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Dedicated to prof. dr. I. C. Popescu
on the occasion of his 70th anniversary

POROUS COLLAGEN SCAFFOLDS FOR BONE REGENERATION

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ABSTRACT. This work presents an innovative procedure to fabricate collagen (COLL) structures with controlled porosity using Langmuir technique of building the self-assembled collagen structures, alone or in the presence of the cross linker APTES [(3-aminopropyl) triethoxy silane], at the interface between air and aqueous saline subphase. Then, at selected surface pressures, the COLL structures were transferred from the interface on solid substrate (glass or mica) by Langmuir-Blodgett (LB) technique, finally obtaining porous COLL scaffolds. The morphology and porosity of COLL scaffolds were characterized by atomic force microscopy (AFM). The porous COLL structures are densified progressively at higher silane concentrations and/or at higher surface pressures. Collagen scaffolds have high potential for use in bone regeneration and tissue engineering.

Keywords: *Collagen type I, APTES, Langmuir-Blodgett technique, porous scaffolds, atomic force microscopy*

INTRODUCTION

The cells interaction with scaffolds is deeply dependent on the surface characteristics, like topography, porosity and composition of scaffolds.

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To mimic the biological environment [1-4], we have chosen the type I collagen (COLL) for fabrication of scaffolds. Undoubtedly, COLL scaffolds possess strong advantages for use in regenerative medicine. However, the relatively reduced mechanical properties of COLL scaffolds appear to limit their utility in orthopedic medicine. This situation can be overcome through addition of a second phase [5-13], like hydroxyapatite (HAP), leading to composite scaffolds, or by using crosslinking approach applied to collagen fibers [14, 15]. However, the fibrous collagen scaffolds, fabricated at controlled surface pressures in the presence of APTES, are not yet reported. Therefore, to better control the pores architecture of scaffolds, in this work, we develop an innovative procedure to fabricate porous collagen scaffolds by self-assembling of collagen molecules, in the absence and the presence of APTES, which is recognized as a cross linker for collagen fibers [14, 15].

First, collagen molecules are self-assembled as a layer at the interface between air and aqueous solution of 2M NaCl, both in the absence and the presence of APTES, and characterized by compression isotherms, in terms of surface pressure versus mean molecular area of collagen [16-19]. Secondly, the collagen interfacial film is then transferred at a controlled surface pressure on the solid surface (like glass or mica) by Langmuir-Blodgett technique [20-22]. Further, the collagen transferred layers are analysed by AFM [23-29], to determine the role of APTES on the structure, morphology and porosity of resulted collagen scaffolds.

RESULTS AND DISCUSSION

The collagen (molecular) aqueous solution of pH 3 is very stable [30, 31]. This COLL acidic solution is spread on the aqueous subphase of 2M NaCl of pH 5.5. By spreading at the interface, the collagen molecules are coming into contact with the aqueous subphase of higher pH, causing them to self-assemble, rather than going into this subphase.

Fig. 1 shows compression isotherms of COLL layers, in the absence (Fig. 1A) and in the presence of APTES (Fig. 1B), spread on 2M NaCl solution. In the inset of Fig. 1B, the compression isotherm is given for APTES solely spread at the same interface air/aqueous solution. These isotherms demonstrate the interfacial behaviour of COLL (Fig. 1A), COLL-APTES (Fig. 1B) and APTES layers (inset of Fig. 1B) on the same saline subphase. Results show that self-assembled layers are highly stable and surface pressures are constant for many hours. Therefore, these layers are appropriate for transfer from the interface to solid substrate at selected surface pressures, generating porous COLL scaffolds.

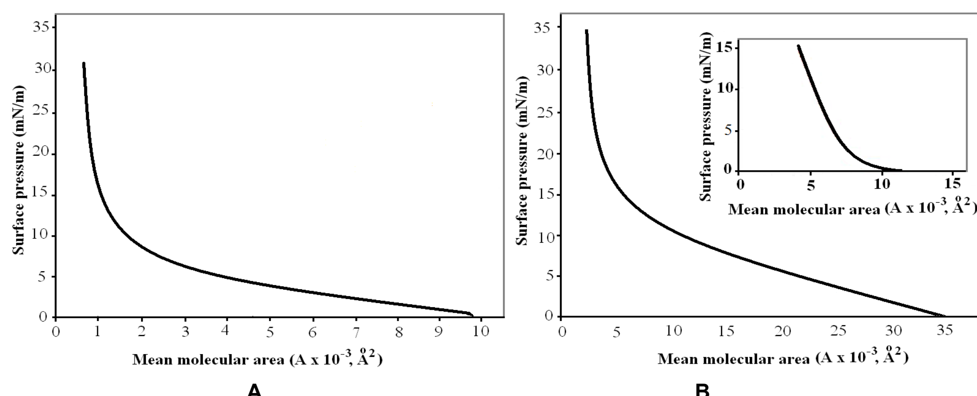

Figure 1. Compression isotherms for self-assembled layers: collagen (Fig. 1A), collagen cross-linked by APTES (Fig. 1B, for volume ratio, 1: 1, COLL-APTES) in aqueous solution used for layer spreading, and APTES (inset in Fig. 1B); each layer was spread at the interface between air and aqueous solution of 2M NaCl, at room temperature.

The compression isotherm (Fig. 1B) for the COLL-APTES layer is highly expanded than that for COLL layer (Fig. 1A). This effect can be related to the interaction of COLL and APTS in interfacial film, either by crosslinking COLL fibrils into COLL fibers or by forming COLL and APTES complexes at the interface. The stability of COLL-APTES layers is higher, as exemplified by the highest surface pressure, like 35 mN/m, which is easily obtained. Additionally, the stable COLL-APTES layers can be also formed directly on water, suggesting that the salting out effect of saline subphase is not important in this case.

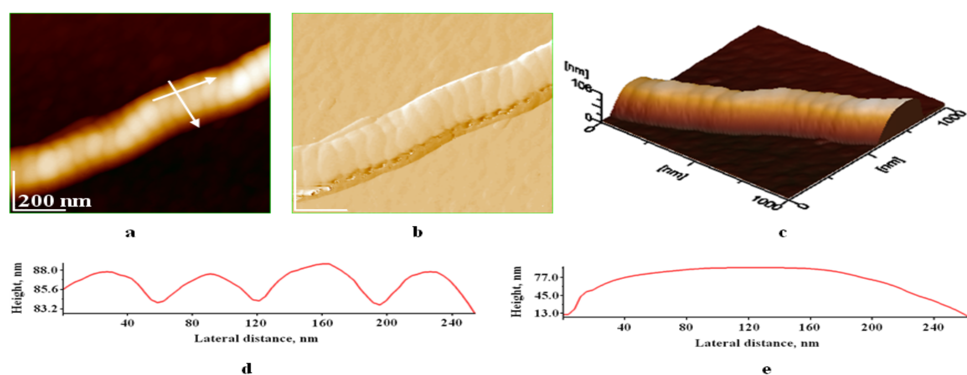

Figure 2. Collagen fiber transferred on glass substrate from the air/aqueous phase of 2M NaCl, at 30 mN/m. 2D topography (a), amplitude (b), 3D-topography (c), cross section profiles along the arrows in image (a), parallel with long axis of COL fiber (d) and perpendicular on COLL fiber (e). Scanned area 1 μm × 1 μm. Surface roughness (RMS) 32.3 nm, on scanned area (a).

The presence of 2 M NaCl in aqueous subphase (pH 5.5) is crucial for high stability of pure collagen self-assembled layers, due to the strong reduction in the COLL solubility in water. Thus, the collagen molecules are kept at the interface and accordingly, they are allowed to self-assemble in COLL fibers, as demonstrated in AFM images (Fig. 2).

The structure of COLL fibers, which are transferred at 30 mN/m from air/water interface is similar to the native banding structure of collagen fibers [24, 28, 29], obtained in different aqueous dispersions. Specifically, COLL molecules form long interfacial fibers (Fig. 2a-c) with strong periodicity of 65 nm along the long axis of COLL fibers (Fig. 2d), in substantial agreement with reported data for native banding in the COLL fibers [24, 28]. Increasing the transfer surface pressures, collagen fibers are better packed at the air/water interface, and consequently in the obtained fibrous COLL scaffolds. The increased surface pressures seem to enhance the native banding in the COLL fibers, rather than to affect the width of the COLL fibers (Fig. 2e).

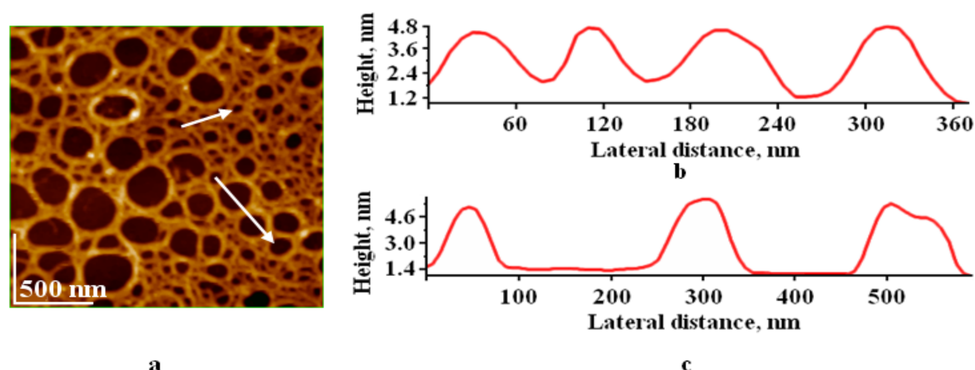

Figure 3A. 2D-topography image (a) on LB layer of collagen-APTES self- assembled patterns for volume ratio, 1:1, used as spreading solution (Fig. 1B), transferred on glass from aqueous phase of 2M NaCl, at 15 mN/m. 2D-topography (a); two cross section profiles (b and c), along the 2 arrows given in image (a); Scanned area 2 μm × 2 μm; RMS 1.7 nm on scanned area (a). Average pore diameters (b and c), from about 90 nm to 150 nm.

In the presence of APTES, collagen molecules formed a complex tight network of strongly interwoven strands, as observed by AFM imaging (Figs. 3-5). The size and the density of pores are strongly dependent on the APTES concentrations in the spreading aqueous solution of collagen, at the air/water interface, and on the surface pressures (Figs 3-5) used to transfer the COLL-APTES self-assembled layers on solid substrates (e.g., glass or mica). The generated interfacial COLL-APTES structures are porous and transferred on solid substrate lead to the COLL-APTES porous scaffolds, which are completely different than those formed from COLL fibers (Fig. 2).

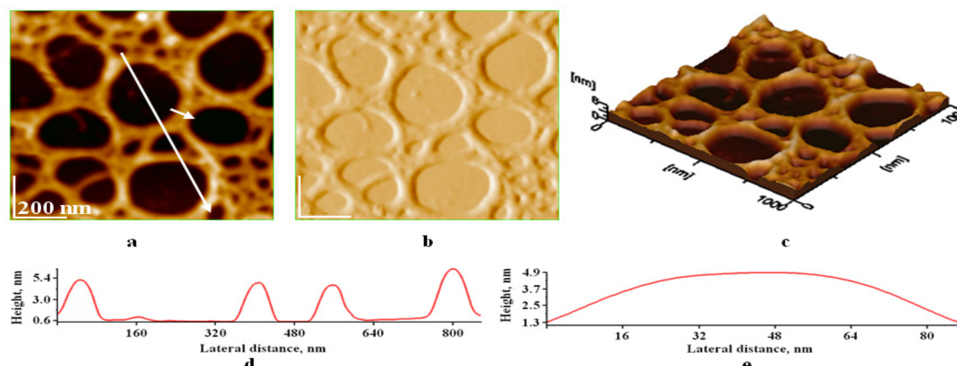

Figure 3B. COLL-APTES self-assembled patterns. 2D- topography (a), amplitude (b), 3D topography (c); two cross section profiles (d and e), along the 2 arrows given in image (a); Scanned area 1μm × 1μm; RMS 1.9 nm on scanned area (a). Details as in Fig. 3A. Porous layer analysis: average pore diameters (d), from about 40 nm, 150 nm to 240 nm. COLL fiber thickness is almost constant of about 70 ± 6 nm (an example is given in (e) cross section profile.

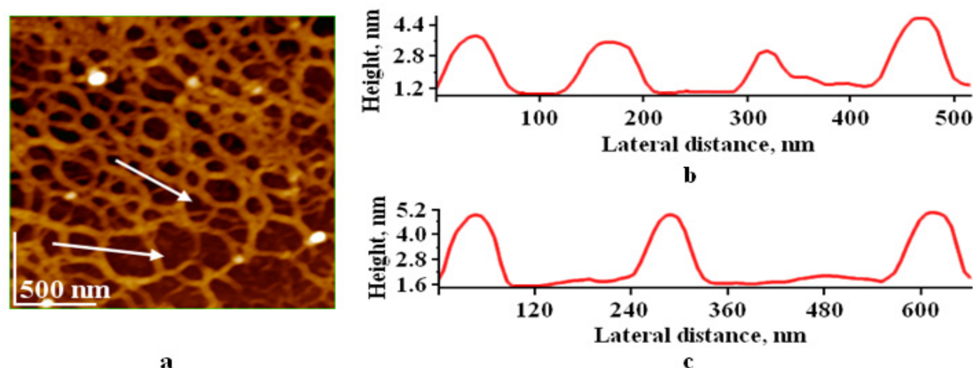

Figure 4. Collagen-APTES self-assembled patterns, for volume ratio of 1:2, used in spreading solution, at the air/water interface. AFM images on LB layer transferred on glass, at 25 mN/m: 2D-topography (a); two cross section profiles (b and c), along the 2 arrows given in image (a). Scanned area 2 μm × 2 μm. RMS 1.5 nm. Average pore diameters (b and c), from about 30 nm, 160 nm to 210 nm. COLL fiber thickness is almost constant of about 70 ± 8 nm.

Another important characteristic of porous COLL scaffolds obtained in presence of APTES is related to their very low surface roughness, as given by the root mean square, RMS, measured by AFM of about 1 or 2 nm, for the outmost layer of scaffold. These porous COLL-APTES scaffolds are rather smooth in comparison with the fibrous COLL scaffolds, where

RMS was much higher (Fig. 2). Thus, by using LB technique it is possible to monitor the morphology, porosity and surface roughness of different types of porous collagen scaffolds.

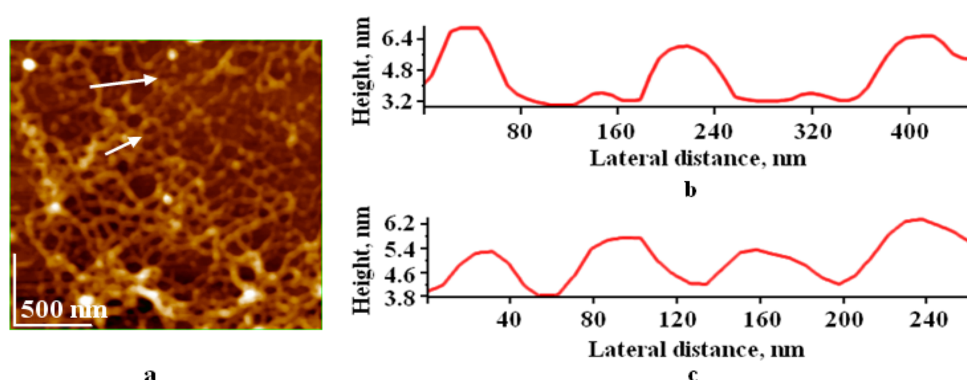

Figure 5. Collagen- APTES self-assembled patterns, for volume ratio of 1:3, used in spreading solution. AFM images on LB layer transferred on glass from air/water interface, at 35 mN/m. 2D-topography (a); two cross section profiles (b and c), along the 2 arrows given in image (a); Scanned area 2 μm × 2 μm; RMS 1.7 nm. Average pore diameters (b and c), from about 20 nm, 80 nm to 140 nm. COLL fiber thickness is almost constant of about 70 ± 10 nm.

CONCLUSIONS

This methodology used to obtain collagen scaffolds of controlled porosity represents an experimental premier in nanotechnology of mimetic scaffolds. The potential of porous collagen scaffolds for use as bone scaffolds will be further assessed in human osteoblasts culture in terms of cell adhesion, proliferation and differentiation. Preliminary results clearly indicate that these scaffolds mimic the extracellular matrix and show a higher cell adhesion, proliferation and differentiation than scaffolds with hydroxyapatite alone. These findings also suggest that the porous combined scaffolds made of nanostructured collagen fibers mineralized with hydroxyapatite and silanized with APTES could have high promises for use in the bone regeneration, hard tissue engineering and orthopedic applications.

EXPERIMENTAL SECTION

Collagen type I, from bovine Achilles tendon (MW 300 kD) and 3-aminopropyl triethoxysilane (APTES), each of the highest purity, were purchased from Sigma-Aldrich (Steinheim, Germany) and used as received. Acetic acid and sodium chloride pro analysis were purchased from Merck

(Darmstadt, Germany). Ultra pure deionized water with a resistivity greater than 18 MΩ·cm at 25°C was obtained from an Elga apparatus and used in all experiments [16-19]. Its pH was 5.5 and its surface tension was higher than 71.8 mN/m at 25°C. Optically polished glass (hydrophilic) and mica (freshly cleaved) were used as solid supports in the Langmuir-Blodgett transfer process, as shown elsewhere [20-22].

Collagen was dissolved in about 0.2 M acetic acid (at pH 3) to a concentration range from 0.5 mg/ml to 1 mg/ml. The collagen aqueous solution was spread drop-wise at the air/aqueous saline solution (e.g., 2M NaCl) interface, directly in Langmuir trough [16-19]. The layer was generally allowed to equilibrate for 10 min up to 1 hour after spreading. Then, the collagen layer at the air/water interface was compressed at constant speed of 10 mm/min, and the compression isotherm was recorded by using the balance KSV 5000 [20-22]. The accuracy of the measurements is ± 0.1 mN/m. At a constant surface pressure, in the range from 15 mN/m to 35 mN/m, the collagen layer was transferred from air/water interface on hydrophilic glass or mica by Langmuir-Blodgett technique [20-22]. Further, the collagen scaffolds comprising glass or mica substrates covered by the self-assembled layer of collagen, in the absence or the presence of APTES, were dried in air and imaged by AFM [23-29].

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