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Oriented collagen nanocoatings for tissue engineering

Laura Pastorino[a], Elena Dellacasa[a], Silvia Scaglione[b,*], Massimo Giulianelli[a], Francesca Sbrana[c], Massimo Vassalli[c], Carmelina Ruggiero[a]

[a] Department of Informatics, Bioengineering, Robotics and Systems Engineering, University of Genova, Via all'Opera Pia 13, 16145 Genova, Italy
[b] IEIIT-CNR, National Research Council, Via De Marini 6, 16149 Genoa, Italy
[c] IBF-CNR, National Research Council, Via De Marini 6, 16149 Genoa, Italy

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ABSTRACT

Collagens are among the most widely present and important proteins composing the human total body, providing strength and structural stability to various tissues, from skin to bone. In this paper, we report an innovative approach to bioactivate planar surfaces with oriented collagen molecules to promote cells proliferation and alignment. The Langmuir–Blodgett technique was used to form a stable collagen film at the air–water interface and the Langmuir–Schaefer deposition was adopted to transfer it to the support surface. The deposition process was monitored by estimating the mass of the protein layers after each deposition step. Collagen films were then structurally characterized by atomic force, scanning electron and fluorescent microscopies. Finally, collagen films were functionally tested in vitro. To this aim, 3T3 cells were seeded onto the silicon supports either modified or not (control) by collagen film deposition. Cells adhesion and proliferation on collagen films were found to be greater than those on control both after 1 ($p < 0.05$) and 7 days culture. Moreover, the functionalization of the substrate surface triggered a parallel orientation of cells when cultured on it. In conclusion, these data demonstrated that the Langmuir–Schaefer technique can be successfully used for the deposition of oriented collagen films for tissue engineering applications.

1. Introduction

Surface design has been emerging as a powerful tool in bioscience and bioengineering fields. The modulation of cell activities and functions through cell-substrate interactions can have a significant impact on biomaterial-based therapies and tissue engineering applications [1,2]. The most intriguing concept in modern biomaterials is thus obtaining materials able to mimic a specific eventually pre-existing microenvironment and, therefore, inducing cells to differentiate in a predetermined manner and to regenerate by themselves the desired tissue according to physiological pathways [3]. The microenvironmental cues become "informative" for cells, since they are able to stimulate specific cellular responses at the molecular level. If the influence of chemical environmental variables on cell activity has been already probed [3], only recently it has been reported that cells are also able to decode the topographic cues of the substrates where they grow [4–7]. In this context, nanostructured biomaterials such as nanoparticles, nanofibers and nanosurfaces have gained increasing interest since they offer a temporary extracellular matrix (ECM) for regenerative cells [8,9]. In particular, the attachment of cells to their environment is mediated by cell surface receptors and corresponding ECM proteins. In this context, advanced manufacturing techniques can be used to functionalize substrates for an enhanced cellular response [10]. Several surface bio-activations have been introduced to improve the substrate properties with the use of biological components, such as protein functionalization. Collagens are the most abundant structural proteins of the ECM [11] and play a pivotal role in the formation of tissues and organs and in maintaining their structural integrity. Moreover it has been demonstrated that collagens influence cell expression [12,13]. At least, 26 genetically distinct collagen types have been isolated, differing in primary structure. Different types of collagen are found in different organs. The most widespread collagen is collagen type I, whose main structural unit is represented by a semi-flexible rod-like molecule, approximately 300 nm long and 1.5 nm in diameter with molecular mass 270–300 kDa, which has the ability to self-assemble into micro-fibrils which form then fibrous assemblies. Due to their properties, collagen type I thin films have been proposed as a mean to control in vitro cell behavior on biomaterials of different nature [14–16]. A fundamental role in controlling cell behavior is played by the conformation and organization of collagen molecules in the film, as these characteristics may influence cell adhesion and

* Corresponding author. Tel.: +39 10 6475 206/+39 10 6475 855 (Lab); fax: +39 10 6475 200.
E-mail address: silvia.scaglione@ieiit.cnr.it (S. Scaglione).

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growth [17,18]. The possibility to deposit layers of ordered collagen molecules with nm-scale precision, reproducing the organized collagen structures, is interesting not only from application point of view but also in a view of understanding cellular mechanisms. Different approaches have been proposed for the deposition of collagen layers, such as the layer-by-layer technique, spin coating and adsorption [19–21]. However by these techniques it is not possible to obtain nm scale layers with controlled orientation of collagen molecules. Recently, the Langmuir–Blodgett (LB) [22–24] technique has been proposed for the study of the properties of collagen monolayers at the air–water interface and for the deposition of oriented collagen layers onto solid supports [25]. In this work the collagen films were transferred from the air–water interface by a vertical lift, the so called LB deposition which is based on the vertical motion of the solid support through the monolayer. Tenboll et al. [25] observed that the LB films presented a preferential orientation which was attributed to re-organization of collagen aggregates during the vertical dipping of the supports. In our work the Langmuir technique was used to form compact and oriented collagen type I based films at the air–water interface. The transfer of the collagen film from the air–water interface was then achieved by the Langmuir–Schaefer (LS) deposition, or horizontal lift [26]. The LS deposition, which implies the horizontal touching of the water surface with the solid support to transfer the floating monolayer, was developed for the protein monolayer transfer as it provides a homogeneous and reproducible coverage of the support [27,28]. The LS deposition was adopted to investigate if the ordering of collagen fibers was achieved during the compression of the water interface. The transfer of collagen films from the air–water interface to the support was characterized by quartz crystal microbalance (QCM) to estimate the mass of transferred protein layers after each deposition step. Collagen films were then deposited onto silanized silicon supports and structurally and functionally characterized. The structural characterization was done by atomic force microscopy (AFM) and scanning electron microscopy (SEM). Moreover, collagen/hyaluronic acid multilayers were deposited onto silicon supports by the layer-by-layer self-assembly technique (LbL) [29] and morphologically characterized by AFM. The obtained images were compared with those obtained for LS collagen layers to gain more information on collagen orientation. Finally, functional characterization was carried out by evaluating in vitro cellular response to the deposited oriented collagen films.

2. Materials and methods

2.1. Reagents

Type I collagen from calf skin was purchased from Sigma–Aldrich. *N*-propanol and acetic acid were from Sigma-Aldrich. Poly(ethyleneimine) (PEI) hyaluronic acid from Sigma-Aldrich were used in the LbL assembly. Reagents for silicon supports activation were from Sigma-Aldrich. All chemicals were used as received without any further purification. Water, used in the experiments for the solutions preparation and washing, was purified by Milli-Q system and had the resistance of 18.2 MΩ cm.

2.2. Langmuir–Blodgett technique

Collagen Langmuir films were obtained by a Langmuir trough from KSV with a size of 150.0 × 475.0 mm. The surface pressure was measured by Wilhelmy balance having an accuracy of 0.2 mN/m. The stock solution of collagen was prepared by dissolving collagen in *n*-propanol/acetic acid solution (1:9 v/v) till the final concentration of 2 μM [30]. Collagen was allowed to dissolve overnight under stirring at 4 °C. Different working conditions were tested in order to find the optimal π–A isotherm. The best result was obtained by compressing immediately 500 μl of protein solution on the water surface. The subphase was pure water and the compression speed was of 20–10 mm/min. The surface pressure of deposition was 15 mN/m and the LS technique was adopted to transfer the protein film from the air–water interface onto the activated silicon supports ($5 \times 5\ mm^2$). Excess water transferred with films was removed by nitrogen flux. Samples with 1 or 3 layers were deposited. It was demonstrated that hydrophobic support surface increases the tendency of collagen to form fibrils in the adsorbed phase. Thus, the deposition was carried out onto hydrophobic silicon supports. Silicon supports were firstly cleaned in concentrated sulphuric acid at 150 °C for 20 min, then extensively washed in bi-distilled water and dried by nitrogen flux. Supports were then treated in dimethyldiclorosilane (2% in hexane) for 20 min and the washed consecutively in hexane, acetone and hexane.

2.3. Layer-by-layer technique

Collagen for LbL deposition was used at a concentration of 0.2 mg/ml in acetic acid solution 0.1 M at pH 4, when collagen is positively charged [31,32]. Collagen was allowed to dissolve overnight under stirring at 4 °C. The obtained solution was then centrifuged at 1000 rpm to remove eventually undissolved material, as suggested by the supplier. Hyaluronic acid at a concentration of 0.5 mg/ml at pH 4 was used as anionic specie in alternation with collagen. The deposition was carried out onto silicon supports cleaned in concentrated sulphuric acid at 150 °C for 20 min and then extensively washed in bi-distilled water. Taking into account that the silicon surface is negatively charged, a first layer of cationic PEI (concentration of 2 mg/ml pH 4) was deposited to impart a positive charge to the surface for the following deposition of the bi-layer (HA/COL).

2.4. Quartz crystal microbalance

Gravimetric measurements were carried out by means of a gauge developed for this purpose using quartz crystal oscillators with a resonance frequency of 10 MHz and stability better than 1 Hz [33]. The collagen film was deposited on both sides of the resonator, and afterwards dried with nitrogen flux; the frequency shift was registered after each covering. The change in resonance frequency was measured after each assembly step and correlated to the adsorbed mass (Δm, ng) and layer thickness (Δt, nm) by the Sauerbrey equation [34].

$$-\Delta F = \left[\frac{2F_0^2}{A\sqrt{\rho_q \mu_q}}\right]\Delta m \quad (1)$$

where F_0 is the resonance frequency of the quartz crystal oscillator, A is the area of the electrode, ρ_q is the quartz density, and μ_q is its shear modulus.

The following equations were derived from [1] and used in the present work:

$$\Delta m = -0.7\ \Delta F \quad (2)$$

$$\Delta t = -0.013\ \Delta F \quad (3)$$

Quartz crystals were washed with acetone and then dried in a nitrogen flux.

2.5. Atomic force microscopy

Samples were air dried at room temperature, and then analyzed at the AFM by using a custom build set-up driven by R9 advanced controller (RHK technology) in air at room temperature.

Data acquisitions were carried out in tapping mode at scan rates between 0.4 and 0.7 Hz, using rectangular Si cantilevers (NCHR,