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Multilayered membranes with tuned well arrays to be used as regenerative patches

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ABSTRACT

Membranes have been explored as patches in tissue repair and regeneration, most of them presenting a flat geometry or a patterned texture at the nano/micrometer scale. Herein, a new concept of a flexible membrane featuring well arrays forming pore-like environments to accommodate cell culture is proposed. The processing of such membranes using polysaccharides is based on the production of multilayers using the layer-by-layer methodology over a patterned PDMS substrate. The detached multilayered membrane exhibits a layer of open pores at one side and a total thickness of 38 ± 2.2 μm. The photolithography technology used to produce the molds allows obtaining wells on the final membranes with a tuned shape and micro-scale precision. The influence of post-processing procedures over chitosan/alginate films with 100 double layers, including crosslinking with genipin or fibronectin immobilization, on the adhesion and proliferation of human osteoblast-like cells is also investigated. The results suggest that the presence of patterned wells affects positively cell adhesion, morphology and proliferation. In particular, it is seen that cells colonized preferentially the well regions.

The geometrical features with micro to sub-millimeter patterned wells, together with the nano-scale organization of the polymeric components along the thickness of the film will allow to engineer highly versatile multilayered membranes exhibiting a pore-like microstructure in just one of the sides, that could be adaptable in the regeneration of multiple tissues.

Statement of Significance

Flexible multilayered membranes containing multiple micro-reservoirs are found as potential regenerative patches. Layer-by-layer (LbL) methodology over a featured PDMS substrate is used to produce patterned membranes, composed only by natural-based polymers, that can be easily detached from the PDMS substrate. The combination of nano-scale control of the polymeric organization along the thickness of the chitosan/alginate (CHT/ALG) membranes, provided by LbL, together with the geometrical micro-scale features of the patterned membranes offers a uniqueness system that allows cells to colonize 3-dimensionally. This study provides a promising strategy to control cellular spatial organization that can face the region of the tissue to regenerate.

1. Introduction

Tissue engineering procedures have often employed porous scaffolds, as three-dimensional (3D) supports for initial cell attachment and subsequent tissue formation both *in vitro* and *in vivo* [1]. Many techniques have been proposed to fabricate such structures,

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where pore size and porosity could be easily controlled [1,2]. For some applications like wound-dressing [3,4], cardiac [5], cornea [6], periosteum [7,8], periodontal [9,10] and nerve regeneration [11], 2-dimensional (2D)-like devices have been explored as supportive structures for cell attachment, growth and differentiation. In flat membranes, cells do not recognize the same pseudo 3D environment as in conventional porous 3D scaffolds [12]. It is well known that cell behavior is highly dependent on the topography of the scaffolds, including mechanical properties [13], roughness [14], width and depth of the substrate pattern [15], as well as the geometry of the exposed sites for cellular adhesion [16–18]. In order to provide geometrical features, the surface of 2D substrata has been patterned with topographic motifs, usually exploring pillars and grooves textures at the nano and sub-micro scale-level [19–22]. The integrin-mediated mechanotransduction takes place through many intracellular molecular pathways that result to changes in the biological outcomes through mechanical forces [23]. The development of patterned scaffolds that can tailor cell-substrate interactions [24–26] will provide and stimulate specific biological recognition pathways to control the cytoskeletal organization of the cells [26,27].

Across the few studies [28,29] that have explored micro-sized patterns in 2D substrates, none have investigated the effect of membranes with tuned well arrays forming pore-like environments in cell function. We hypothesize that the existence of such structures could help cells to organize adequately towards a new tissue formation.

It is widely known that the pore size of 3D scaffolds influences cellular behavior. For example, in collagen scaffolds with pores diameter ranging between 85 μm and 325 μm, more adhered cells were observed in the structures with the biggest pores size [30]. Although several studies were already reported about the pore size effect [30–33], significant less investigation has been addressing the effect of geometry and curvature on cellular adhesion and proliferation. Still, recent literature [34–36] has been reporting the influence of scaffolds surface curvature on the kinetics of tissue formation; *in vitro* and *in vivo* studies comparing tissue growth in natural bone structures and in hydroxyapatite scaffolds showed that the tissue formation occurs preferentially on the concave areas of the scaffolds [35].

Herein, we propose a new concept of quasi-3D freestanding membranes that exhibit an open pore layered organization in one side of the membrane, with a well-defined geometrical feature to control cellular behavior. For the proof of concept, we patterned wells with 500 μm of diameter and about 15 μm of depth in nanostructured multilayered films. We explored the possibility of controlling cell function by tuning the geometry of the pores, which is not straightforward in 3D scaffolds. The strategy herein proposed offers versatile possibilities for adapting the membranes morphology and pattern to the desired application. To prepare such kind of membranes made of non-meltable natural-based materials, solution-based processing techniques must be preferentially used. Solvent casting is a widely used method to prepare films, but it might not be adequate to process the envisaged patterned membranes, especially if the height of the geometrical features is at the same size level (or higher) of the thickness of the membranes [37,38]. In this study, we propose the use of Layer-by-Layer (LbL) as it allows the assembly of oppositely charged elements over any substrate with a geometrical control at the nano-scale-level [39,40]. It was already shown that LbL could be used to coat textured surfaces with micrometer-scale features to enhance the performance of biomedical devices [41,42]. On the other hand, LbL was also used to process flat freestanding membranes based on chitosan (CHT) and alginate (ALG) [12,43]. We combined these two possibilities to produce for the first time easily detachable freestanding multilayer membranes patterned with well-defined geometrical motifs and uniquely composed by natural-based polymers. We are particularly interested in generating an array of pores with controlled geometry, size, and depth that could be adequate to accommodate cell colonization, organization and proliferation.

In this work, low surface energy polydimethylsiloxane (PDMS) templates, fabricated by UV photolithography, are used as supportive substrates to prepare patterned membranes. In the end we expect to produce polymeric membranes with well-defined geometries that can replicate the patterning of the supportive substrate with a high geometrical control, while maintaining the nano-scale control of the polymer organization along the thickness of the membrane. Such devices will be investigated as single patterned membranes but we expect that they can be seen as building blocks to produce thicker tissues upon stacking different membranes, as proposed before with cell sheets [44].

2. Materials and methods

2.1. Materials

CHT of medium molecular weight (Mw = 190–310 kDa, 75–85% degree of deacetylation (DD), viscosity 200–800 cps) and ALG (Mw = 538 kDa) were purchased as powder from Sigma Aldrich. CHT was submitted to a purification process, described elsewhere [45]. The polypropylene (PP) substrates used for the flat (F) control membranes were obtained from Firmo-Papéis e Papelarias S.A as A4 sheets and cut into rectangles with (10x4cm^2). Genipin (G) was supplied by Waco Chemicals GmbH in Germany and Fibronectin from Human Plasma (Fn) (1 mg.ml^{-1}) was purchased from Millipore S.A.S France. Ethanol was supplied by Fisher Chemical. Sodium Chloride (NaCl), phosphate buffered saline (PBS), dymethil sulfoxide (DMSO), N-(3-Dimethylaminopropyl)-N′-ethylcarbodi mine hydrochloride (EDC) and N-Hydroxysulfosuccinimide sodium (NHS) were obtained from Sigma Aldrich. SU-8 100 (epoxy-based negative photoresist) and SU-8 developer were purchased from Microchem. Kit Sylgard® and 184 Silicone Elastomer (Polydimethylsiloxane – PDMS) was purchased from Dow Corning.

2.2. Build-up and real-time characterization of CHT/ALG films

The build-up process of CHT and ALG multilayer was evaluated using a quartz crystal microbalance with dissipation monitoring (QCM-D, Q-Sense, Sweden), with a gold coated sensor excited at a fundamental frequency of 5 MHz and eleventh overtone (55 MHz). The crystals were cleaned by a successive washing steps with acetone, ethanol and isopropanol, in an ultrasound bath at 30 °C. CHT 0.2% (w/v) and ALG 0.2% (w/v) solutions were prepared in sodium acetate buffer (0.1 M) in the presence of additional salt (0.15 M of NaCl solution). The pH of both solutions was adjusted to 5.5. The CHT solution was injected with a constant flow rate of 50 μL.min^{-1} into the system, standing for 10 min to allow the adsorption equilibrium at the surface of the crystal. Then a sodium acetate buffer (0.15 M of NaCl, pH 5.5) solution was pumped into the system for 10 min. The same procedure was followed with the ALG solution for the desired number of layers. The frequency and dissipation were monitored in real time until the number of the desired layers was successfully accomplished. The Fn absorption onto the resulting system was also evaluated. A 10 μg.ml^{-1} Fn solution was injected for approximately 2.5 h in the system and then washed with NaCl solution for 20 min. The thickness of the film was estimated by the Q-Tools software, from Q-Sense, using the Voigt model.