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Supramolecular organization of collagen and anti-cancer drugs

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We have studied several biosystems comprising a fibrous protein, namely type 1 collagen (COL), which co-assembles with an anti-cancer drug, such as 5-fluorouracil (FLU) or doxorubicin (DOX), to form ordered films on glass or mica substrate, as visualized by atomic force microscopy. The anti-cancer drugs appear to lead to supramolecular collagen structures, with a remarkable nanoscale order, that mimics natural protein assemblies. Direct incorporation of such small molecules into the collagen assemblies represents a step toward rational design of nanostructured materials for a wide range of applications in synthetic biology and medicine, including the design of new drug delivery systems.

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1. 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, where the organization of proteins can influence the signal transduction and the cellular response [11, 13]. Several factors drive the nano scale organization of protein layers, such as the characteristics of the solid substrate surface, the structural rearrangements within the protein molecules [17, 18] and the spatial organization at the supramolecular scale [1, 2, 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]. However, little is known about the effect of adsorption and deposition time that influences the lateral mobility of adsorbed biomolecules and their 2D- and 3D-organization within the layers at the solid surface.

Among proteins, type I collagen became an interesting model compound particularly for atomic force microscopy (AFM) studies, due to its auto-associative properties [8, 11, 13, 16]. Type I collagen is the major fibrillar protein in the extracellular matrix and in connective tissues [7]. It is abundant in bone, cartilages, ligaments, tendons and skin [8].

The collagen plays an important role in mechanical reinforcement of tissues, and in proliferation, migration, and signal transduction of adjacent cells. Consequently, collagen appears to be a vehicle for drug delivery of anti-cancer drugs to cancer cells. Since the collagen surface is involved in cell-collagen interactions, it is crucial to understand the supramolecular organization of collagen and anti-cancer drugs as well as the physical and biochemical properties of the collagen fibril surface.

The goal of this work is to study the supramolecular organization of type I collagen (COL) layers obtained by adsorption and deposition methods, from aqueous collagen solutions on two solid substrates. Also, we attempt to explain the influence of experimentally conditions on the morphology of COL layers both in the absence and the presence of two anti-cancer drugs, doxorubicin (DOX) and 5-fluorouracil (FLU). For this purpose, type I collagen fibrils were regenerated from aqueous dispersions of collagen and the surface topography was analyzed using the tapping mode AFM.

The major tool for this investigation is AFM, which offers a high resolution to analyze in situ a broad range of biological objects [1, 2, 8, 12, 13, 16].

To our knowledge, this work is the first time investigation of the supramolecular organization of collagen molecules in the presence of anti-cancer drugs assembled on hydrophilic solid substrates.

2. Experimental

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

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solution at 4 ^{o}C and an acidic 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 for 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) and 5-fluorouracil (FLU, minimum 99% by TLC), both 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. 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 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 micro-fibrils assembly. The final suspensions of collagen were further used to prepare the thin films deposited on glass or mica 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 agreement with findings on type I collagen, from calf skin [8, 12, 13].

The used hydrophilic substrates are glass plates and mica of 2 cm x 2 cm surface area, each. The glass plates, optically polished, were sequentially washed with methanol and water, and mica surface was freshly cleaved, before deposition of collagen layers with or without anti-cancer drugs.

Then, at room temperature, the final collagen dispersions (about 2 ml) both in the absence and in the presence of anti-cancer drugs was delivered onto the horizontal solid substrate, i.e. glass or mica, which enabled the collagen, as well as the collagen with anti-cancer drug, to anchor on substrate surface.

Two series of samples were prepared starting from final stock collagen solutions 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 aluminum 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 × 20 μm^2 to 1 × 1 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].

3. Results and discussion

AFM images were obtained in the tapping mode, using the AFM, JEOL 4210, at a scanning rate of 1-2 Hz. Height AFM images, 2D topographies, were obtained and give information on the sample topography (Fig. 1, Fig. 2A, and Fig. 3). In some cases, so-called amplitude image was obtained using the error signal (Fig. 2B). This amplitude image allows fine details to be visualized, particularly on features presenting large topographic variations, as shown in Fig. 2A.

Fig. 1 shows representative 2D-topographies obtained on COL samples on glass (Fig. 1A) or mica (Fig. 1B), COL:DOX on glass (Fig. 1C) or mica (Fig. 1D) and COL:FLU samples on glass (Fig. 1E) or mica (Fig. 1F). These samples were obtained from two series of stock collagen solution aboth in the absence (Fig. 1A and 1B) and in the presence of anti-cancer drugs (Fig. 1C-1F), aged for 2 h at 37 ^{O}C, and for an adsorption time of 30 min on hydrophilic solid substrates at room temperature

Analyzing the Fig. 1, it is clear that the collagen layers present different morphologies according to the sample history. For example, almost homogeneous layers of collagen molecules are observed after adsorption and layer deposition on solid substrates from acidic collagen aqueous solutions (Fig. 1A and 1B). Undoubtedly, taking into account that glass and mica are hydrophilic substrates, negatively charged, and the collagen is positively charged in acidic aqueous dispersions (pH about 3), the electrostatic interactions contribute to a rather high stability of these layers. These observations found for samples of type I collagen, from bovine Achilles tendon, on glass or mica are similar to those obtained on dry samples of type I collagen, from calf skin, on hydrophilic substrate, such as plasma-oxidized polystyrene [8], in appropriate experimental conditions.

In the case of collagen layers obtained on glass (Fig. 1C and 1E) and on mica (Fig. 1D and 1F) in the presence of anti-cancer drugs, their nanostructure shows a better order than in the case of pure collagen layers (Fig. 1A and 1B). This situation is due to the supramolecular organization of collagen in the presence of doxorubicin (COL:DOX, Fig. 1C and 1D) or of 5-fluorouracil

(COL:FLU, Fig. 1E and 1 F). Presumably, the collagen co-assembles with the anti-cancer drug, such as FLU or DOX, to form ordered films. Apparently, anti-cancer drugs facilitate supramolecular collagen structures, with a remarkable nanoscale order, particularly for COL:FLU (Fig. 1E and 1F). We suggest that these structures can mimic the reconstruction of natural collagen assemblies.

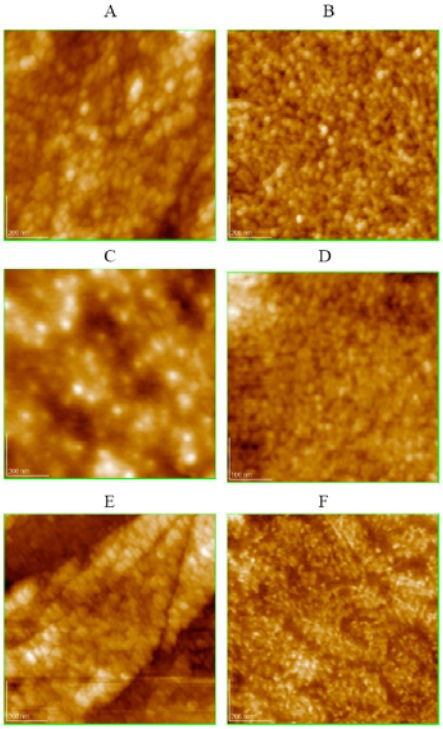


Fig. 1. 2D-topographies for COL films (A, on glass; B, mica), COL:DOX (B, glass; D, mica) and COL:FLU (E, glass; F, mica). Scannned area 1 μm x 1 μm.

Thus, a direct incorporation of such small molecules as FLU and DOX into the collagen assemblies, probably in their most disordered areas, such as non-helical parts known as telopeptides, leads to a better two- and three-dimensional collagen organization.

Also, the binding of anti-cancer drug molecules on the collagen fibril surface can not be excluded. Definitely, the binding of anti-cancer drugs within collagen fibrils and on the surface of collagen fibrils is important for the design of new drug delivery systems. Anyhow, in the presence of DOX (Fig. 1C and 1D) or FLU (Fig. 1E and 1F) a higher level of supramolecular organization of collagen is observed.

On the other hand, the morphology of collagen films is changed depending on the solid substrate characteristics, like roughness and the charge surface. For instance, glass substrates are negatively charged and present a roughness, indicated by rms values, of 5 – 7 Å, obtained by AFM. The mica surface is atomically flat and more negatively charged than glass. In the case of hydrophilic mica substrate, smooth collagen layers, showing only a slight granular structure, are observed (Fig. 1B). In the presence of anticancer drugs, the collagen structure on mica is slightly different for COL:DOX (Fig. 1D) and some characteristic features are observed for COL:FLU (Fig. 1F). Comparing the AFM images of collagen layers on glass (Fig. 1A, 1C, 1E) with those corresponding on mica (Fig. 1B, 1D, 1F), it is clear that the interaction of layers with the mica is stronger and generally the layers are better organized.

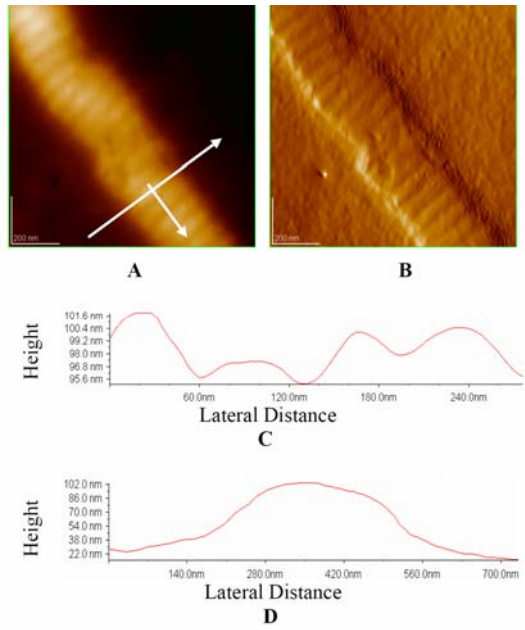
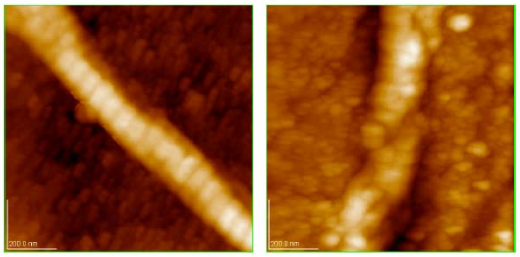


Fig. 2. Collagen fiber on glass, re-assembled from aqueous solution of collagen. A) 2D-topography; B) amplitude image; C) cross section perpendicular on fiber, D) cross section along the fiber, the arrows in panel A. Scanned area : 1 μm x 1 μm.

When the aggregation of collagen in the acidic solution is increased (ageing for 3 or even 5 days at 37 ^{o}C), and for the adsorption time of 1 h, the collagen fibril appears (Fig. 2A). The observed collagen fibril is made of collagen aggregates, as a thread like structure (Fig. 2A). Further, the amplitude (Fig. 2B) image gives fine details on the lateral structure of collagen fibril and Fig. 2C shows the cross profile along the collagen fibril axis (see the arrow in Fig. 2A). As shown in Fig. 2A the collagen fibril has a distinct banding pattern consisting of transversally running high ridges and shallow grooves. The periodicity of the banding was determined to be between 60 and 70 nm by height profile analysis along the fibril axis with a ridge height of 2-5 nm (Fig. 2C). The periodicity value found by us for type I collagen, from bovine Achilles tendon, is in substantial agreement with the value of 65 nm reported for fibrils of type I collagen, from calf skin [13]. This finding shows the resemblance between auto-associative properties of the type I collagen, independent of natural source of provenance. Furthermore, the collagen fibril height is about 70-80 nm, width (apparent diameter) about 300 nm (Fig. 2D and the length is of several μm. The collagen fibril is probably made by the assembly of collagen molecules and collagen aggregates.



A B

Fig. 3. Collagen fiber on glass, re-assembled from collagen aqueous solution in the presence of anti cancer drugs, 2D-topography: A) COL:DOX; B) COL:FLU. Scanned area: 1 μm x 1 μm.

In the case when the aggregation of collagen takes place in the presence of anti-cancer drugs, in acidic solutions, for an increased period of ageing, for 3 or even 5 days at 37 ^{o}C, and for the adsorption time of 1 h, the collagen fibril on glass also appears (COL:DOX, Fig. 3A, and COL:FLU, Fig. 3B). From Fig. 3, the mixed collagen fibrils have a similar form as the collagen fibril obtained in the absence of anti-cancer drugs (Fig. 2).

Using anti-cancer drugs to self assemble with collagen molecules, it appears that a more control over the collagen assembly process is gained. In this case the mixed fibril formation is due to a competitive adsorption of collagen monomers and aggregates with bound anti-cancer drugs, followed by their supramolecular organization on solid substrate surface. The ability of anti-cancer drugs to control collagen assembly (Fig. 1C-1E and Fig. 3) brings further utility to these systems, because it allows the morphology of collagen to be engineered. 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 nanoscale engineering of self-assembling systems. Also, the biosystems developed in this work can have an important impact on the design of new drug delivery systems targeted to the cancer cells.

4. Conclusion

The supramolecular organization of type I collagen, from bovine Achilles tendon, in the absence and in the presence of two anti-cancer drugs was monitored on different solid supports, such as glass and mica, by atomic force microscopy, operating in tapping mode. The collagen assemblies were engineered by deposition method (e.g, casting method) and adsorption from acidic aqueous solutions, aged at high temperature of 37 ^{o}C, for different periods of time. The results indicate that the AFM image resolution was improved by stabilizing the collagen molecules and aggregates through interactions with anti-cancer drugs, such as doxorubicin or 5-fluorouracil. Also, these data demonstrate the nanostructure of the collagen fibril surface.

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References

[1] M. Tomoaia-Cotisel, In Convergence of Micro-Nano-Biotechnologies, Series in Micro and Nanoengineering, Vol. 9, Editors: M. Zaharescu, E. Burzo, L. Dumitru, I. Kleps, D. Dascalu, Romanian Academy Press, Bucharest (2006) pp. 147 - 161.
[2] M. Tomoaia-Cotisel, A. Tomoaia-Cotisel, T. Yupsanis, Gh. Tomoaia, I. Balea, A. Mocanu, Cs. Racz, Rev. Roum. Chim. **51**, 1181 (2006).
[3] O. Horovitz, Gh. Tomoaia, A. Mocanu, T. Yupsanis, M. Tomoaia-Cotisel, Gold Bull. **40**, 213 (2007).
[4] J. T. Elliott, J. T. Woodward, A. Umarji, Y. Mei, A. Tona, Biomaterials **28**, 576 (2007).
[5] M. Brama, N. Rhodes, J. Hunt, A. Ricci, R. Teghil, S. Migliaccio, C. Della Rocca, S. Leccisotti, A. Lioi, M. Scandurra, G. De Maria, D. Ferro, F. Pu, G. Panzini, L. Politi, R. Scandurra, Biomaterials **28**, 595 (2007).
[6] S. Rammelt, T. Illert, S. Bierbaum, D. Scharnweber, H. Zwipp, W. Schneiders, Biomaterials **27**, 5561 (2006).
[7] E. Gurdak, P. G. Rouxhet, Ch. C. Dupont-Gillain, Colloids and Surfaces B: Biointerfaces **52**, 76 (2006).
[8] Ch. C. Dupont-Gillain, I. Jacquemart, P. G. Rouxhet, Colloids and Surfaces B: Biointerfaces **43**, 178 (2005).
[9] I. J. E. Pamula, V. M. De Cupere, P. G. Rouxhet, Ch. C. Dupont-Gillain, J. Colloid and Interface Sci. **278**, 63 (2004).
[10] E. Pamula, V. De Cupere, Y. F. Dufrene, P. G. Rouxhet, J. Colloid and Interface Sci. **271**, 80 (2004).
[11] F. A. Denis, P. Hanarp, D. S. Sutherland, J. Gold, Ch. Mustin, P. G. Rouxhet, Y. F. Dufrene, Langmuir **18**, 819 (2002).
[12] V. M. De Cupere, P.G. Rouxhet, Surface Science **491**, 395 (2001).
[13] K. Kato, G. Bar, H.-J. Cantow, Eur. Phys. J., E **6,** 7 (2001).
[14] R. Usha, R. Maheshwari, A. Dhathathreyan, T. Ramasami, Colloids and Surfaces B: Biointerfaces **48**, 101 (2006).
[15] K. Poole, K. Khairy, J. Friedrichs, C. Franz, D. A. Cisneros, J. Howard, D. Mueller, J. Mol. Biol. **349**, 380 (2005).
[16] Y. F. Dufrene, T. G. Marchal, P. G. Rouxhet, Langmuir **15**, 2871 (1999).
[17] I. Bratu**,** M. Tomoaia–Cotisel**,** G. Damian, A. Mocanu, J. Optoelectron. Adv. Mater. **9**, 672 (2007).
[18] M. Tomoaia-Cotisel, A. Mocanu, N. Leopold, M. Vasilescu, V. Chiş, O. Cozar, J. Optoelectron. Adv. Mater. **9**, 637 (2007).

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