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Differential neuronal and glial behavior on flat and micro patterned chitosan films

Marta Mattotti[a,b,c,1], Zaida Alvarez[a,b,c,e,1], Luis Delgado[b,c], Miguel A. Mateos-Timoneda[a,b,c], Conrado Aparicio[d], Josep A. Planell[a], Soledad Alcántara[e], Elisabeth Engel[a,b,c,*]

[a] Institute for Bioengineering of Catalonia (IBEC), The Barcelona Institute of Science and Technology, Barcelona, Spain
[b] Department of Materials Science and Metallurgy, Technical University of Catalonia (UPC), Barcelona, Spain
[c] CIBER en Bioingeniería, Biomateriales y Nanomedicina (CIBER-BBN), Barcelona, Spain
[d] Minnesota Dental Research Center for Biomaterials and Biomechanics, School of Dentistry, University of Minnesota, Minneapolis, MN, USA
[e] Department of Pathology and Experimental Therapeutics, Faculty of Medicine and Health Sciences (Bellvitge Campus), University of Barcelona, Barcelona, Spain

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ABSTRACT

Chitosan is a biodegradable natural polysaccharide that has been widely studied for regenerative purposes in the central nervous system. In this study we assessed the *in vitro* glial and neuronal cells response to chitosan either flat or patterned with grooves in the micrometric range. Chitosan demonstrated to be a good substrate for the attachment and growth of both neurons and glial cells. Chitosan micropatterns promoted glial cell maturation, suggesting astroglial activation. Nevertheless, those mature/reactive glial cells were permissive for axonal growth. Axons aligned and organized along the patterned grooves and the size of the linear topographic patterns is also affecting neurite and cell response. Patterns with 10 μm width induced fasciculation of axons, which can be useful for CNS tissue engineering substrates when precise orientation of the axonal outgrowth is desired.

1. Introduction

In order to help regeneration in central nervous system, the final aim of implanting biomaterials is to encourage a permissive environment for neurons that attempt to restore connections. Among the biomaterials used in investigation for this purpose, chitosan outstands due to its good affinity for nerve cells [1–4].

Chitosan is a natural polycationic linear copolymer of beta (1–4)-D-glucosamine and is the second most abundant polysaccharide in nature. Chitosan is biodegradable, water soluble, sustains the release of molecules and supports the attachment and proliferation of different cell types, and thus it has recently found a range of applications in tissue engineering [5,6]. Moreover, chitosan does not provoke immune rejection, has good and tunable mechanical properties and has hemostatic and antibacterial properties [7]. Chitosan can be easily microstructured by soft lithography techniques up to the nanometric scale [8,9]. Previous studies involving neural cells growing on line patterned chitosan showed that cells followed and aligned to the pattern [10,11].

Although many studies have been published regarding chitosan for nerve tissue engineering, all of them apply surface modifications, either chemical or biochemical. In this study we used neural cells from the cerebral cortex as an *in vitro* model to analyze the effect of topography on non-chemical modified chitosan surfaces and performed biochemical characterization of cell-biomaterial interactions. Most of the published studies are limited to adhesion, morphology and viability assessment of neurons, iPSC derived neurons and neural embryonic stem cells [12,13]. However, in order to interpret the interaction between cells and biomaterials, a deeper understanding of cells response is required.

The functional units of the central nervous system (CNS) are neurons, which are unique in their ability to transmit rapid electrical signals in the form of action potential (Allen and Barres, 2009), yet they are vulnerable to injury. Most mature neurons are postmitotic and are identified by the expression of specific markers such as β-III-tubulin (Tuj-1), microtubule associated protein 2 (MAP-2) among others. On the contrary, the most abundant cell type that guide regeneration is astrocytes. Astrocytes play crucial roles in

* Corresponding author.
E-mail address: eengel@ibecbarcelona.eu (E. Engel).
[1] These authors contributed equally.

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the CNS during normal homeostasis, during development and after injury [14]. Astrocytes constitute a heterogeneous group of cells, which show different morphology and molecular patterns as development proceeds, such as nestin and brain lipid binding protein (BLbP) that are expressed glial precursors and immature astrocytes, and glial fibrillary acidic protein (GFAP), which is a marker of mature astrocytes [14]. They serve as physical and trophic support for neurons and they can be either permissive for their growth and guidance, like during cerebral cortex development, or can be extremely inhibitory, like the glial scar formed after injury [14]. For this reason, when designing biomaterials for CNS tissue engineering applications, the study of not only neuronal response, but also glial cell response and the interaction between the two cell types is extremely important.

Previous studies in our group have demonstrated the relevance of the biomaterial and the topography to elicit cell phenotype reprograming [15,16]. Thus, the purpose of our study was to evaluate *in vitro* glial and neuronal responses to flat and micro patterned chitosan in terms of cell morphology and differentiation state.

2. Materials and methods

2.1. Preparation of flat and micropatterned films of chitosan

Chitosan films were prepared as previously described [8]. Briefly, medium molecular weight chitosan (75–85% deacetylated, 200–800 cps viscosity), derived from crab shell, was purchased from Aldrich (Sigma-Aldrich Chemical, USA). A 2% (w/v) polymer solution was produced by dissolving the chitosan in dilute glacial acetic acid (1% v/v). The chitosan polymer solution was centrifugated at 15000 rpm to get rid of un-dissolved particles.

The solution was casted on flat or patterned moulds. Micropatterns consisted of 2 μm and 10 μm wide grooves (ln2 and ln10), all 1 μm deep/tall. The silicon moulds were provided by AMO GmbH (Aachen, DE) and consisted of 1.5" × 1.5" silicon squares. The solution was dried for 8 h at 37 °C and neutralized with 3w/v% NaOH. The films were rinsed in MilliQ water (Millipore, USA). For cell studies, chitosan films were neutralized under sterile conditions, using sterile water and operating under the hood. Standard borosilicate glass (Knittel Glaeses, Germany) and tissue culture plastic (TCP) have been used as control materials, respectively for imunocytochemistry (ICC) or Western blotting, respectively. Control materials were used nude or coated with poly-D-lysine (Sigma-Aldrich, Saint Louis, MO) to allow the adhesion of neurons. Glass was autoclaved as standard procedure at 120 °C for 20 min.

2.2. Material characterization

Wettability of the materials was characterized *via* contact-angle measurements using an OCA 20 system (Dataphysics, GmbH, Germany). The captive bubble method was performed using a custom made PMMA chamber filled with ultrapure water. A 2 μl bubble of room air was dispensed and allowed to make contact with an upside down sample located at the top of the chamber. The reported value for the contact angle is the complementary angle of the one measured between the air-bubble and the surface of the tested material. A minimum of four different measurements was performed on at least four different samples.

ζ-Potential measurements of chitosan, Glass and LysGlass were carried out using a SurPASS apparatus with VisioLab software (Anton Paar Ltd. – UK). All the measurements were performed 4 times at the pH of the electrolyte (KCL 1 mM, pH 5.5) after 2 h of equilibration using the Adjustable Gap Cell for small samples (20 mm x 10 mm).

The percentage of water absorption was calculated weighting the samples in dry and wet state. Mechanical properties of chitosan films were determined using an uniaxial testing machine (Adamel Lhomargy DY34) equipped with a 100-N load cell under a cross-head speed of 20 mm/s (ASTM D882 method). Measurements were taken at room temperature, maintaining the films in the wet state. All samples were cut into rectangular shape with dimensions of 210 ± 20*24 ± 2 mm. At least five samples were tested.

The *in vitro* degradation of chitosan films was followed at 37 °C over a period of 5 weeks. Four samples (~20 mg) were immersed in PBS (250 ml PBS: 1 g sample), retrieved weekly, dried during 6 h at 37 °C and weighted with an analytical balance. The percentage of mass loss after certain time (t) of immersion in PBS, % Deg(t), was calculated using the following formula;

$$\%\mathrm{Deg}(t) = 100(M0 - M(t))/M0,$$

where M0 is the dried weight of the sample at time t = 0 and M(t) is the dried weight at a time = t of immersion in PBS.

The topographical characterization of the surface of patterned chitosan films was performed on a surface of 194 × 94 μm using a WYKO NT1100 white light interferometer and the software Vision 32 V2.303 (Veeco Instruments, Inc, USA).

2.3. In vitro *studies*

All animal housing and procedures were approved by the Institutional Animal Care and Use Committee in accordance with Spanish and EU regulations. Neurons were obtained from embryonic brains like described previously [17]. Cerebral cortices from E16 mice were dissected out free of meninges in dissection buffer (PBS 0.6% glucose (Sigma), 0.3% BSA (Sigma)), digested with trypsin-DNAse I (Biological Industries and Sigma respectively), dissociated and preplated for 30 min in preplating medium (CO2-equilibrated NeurobasalTM (NB) supplemented with 5% normal horse serum (NHS), 1% Pen-Strep, 0.5 mml-glutammine, 5.8 μl/ml NaHCO3 (Sigma–Aldrich, Saint Louis, MO)). Embryonic cortical cells were then recovered from the supernatant, centrifuged and resuspended in neuronal serum free culture medium (NB, 1% Pen-Strep, 0.5 mml-glutamine, 1 x B27, 5.8 μl/ml NaHCO3). Neurons were plated at a density of 2.5 105 cells/cm2 on chitosan films and on poly-lysine coated control surfaces and cultured for 5 days *in vitro* (DIV). The medium was supplemented with 1% NHS during the first 24 h and then changed for fresh neuronal serum free medium. For mixed glial-neurons co-cultures, neurons were seeded directly on top of 5DIV glial cell cultures and cultured during 5 more days in serum free neuronal culture medium. Glial cells were derived from brain cortex of newborn mice like described elsewhere [17]. Briefly, cerebral cortices were dissected out free of meninges in dissection buffer and digested with trypsin-DNAse I for 10 min at 37 °C. The tissue was dissociated in Dulbecco's Modified Eagle Medium (DMEM, Biological Industries), 10% Normal Horse Serum (NHS, GIBCO), 1% Penicillin-Streptomycin (Pen-Strep, Biological Industries) and 2 mM L-Glutamine (Biological Industries), referred to in this text as growing medium (GM). After centrifugation and re-suspension, cells were plated and grown to confluence at 37 °C, 5% CO2 (approximately 25–30 DIV). All the experiments were performed using glial cells from the first passage (Ps1). Glial cells were cultured at a density of 2 × 105cells/cm2 either on chitosan films or on control surfaces for 5DIV in NB, 3%NHS, 1% Pen-Strep, 2mML-Glutamine (Culture Medium, CM). The samples were either fixed in 4% PFA for 15 min at RT for immunochemistry procedures or used for protein extraction and western blot analysis.