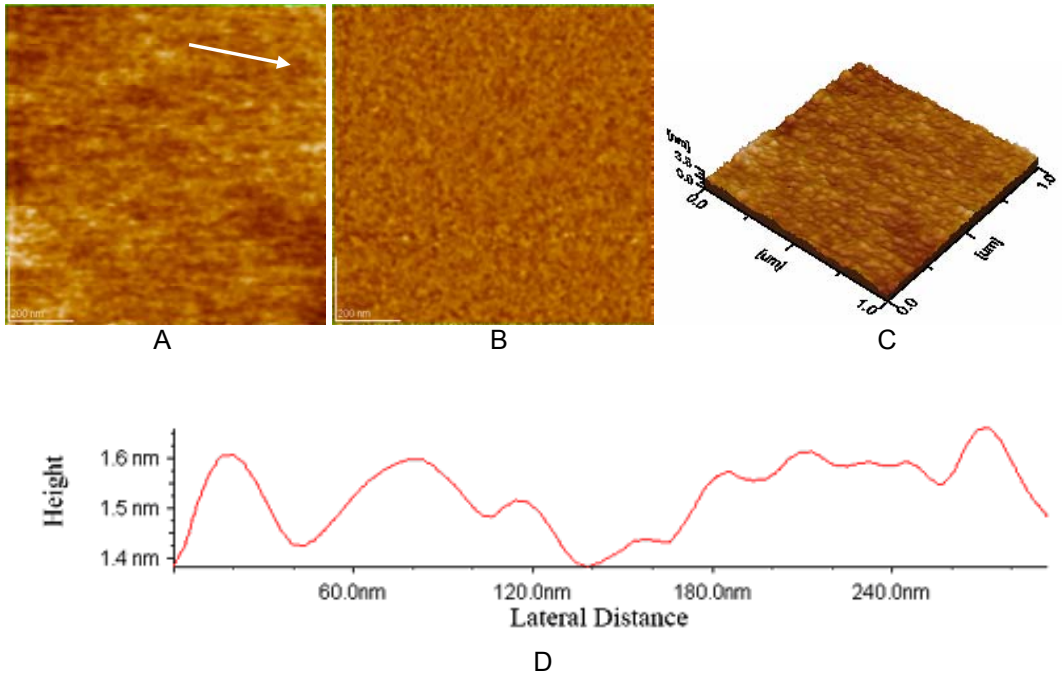


Fig. 7 – Collagen with lipoic acid film on mica: A) 2D – topography; B) phase image; C) 3D-topography; D) profile of the cross section along the arrow in Fig. 7A. Scanned area: 1 µm x 1 µm.

From this investigation, we suggest that the anti-cancer drugs FLU, DOX and LA 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, telopeptides, and anti-cancer drug leading to more ordered mixed networks. The mixed COL and FLU, COL and DOX or COL and LA layers present a good adhesion on mica surface, which in turn gives a high stability to these nanostructures.

The assemblies 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 nanostructure organization of type I collagen in the absence and in the presence of three anti-cancer drugs (FLU, DOX and LA) was investigated by adsorption from bulk solutions on mica support by AFM operated in tapping mode. The AFM enabled the examination of topography, thickness and surface roughness of these layers at the nanometer scale and confirmed that these biomolecules form supramolecular associations and nanostructured film matrices on mica surface.

The investigation shows that the interaction of collagen-anticancer drug layers with mica surface is strong and generally the layers are better organized than those adsorbed on glass substrate.[21,22] The difference in the nanostructured organization of these layers is attributed, at least in part, to the electrostatic interaction with the substrate surface, mica surface being more negatively charged than glass.

The specific interactions between these molecules within the adsorbed layers on mica surface could be explained by the molecular recognition and hydrogen bonds. Also, the simple entrapment of drug molecules into the collagen matrix can not be rulled out. The formation of hydrogen bonds between the anti-cancer drugs and the collagen matrix might be essential for the high stability of the mixed networks observed in AFM images.

Moreover, these data are the basis for future studies in our laboratories on various collagen structures obtained in different conditions, with the aim to find out the formation of self assemblies and how they could be used in nanotechnology and nanomedicine. The ability of anti-cancer drugs to control the COL assembly might suggest the utility of these systems in engineering the morphology and function of protein.

Due to current attention given to the design and production of novel biomaterials for applications in nanoscience and nanobiotechnology, our findings could offer a strong promise for nanometer scale engineering of collagen self-assembling systems. 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. Undoubtedly, the high stability of collagen nanostructures and their strong binding to mica surface make them suitable for the use for cellular interactions.

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