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In memoriam prof. dr. Ioan A. Silberg

SUPRAMOLECULAR ORGANIZATION AND NANO STRUCTURATION OF COLLAGEN AND ANTI CANCER DRUGS

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ABSTRACT. Several mixed biosystems comprising a fibrous protein, namely type 1 collagen (COL), which co-assemble with an anti-cancer drug, such as fluorouracil (FLU), doxorubicin (DOX) or lipoic acid (LA), to form ordered films on glass substrate, were visualized by atomic force microscopy (AFM). The obtained nanostructures show different morphology and stability of mixed assemblies made of COL:FLU, COL:DOX and COL:LA. The anti-cancer drugs appear to lead to supramolecular collagen structures with a remarkable level of nanoscale order on glass, which mimics natural protein assemblies. The obtained patterns, especially for COL:FLU biosystem, reflect a high level of internal order within the ordered molecules network. In these cases, the external order reflects high internal organization within these highly evolved systems. Using anti-cancer drugs to self assemble with collagen molecules we have more control over the collagen assembly process. This ability of anti-cancer drugs to control COL assembly brings further utility to the system as it allows additional compounds to be added to self assembly mixtures. In turn, it allows morphology and function of protein to be engineered. Due to current attention given to the design and production of novel bio-inspired materials for applications in nanoscience and nanobiotechnology our findings could offer a strong promise for nanoscale engineering of self-assembling systems. Direct incorporation of small molecules into the collagen assemblies represents a step toward rational design of nanostructured materials for potential applications in industry, medicine and synthetic biology, drug delivery systems and nanobiotechnology.

Keywords*: type I collagen; doxorubicin; 5-fluorouracil; lipoic acid; nanostructure; AFM.*

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INTRODUCTION

The organization of proteins at surfaces is of increasing importance [1-16] in a wide range of applications, including implant biocompatibility, cell adhesion and growth [5, 6], and biomaterials design [4, 5]. 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 [11] and the cellular response [11, 13]. Several factors drive the nano scale organization of protein layers, such as distribution of charged groups in the protein interfacial layer, the characteristics of the substrate surface, the structural rearrangements in the protein molecules [17, 18] and the spatial organization at the supramolecular scale [4, 7]. Among the various investigated parameters, the influence of the protein nature and of the solid substrate has received a considerable attention [1, 2, 4, 7-14].

In particular, type I collagen is the major fibrillar protein in the extracellular matrix and in connective tissues [7]. It is a protein of molecular mass about 300 Kg/mol, length about 300 nm, diameter around 1.5 nm, abundant in bone, cartilages, ligaments, tendons and skin [8]. Structurally, it consists of three chains, twisted to form a semi-rigid helical structure, the so called collagen monomer. Non-helical parts (telopeptides) are found at the extremities of each monomer. It contains regions which are specifically recognized by cell-surface receptors, being therefore involved in biorecognition processes.

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 hydroxyproline in Y position. A characteristic of type I collagen is that, among the three polypeptide chains, two (α_1) are identical and one (α_2) is different. For instance, in calf skin collagen, the α_1 chains are made of 1056 amino acids residues each and the α_2 chain of 1038 residues [7]. Telopeptides participation seems to be catalytic rather than constitutive and they facilitate the appropriate packing into fibers.

By the assembly of collagen monomers, characteristic band patterns with a periodicity of 67 nm (D banding pattern) along the fibril length are formed. Further linear (end to end) and lateral association (entwining of the structures) gives rise to microfibrils which can further assembly into large fibrillar structures and finally into fibers [19].

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 from diluted acidic collagen aqueous solutions. In contrast, fibrillar or fiber structures

were obtained at high concentrations [19]. It was also indicated that the morphology of collagen films can be changed upon drying process [12] depending on the solid substrate characteristics, like roughness, hydrophilicity or the charge surface.

The collagen supramolecular assembly plays an important role in mechanical reinforcement of tissues, and in proliferation, migration, and signal transduction of adjacent cells. Since only the fibril surface is available for cell-collagen interactions, it is crucial to understand the physical and biochemical properties of the fibril surface. However, the surface structure of collagen fibrils and its implications in cellular interactions have not yet been fully understood, primarily due to technical limitations in analyzing in situ the collagen fibril surface at high resolution.

During the last decade, it is recognized that atomic force microscopy (AFM) observations [1, 8, 12, 13, 16, 19] allow a better understanding of biomolecule layer organization. The atomic force microscope has received considerable attention due to its potential to analyze in situ a broad range of biological objects. It can be also expected that the AFM serves to gain deeper insight into the surface properties of collagen auto-associative properties.

In this study, we explore how the anti-cancer drugs influence the formation of supramolecular organization and nano structuration of collagen adsorbed on glass surface using AFM. The preliminary AFM analysis of the following systems consisting of collagen (type I, COL) and anti-cancer drugs (fluorouracil: FLU, doxorubicin: DOX or lipoic acid: LA) indicates the presence of specific molecular interactions with the development of supramolecular associations. Thus, it is of great interest to deeply investigate the molecular and colloidal self association of collagen from aqueous solutions within these systems containing anti-cancer compounds.

As an effect of self association of pure compounds (FLU, DOX, and LA), as well as of their supramolecular associations with collagen, the multifunctional bionanostructures are formed by molecular or colloidal self-assembly on glass solid surfaces by adsorption from aqueous solutions on glass surface. The nanostructures obtained on glass were studied by AFM, in order to determine what kind of structures are formed in vitro under various conditions in the presence of anti-cancer drugs.

AFM imaging offers the advantage of giving both topographic and phase images, as well as the surface roughness of the nanostructured mixed films obtained. In the following we will give an account of a systematic study using atomic force microscopy for collagen and anti- cancer drugs.

By the use of AFM images, structure changes from a pure component to another are visualized (topographic images), and at the same time the structural characteristics of their supramolecular associates are shown.

Simultaneously, the phase images reveal the viscoelastic properties of the obtained bionanostructures and help to identify supramolecular self-aggregates, which could modify the physical and chemical properties of biomolecules, with important biological and medical effects.

The structure of collagen films is not yet well known. Here, we use AFM investigations for evidencing the 2- and 3-dimensional organization of collagen molecules in the presence of anti-cancer drugs. Collagen films might be used to cover the implants in nanomedicine, due to their biocompatibility with natural bone structures. Further, the collagen films mixed with anti-cancer drugs could be new drug delivery systems for the treatment of bone cancer.

EXPERIMENTAL PART

A strategy in three stages was followed:

- first: the use of collagen solutions presenting different states of aggregation; collagen solutions were prepared at different pH values
- second: solutions were aged for varying periods of time; the aggregation of collagen in solution was monitored by using UV-Vis spectrophotometry
- third: collagen solutions with different states of aggregation were used for: a) adsorption/deposition on hydrophilic glass substrates; b) the supramolecular organization of the obtained adsorbed collagen layers was investigated using a combination of self-assembly layers and atomic force microscopy (AFM).

Type I collagen (COL, from bovine Achilles tendon) was purchased from Sigma-Aldrich Chemical, Co., St. Louis, MO. Collagen was dissolved in 0.167 M acetic acid solution at 4 ^{o}C and an acidic aqueous dispersion of collagen concentration of 0.5 mg/ml was obtained (pH ≈ 3). After sonication for 30 min, the collagen dispersion was filtered through a 0.45 μm Millipore filter, to remove pre-aggregated collagen oligomers.

From this initial collagen solution, two series of stock collagen solutions were prepared, namely 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 in this case, 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) and lipoic acid, all purchased from Sigma- Aldrich Inc., St. Louis, MO. The aqueous solutions of DOX (in 0.3 M NaCl) and FLU (in ethanol:water, 1:1 v/v, containing 0.3 M NaCl), of the initial concentration in anti-cancer drug about 0.1 mM, were obtained. A 0.1 mM solution of lipoic acid in ethanol was prepared. Ethanol was pro analysis purchased from Merck. Ultra pure

deionized water was used (pH 5.6) in all experiments. In the resulted final suspensions of collagen or of collagen and anti-cancer drugs, the collagen concentration of about 250 μg/ml was obtained.

The final collagen suspensions both in the absence and in the presence of anti-cancer drugs were allowed to stand at 37 ^{o}C, for 48 h, 3 days or even 5 days, to let the association of collagen monomers in solution and probably the formation of collagen supramolecular assembly.

The final suspensions of collagen were further used to prepare thin films deposited or adsorbed on glass surface at room temperature. By using the above experimental strategy, the aggregation of collagen was 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 [8, 12, 13].

The used hydrophilic substrates are glass plates (2.5 x 2.5 cm^2). The glass plates, optically polished, were sequentially cleaned with sulfochromic mixture and washed with methanol and water, before deposition of collagen layers with or without anti-cancer drugs.

Then, at room temperature, the final collagen dispersion (about 2 ml) both in the absence and in the presence of anti-cancer drugs, was delivered onto the horizontal glass substrate. After the solvent evaporation the collagen, as well as the collagen with anti-cancer drug, are adsorbed and self assembled on substrate surface.

Two series of samples were prepared starting from final stock collagen solutions, namely in the absence and in the presence of anti-cancer drugs. For all samples the adsorption time lasted 30 min at room temperature, or otherwise specifically mentioned. Then, gentle water rinsing was performed on slightly tilted substrates, with said adsorbed layers on them, in order to eliminate the salt and other solution ingredients. The resulted samples were dried slowly in air for AFM examination.

Atomic force microscopy (AFM) investigations were executed 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, 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 x 20 μm^2 to 0.5 x 0.5 μm^2 of the same collagen 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 ^{o}C) as previously reported [1, 2].

RESULTS AND DISCUSSION

Self assemblies of collagen

In Figs. 1-4 are given AFM images for the *pure COL film* deposited on glass from the 0.5 mg/ml aqueous solution, for a scanned area 1x1 μm^2 (Figs. 1, 3, 4) and 500 x 500 nm^2 (Fig. 2).

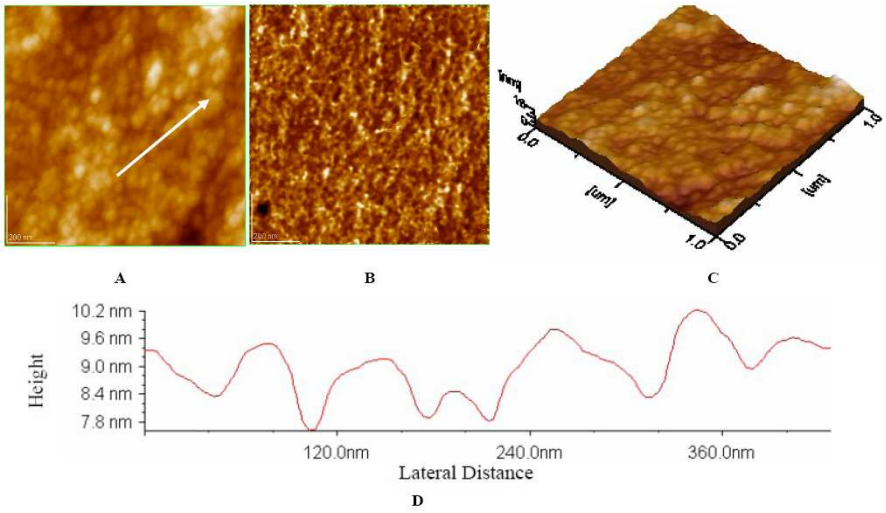


Fig. 1. Collagen film on glass, zone 1. A) 2D – topography B) phase image C) 3D-topography, D) profile of the cross section along the arrow in Fig. 1A. Scanned area: 1 μm x 1 μm.

The topographic images of the nanostructured pure COL film, present the self-organization of the COL molecules as rows, linear or curved (Figs. 1A, 2A, 3A), or assembled in star-shaped configurations (Fig. 4A). The organization of COL molecules is observed in the phase images, namely in linear rows (Figs. 1B, 2B), in round forms or circles (Fig. 3B), or star-shaped (Fig. 4B). Similar structures are also observed in 3D-topographies (Figs. 1C - 4C).

From the cross-section profiles (Figs. 1D, 3D, 4D) one can identify formations of COL fragments about 60-70 nm in length (Fig. 1D), in good agreement with literature data, where a 67 nm size is reported for the ordered regions, axially repeated on the collagen micro-fibrils [19]. The height of collagen formations in the outermost film is in the range from 1.4 to1.6 nm (Figs. 1D-4D), in good agreement with the 1.5 nm value reported in literature [7, 8, 19] for the diameter of the collagen molecule (triple helix)

The film roughness (Table 1) estimated from the cross sections profiles (Figs. 1D-4D) presents low values, between 0.4 and 0.6 nm, suggesting a high supramolecular organization in the surface of the COL

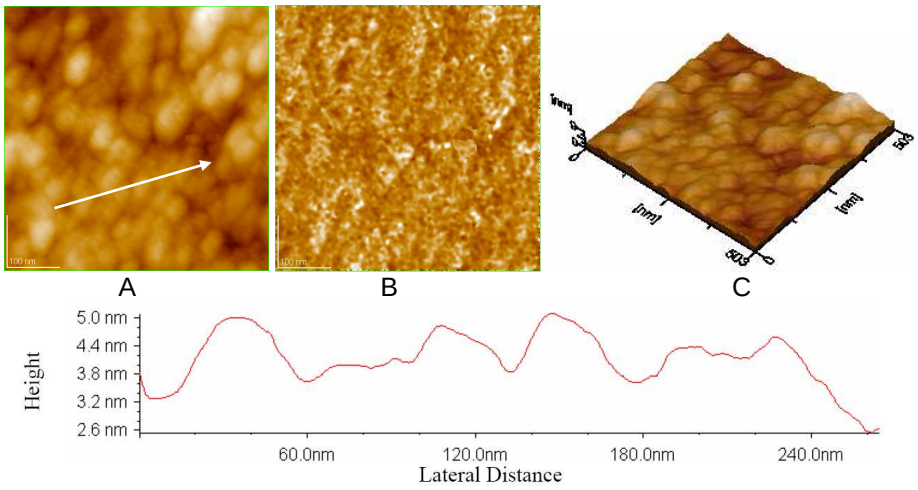


Fig. 2. Collagen film on glass, zone 1: A) 2D – topography B) phase image C) 3D-topography, D) profile of the cross section along the arrow in Fig. 2A. Scanned area: 0.5 μm x 0.5 μm.

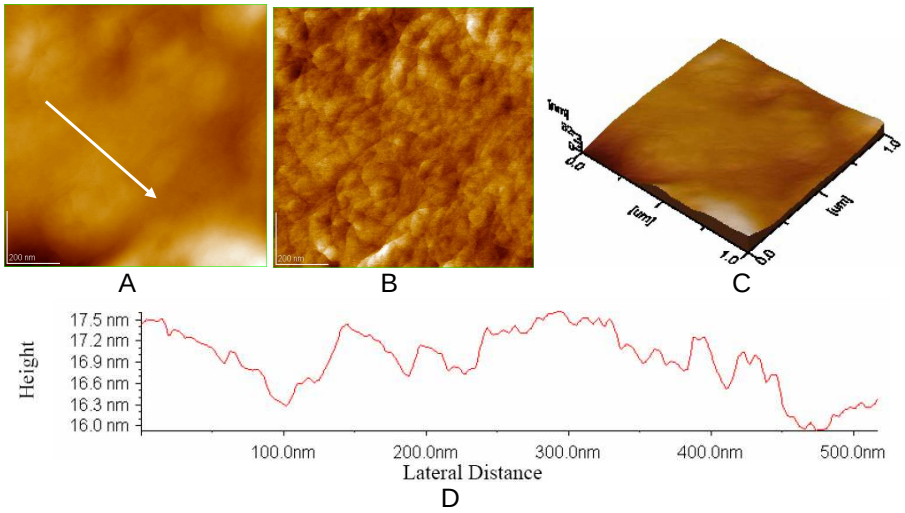


Fig. 3. Collagen film on glass, zone 2: A) 2D – topography B) phase image C) 3D-topography, D) profile of the cross section along the arrow in Fig. 3A. Scanned area: 1 μm x 1 μm.

film deposited on glass. By the examination of the profile in Fig. 3D, large supramolecular associates up to 300 nm are visualized, formations referred as tropocollagen or simply collagen monomer. The collagen fragments are much shorter. The disposition of collagen fragments or collagen monomers can be linear, ramified or curved, even concentric.

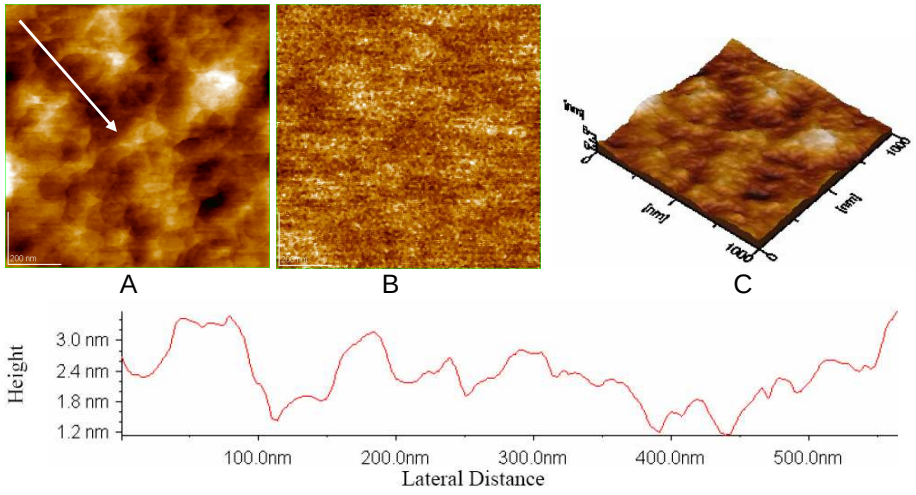


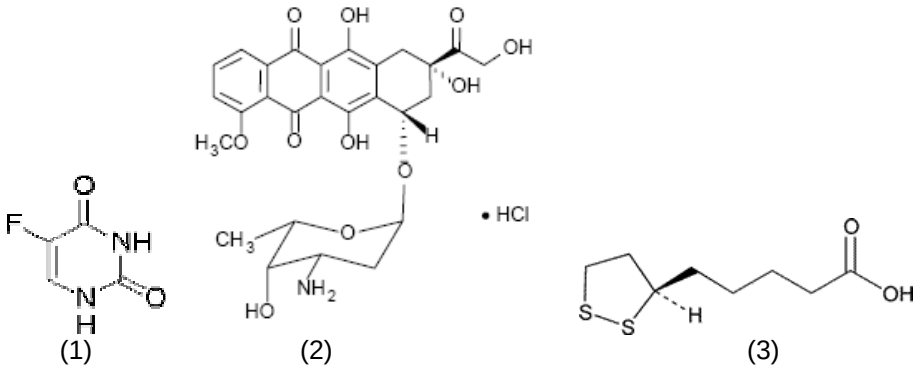
Fig. 4. Collagen film on glass, zone 3: A) 2D – topography B) phase image C) 3D-topography, D) profile of the cross section along the arrow in Fig. 4A. Scanned area: 1 μm x 1 μm.

The collagen fragments or monomers aggregate (associate) in a supramolecular lattice with an ordered zone of about 67 nm, axially repeated to finally form collagen assembly.

This complex morphology suggests that the self-assemblies can order themselves into various arrangements, even in linear or concentric features; they are not always parallel to one another and cannot retain the same neighbours among arrangements.

Self assembly of anticancer drugs

Three anti-cancer drugs are studied and their formulas are given in Scheme A.



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

AFM images are given for the pure anti cancer drugs as follows: pure fluorouracil (FLU) film (Fig. 5), deposited on glass from a 10^{-4} M solution in ethanol:water, by adsorption, for a scanned area 1x1 μm^2; AFM images for the doxorubicin, (DOX film) are presented in Fig. 6. Finally, Fig. 7 shows the lipoic acid (LA) film, also deposited on glass from its ethanolic solution.

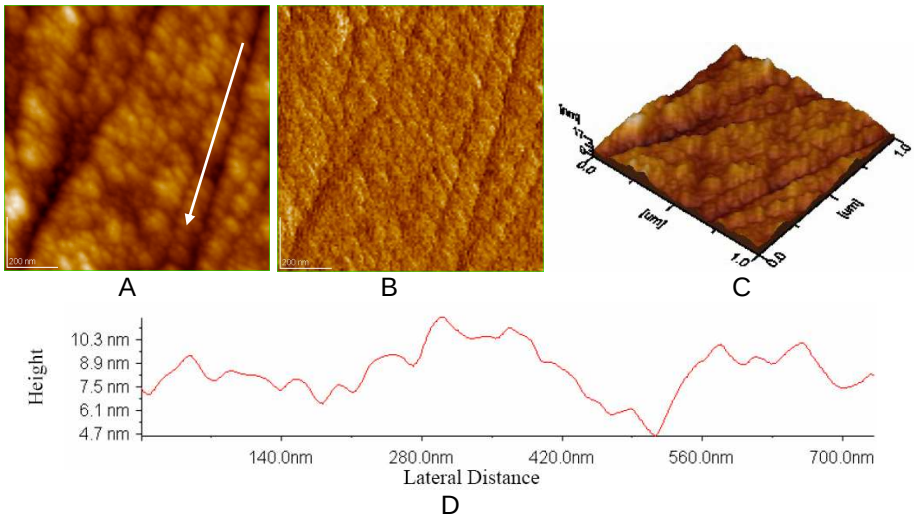


Fig. 5. Fluorouracil film on glass: A) 2D – topography; B) phase image; C) 3D-topography; D) profile of the cross section along the arrow in Fig. 5A. Scanned area: 1 μm x 1 μm.

The topographic images corresponding to the nanostructured films of 5-fluorouracil indicate the self aggregation of FLU molecules in self-associations, in long parallel rows, apparently close-packed, as visualized in Fig. 5A. DOX molecules aggregates present a rather round shape, with a slight tendency to row arrangements (Fig. 6A).

As for the lipoic acid (LA) film, it shows an association in large aggregates, arranged in distant rows (Fig. 7A). The phase images (Fig 5B-7B), the 3-dimensional images (Figs. 5C-7C), as well as the cross sections profiles (Figs. 5D-7D) support the arrangements of supramolecular aggregates, as described by the topographic images (Figs. 5C-7C). The roughness of anti-cancer drugs films, as described by the rms values on the film surface (Table 1) is about 2.5 nm (FLU), 3.8 nm (DOX) and 1.4 nm (LA), and on the cross section profile 1.5 nm (FLU), 0.9 nm (DOX) and 0.8 nm (LA). It seems that FLU, DOX and LA films generally present a somewhat higher roughness than collagen films.

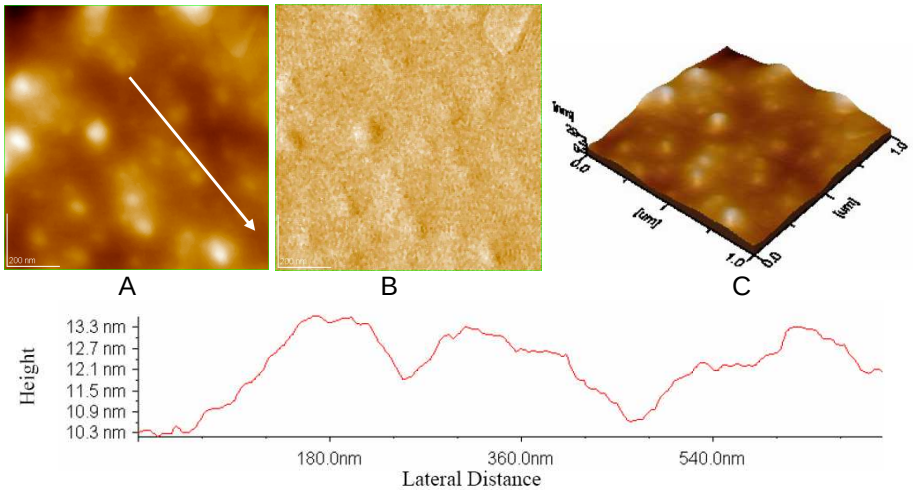


Fig. 6. Doxorubicin film on glass: A) 2D – topography; B) phase image; C) 3D-topography; D) profile of the cross section along the arrow in Fig. 6A. Scanned area: 1 μm x 1 μm.

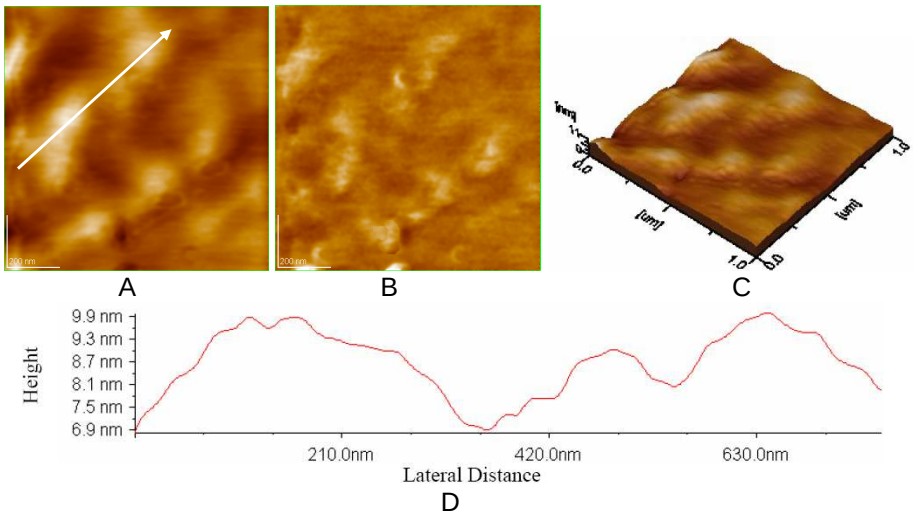


Fig. 7. Lipoic acid film on glass: A) 2D – topography; B) phase image; C) 3D-topography; D) profile of the cross section along the arrow in Fig. 7A. Scanned area: 1 μm x 1 μm.

Nanostructured organization of collagen and anti cancer drugs

In Fig. 8 AFM, images are given for the *mixed COL + FLU film*, deposited on glass from the liquid mixture, (see the experimental part), for a scanned area of 1x1 μm^2. Similarly, in Fig. 9, AFM images for the *COL + DOX film*, deposited on glass from the mixture of solutions are given, while Fig. 10 presents the *COL + LA film*, also deposited on glass from their mixed solution.

Fig. 8 (A, C) allows us to state that mixed COL+FLU films present an advanced fibril structure long over 1 μm and with a width over 0.5 μm, while the height fluctuates between 1.5 and 3.5 nm.

From Fig. 9 (A, C) it is evident that mixed COL+DOX films show a different structure than the pure DOX film (Fig. 6) or the pure COL film (Fig. 1-4). In these figures we observe the presence of domains formed by high, rounded supramolecular associations, showing an apparent hexagonal packing. These domains are about 0.3 μm long and present a nanostructure, consisting of spherules about 60-70 nm in diameter. Between the domains there are bondings through these isolated spherules.

The phase image in Fig. 9B indicates a rather uniform film, compact and with a good adherence to the glass support.

The mixed COL+LA film in Fig. 10 (A, B, C) presents a homogeneous structure, quite different from that of the COL+FLU film (Fig. 8) and the COL+DOX film (Fig. 9). The LA molecule includes a ring, containing two sulphur atoms and a hydrocarbon chain, presenting a carboxyl group (see formula). The completely different structure built up by

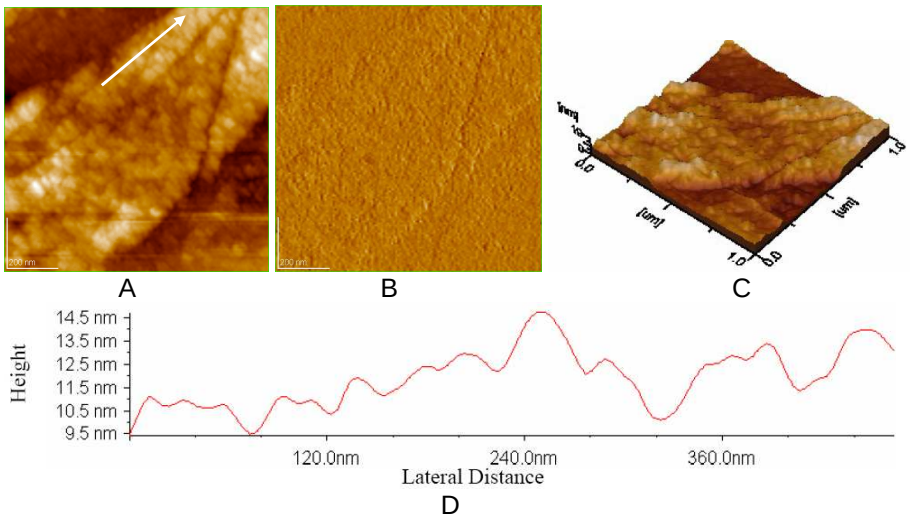


Fig. 8. Collagen with 5-fluorouracil film on glass: A) 2D – topography; B) phase image; C) 3D-topography; D) profile of the cross section along the arrow in Fig. 8A. Scanned area: 1 μm x 1 μm.

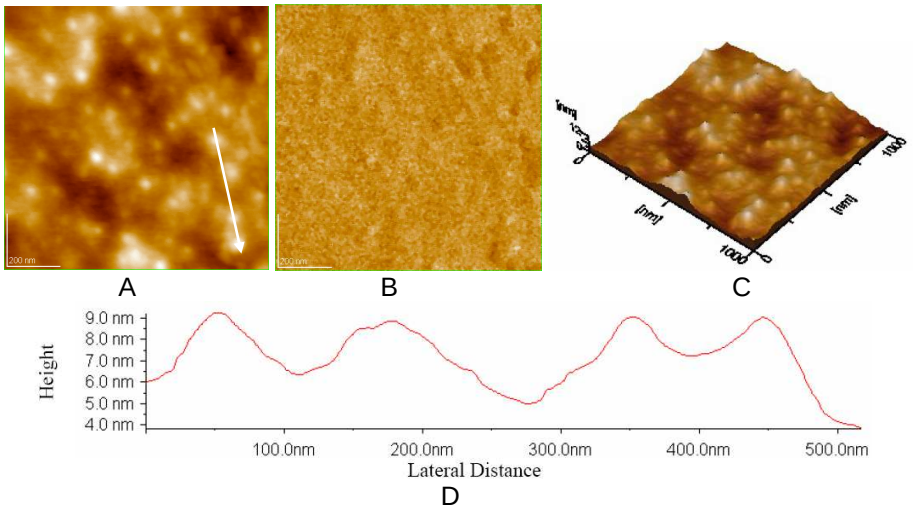


Fig. 9. Collagen with doxorubicin film on glass: A) 2D – topography; B) phase image; C) 3D-topography; D) profile of the cross section along the arrow în Fig. 9A. Scanned area: 1 μm x 1 μm.

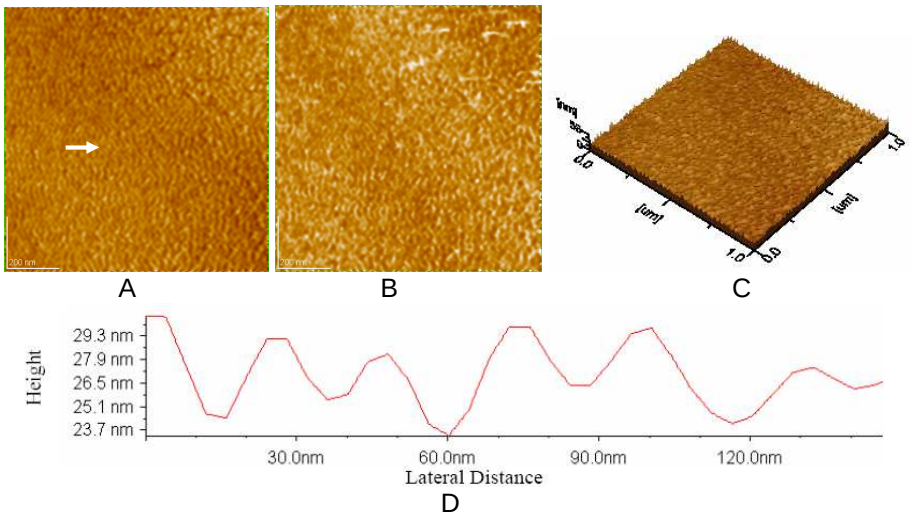


Fig. 10. Collagen with lipoic acid film on glass: A) 2D – topography; B) phase image; C) 3D-topography; D) profile of the cross section along the arrow in Fig. 10A. Scanned area: 1 μm x 1 μm.

Table 1.

RMS values for collagen and collagen mixtures with anti cancer drugs, from AFM cross sections on glass, as a measure for surface roughness of the films

Fig.	Sample	Support	RMS area, nm	RMS profile, nm
1	Collagen 1/1 μm, zone 1	glass	1.8	0.6
2	Collagen 0.5/0.5 μm, zone 1	glass	1.3	0.6
3	Collagen 1/1 μm, zone2	glass	1.0	0.4
4	Collagen 1/1 μm, zone 3	glass	1.1	0.5
5	5-Fluorouracil 1/1 μm	glass	2.5	1.5
6	Doxorubicin 1/1 μm	glass	3.8	0.9
7	Lipoic acid 1/1μm	glass	1.4	0.8
8	Collagen + 5-Fluorouracil, 1/1 μm	glass	3.4	1.2
9	Collagen +Doxorubicin,1/1μm	glass	1.9	1.4
10	Collagen + Lipoic acid, 1/1μm	glass	3.3	1.7

LA with COL in comparison to COL+FLU or COL+DOX could be caused by the specific interaction between LA and COL, leading to the LA spreading on the COL surface, filling out the gaps between the COL fragments and building on oriented LA film on the COL surface. An arrangement of this kind could explain the greater thickness of the mixed COL+AL film against the COL+FLU or COL+DOX films. But this assumption will be verified, in future studies, by building a system containing a COL layer with a superposed LA layer. From the cross section profile (Fig. 10 D), domains are evidenced of about 35-40 nm width and about 3.5 - 4.2 nm high. We also suggest that in the COL+FLU and COL+DOX systems there are specific lateral interactions between components.

Thus, the supramolecular organization of collagen molecules in presence of anti-cancer drugs molecules shows that the anti-cancer drug is strongly bound to collagen fragments. We suggest that the binding between collagen and anti-cancer drug takes place through molecular recognition between the least ordered zone of collagen, named telopeptides, and anti-cancer drug leading to more ordered mixed networks.

CONCLUSIONS

The present study confirms the formation of supramolecular associations and their assembly in nanostructured collagen films deposited on glass support, both in pure state and mixed with anti-cancer drugs: FLU, DOX and LA. The specific interactions between the molecules in the oriented films on glass support could be explained by electrostatic interactions coupled

with molecular recognition interfacial phenomena. Evidently, the formation of hydrogen bonds between the anti-cancer drugs and the collagen matrix is also essential for the stability of the mixed networks observed in AFM images.

This investigation is the basis for future investigations on various collagen structures in different conditions, with the aim to find out the formation of self assemblies and how they could be used in nanotechnology and nanomedicine.

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