Biomaterials 48 (2015) 56–65

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Layer-by-layer assembled cell instructive nanocoatings containing platelet lysate

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A R T I C L E I N F O

Article history:
Received 15 September 2014
Accepted 20 January 2015
Available online 10 February 2015

Keywords:
Layer-by-layer assembling
Instructive surfaces
Platelet lysate
Growth factors
Cell behavior
Platelet derivative

A B S T R A C T

Great efforts have been made to introduce growth factors (GFs) onto 2D/3D constructs in order to control cell behavior. Platelet lysate (PL) presents itself as a cost-effective source of multiple GFs and other proteins. The instruction given by a construct-PL combination will depend on how its instructive cues are presented to the cells. The content, stability and conformation of the GFs affect their instruction. Strategies for a controlled incorporation of PL are needed. Herein, PL was incorporated into nanocoatings by layer-by-layer assembling with polysaccharides presenting different sulfation degrees (SD) and charges. Heparin and several marine polysaccharides were tested to evaluate their PL and GF incorporation capability. The consequent effects of those multilayers on human adipose derived stem cells (hASCs) were assessed in short-term cultures. Both nature of the polysaccharide and SD were important properties that influenced the adsorption of PL, vascular endothelial growth factor (VEGF), fibroblast growth factor b (FGFb) and platelet derived growth factor (PDGF). The sulfated polysaccharides-PL multilayers showed to be efficient in the promotion of morphological changes, serum-free adhesion and proliferation of high passage hASCs (P > 5). These biomimetic multilayers promise to be versatile platforms to fabricate instructive devices allowing a tunable incorporation of PL.

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1. Introduction

The design of cell instructive surfaces is of major interest in the field of tissue engineering and regenerative medicine. Cell behavior is dictated by the interactions occurring between cell surface macromolecules, e.g. transmembrane proteins, glycolipids, glycoproteins and carbohydrates with the extracellular environment [1–4]. Cells are embedded in extracellular matrix (ECM), which is rich in glycosaminoglycans, proteoglycans, water and growth factors (GFs), and confers mechanical support and anchorage points. GFs are cytokines present either in soluble form or non-covalently bound to ECM polysaccharides presenting various sulfation degrees. GFs bind to cell tyrosine kinase receptors triggering intracellular events, making them very attractive molecules for cell behavior manipulation [5]. Cell behavior is affected by the concentration of GFs, as well as, by the presence or absence of their conjugations with sulfated polysaccharides [6]. GFs have a short half-life and so, strategies for their stabilization and exposure to cells at adequate doses, to trigger the adequate cell responses, are very important for many therapeutic applications [7,8]. Several methodologies for the introduction of one or two different recombinant GFs (rGFs) have been used: covalent binding [9,10], absorption [10], biotinylated heparin-avidin bonding [11], layer-by-layer (LbL) assembling [12], GF absorption onto pre-built LbL multilayers [13,14], and electrostatic binding with heparin [15,16]. However, more translational developments in this area have been hindered by the high cost of rGFs, and difficulties to introduce simultaneously several GFs.

Platelets arise as a cost-effective autologous source of multiple GFs and other bioactive proteins – Scheme 1a. Among them, some have a proven ability to improve, for instance, adhesion, mitogenesis and cell differentiation [17–19]. The instructive potential of

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http://dx.doi.org/10.1016/j.biomaterials.2015.01.020
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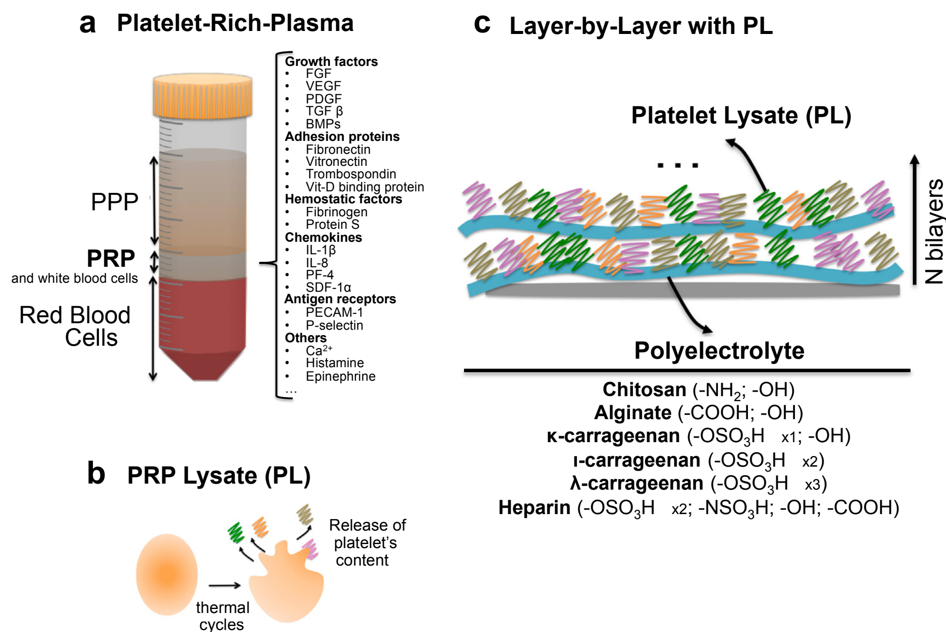

Scheme 1. Main steps for the preparation of PL/Polysaccharides Layer-by-Layer assembled nanocoatings. a) Platelet isolation from human blood as Platelet-Rich-Plasma (PRP) and examples of bioactive proteins than can be found in the enriched protein cocktail. b) PL preparation: PRP activation by platelet disruption induced by thermal cycles for the release of the inner content. c) Layer-by-Layer deposition combining PL with several PEs which respective functional groups and content are indicated.

platelet derivatives as a media supplement has been shown to vary with the GF content and also displays variability associated with the donor [20–22]. Platelet's derived GFs have been successfully included onto surfaces employing adsorption [23,24] and antigen specific GF recruitment from platelet lysate (PL) [25]. Sole absorption of GFs only allows for a short-term control over cells since, typically, they are quickly released. Covalent binding of a GF allows a more stable presentation to the cells. However, its activity may be compromised by changes in the GFs conformation, masking the active sites, or by GF-receptor complexes cell internalization inhibition [26].

LbL is a simple and versatile technology that has been employed in the development of a large variety of biomedical devices [27,28]. It is often based on a simple alternated deposition of negatively and positively charged polyelectrolytes (PEs). LbL can be performed in protein-mild conditions on virtually any substrate, ranging from nanoparticles to 3D scaffolds and hydrogels [27,28]. There are reports on the beneficial use of one or two rGFs in LbL approaches [12,29,30].

We believe that it would be advantageous to include multiple GFs from human sources as structural components of the multilayers, increasing both the complexity of the structures and the similarity with the ECM. We propose the use of LbL assembling for the development of multilayers nanocoatings containing PL in their structural composition, in which cell instructive cues can be preserved. PL was combined with several polyelectrolytes (PEs) presenting different SD (from 0 to 3 sulfate groups per sugar unit), different charges, functional groups conformation, and compared to the gold standard for protein stabilization – heparin. Heparin is a highly sulfated polysaccharide that has been widely used to stabilize and attract rGFs [15,16,31,32] and has been inspiring the synthesis of new polymeric matrices with heparin-analog ending groups [33]. PEs were assessed for their capability to adsorb PL, including the following specific GFs: VEGF, PDGF and FGFb. The role of the PE used and the bioactivity of those multilayers were evaluated by assessing its mitogenic, morphological and phenotypic effects on human adipose derived stem cells (hASCs).

2. Materials and methods

2.1. Materials

Medium molecular weight chitosan (Chi), with a degree of deacetylation of 80% (Sigma Aldrich, MKBB0566), was purified by a re-precipitation method. Briefly, Chi powder was first dissolved in 2% (v/v) acetic acid solution at a 1% (w/v) concentration. The mixture was maintained under stirring overnight at room temperature. The impurities were removed by four filtration cycles. Then, Chi was precipitated by addition of 1 M NaOH while stirring. Final steps consisted of washing Chi with distilled water until reaching a neutral pH and of dehydration by washing with ethanol–water mixtures with increasing ethanol content (20–100% v/v). Chi was freeze-dried for 3 days and grinded. κ- (Sigma–Aldrich, 22048), ι- (Fluka, 22045), λ-carrageenan (Car; Sigma–Aldrich, 22049), sodium heparin (Hep; Sigma–Aldrich, H3149), sodium alginate (Alg; Sigma Aldrich, 250 cP), and poly(ethyleneimine) solution (PEI; Sigma–Aldrich, P3143) were used as received.

2.2. Materials preparation

2.2.1. Preparation of platelet lysate

Platelet concentrates were obtained from different platelet collections performed at Instituto Português do Sangue (IPS, Porto, Portugal), under a previously established cooperation protocol. The components were obtained using either the Trima Accel® Automated Blood Collection System. All the platelet products were biologically qualified according to the Portuguese legislation. The platelet count was performed at the IPS using the COULTER® LH 750 Hematology Analyzer and the sample volume adjusted to 1 million platelet.μL^{-1}. The collected samples were subject to three repeated temperature cycles (frozen with liquid nitrogen at −196 °C and heated at 37 °C) and frozen at −20 °C until further use. The remaining platelets were eliminated by centrifugation at 1400 *g* for 10 min. Aliquots of platelet lysate (PL) were stored at −20 °C until final use.

2.2.2. Polyelectrolytes solutions

κ-,ι-, λ-Car, Hep and Alg were prepared in 1 M Tris HCL 40 nM NaCl pH 7.4 with a concentration of 0.5 mg.mL^{-1}. Chi was dissolved in sodium acetate buffer with a concentration of 0.5 mg mL^{-1}. All the solutions were gently stirred overnight. PL was 10-fold diluted with Tris HCL buffer or in 1 M sodium acetate 40 mM NaCl pH 6 when to be combined with Chi.

2.2.3. QCM-D monitoring

A Q-Sense E4 quartz crystal microbalance (QCM-D, Q-Sense AB, Sweden) with dissipation was used for *in situ* monitoring the deposition of PE/PL bilayers at the surface of 100 nm gold-coated crystals. The crystals were first cleaned in an ultrasound bath at 30 °C, and immersed successively in acetone, ethanol, and isopropanol. All crystals were initially modified with PEI to confer an initial positive charge. A 0.5% w/v PEI solution (Mw 750,000, Sigma–Aldrich) was pumped for

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