Colloids and Surfaces B: Biointerfaces 116 (2014) 93–103

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

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

Study on multilayer structures prepared from heparin and semi-synthetic cellulose sulfates as polyanions and their influence on cellular response

Neha Aggarwal[a], Noomi Altgärde[b], Sofia Svedhem[b], Kai Zhang[c], Steffen Fischer[d], Thomas Groth[a,*]

[a] Biomedical Materials Group, Institute of Pharmacy, Martin Luther University Halle-Wittenberg, Heinrich-Damerow-Strasse 4, 06120 Halle (Saale), Germany
[b] Department of Applied Physics, Chalmers University of Technology, 412 96 Gothenburg, Sweden
[c] Institute of Macromolecular Chemistry and Paper Chemistry, Technische Universität Darmstadt, Petersenstr. 22, D-64287 Darmstadt, Germany
[d] Institute of Plant and Wood Chemistry, Dresden University of Technology, Pienner Str. 19, D-01737 Tharandt, Germany

ARTICLE INFO

Article history:
Received 17 October 2013
Received in revised form
15 December 2013
Accepted 20 December 2013
Available online 31 December 2013

Keywords:
Chitosan
Heparin
Cellulose sulfate
Layer-by-layer
Cell adhesion
Cell growth

ABSTRACT

Multilayer coatings of polycationic chitosan paired with polyanionic semi-synthetic cellulose sulfates or heparin were prepared by the layer-by-layer method. Two different cellulose sulfates (CS) with high (CS2.6) and intermediate (CS1.6) sulfation degree were prepared by sulfation of cellulose. Multilayers were fabricated at pH 4 and the resulting films were characterized by several methods. The multilayer 'optical' mass, measured by surface plasmon resonance, showed little differences in the total mass adsorbed irrespective of which polyanion was used. In contrast, 'acoustic' mass, calculated from quartz crystal micro balance with dissipation monitoring, showed the lowest mass and dissipation values for CS2.6 (highest sulfation degree) multilayers indicating formation of stiffer layers compared to heparin and CS1.6 layers which led to higher mass and dissipation values. Water contact angle and zeta potential measurements indicated formation of more distinct layers with using heparin as polyanion, while use of CS1.6 and CS2.6 resulted into more fuzzy intermingled multilayers. CS1.6 multilayers significantly supported adhesion and growth of C2C12 cells where as only few cells attached and started to spread initially on CS2.6 layers but favoured long term cell growth. Contrastingly cells adhered and grew poorly on to the layers of heparin. This present study shows that cellulose sulfates are attractive candidates for multilayer formation as potential substratum for controlled cell adhesion. Since a peculiar interaction of cellulose sulfates with growth factors was found during previous studies, immobilization of cellulose sulfate in multilayer systems might be of great interest for tissue engineering applications.

1. Introduction

In the field of tissue engineering and implantable biomaterials, the appropriate design of biomaterials at micro and nanometer scale to control their bulk and surface properties to direct cell fate is a great challenge. Apart from bulk composition, which controls mechanical and other properties, the biomaterial surface features are of prime importance in dictating the interaction of the material with its environment [1,2]. Hence, surface modification and functionalization of biomaterials have become very important for biomaterials research and a large number of techniques have been developed for this purpose [3,4]. A physical surface modification technique called the layer-by-layer (LbL) technique, introduced by Decher and co-workers [5], has been adopted for biomaterials surface modification to improve their biocompatibility during the last years [6]. The LbL technique is based on electrostatic attraction and ion-pairing of oppositely charged polyelectrolytes that are alternatingly adsorbed onto charged substrata [7]. It is important to note that the conditions during complexation of the polyelectrolytes, e.g. temperature, pH and ionic strength, control the multilayer properties, particularly when weak polyelectrolytes are involved [8]. The selection of appropriate polyelectrolytes and complexation conditions allows the design of material coatings with controlled intrinsic (bulk) and extrinsic (surface) properties [9]. While the majority of research with the layer-by-layer method is based on fully synthetic polyelectrolytes, multilayer films consisting of charged polysaccharides have gained an increasing attention during the last years [10,11].

Glycosaminoglycans (GAGs), a class of polysaccharides, are an important component of the extracellular matrix (ECM). The GAGs are built from sugar rings, which are linked by glycosidic bonds and

* Corresponding author. Tel.: +49 345 55 28 460/+49 15228627911; fax: +49 345 55 27 379..
E-mail address: thomas.groth@pharmazie.uni-halle.de (T. Groth).

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http://dx.doi.org/10.1016/j.colsurfb.2013.12.043

possess various charged functional moieties, like amino, sulfate or carboxylic groups. Many GAGs like heparin and chondroitin sulfate are strong polyanions due to the presence of sulfate groups. In addition, they have a bioactivity that is expressed by specific interactions with proteins and cells. For example, heparin possesses a multitude of binding partners such as adhesive proteins (e.g. fibronectin) and growth factors (e.g. bone morphogenic proteins), which regulate adhesion, movement, growth and differentiation of cells [12]. Therefore heparin has been used as a component of multilayer coatings on biomaterial surfaces [13,14]. Despite the exceptional multifunctionality of heparin, it is associated with certain drawbacks like the isolation from animal sources, e.g. porcine mucosa or bovine lung, which limits availability in larger quantities and contributes also to chemical heterogeneity and variability of physiological activity [12,15]. Therefore a chemical modification of more abundantly occurring polysaccharides like cellulose to achieve heparinoid features has been suggested previously [16]. In this regard, regioselective sulfation of cellulose has been shown particularly effective to achieve a bioactivity comparable to that of heparin with respect to growth factors and cell interactions [17,18]. Because of the inherent charge density depending on the degree of sulfation, cellulose sulfates are interesting candidates for the LbL technique, and such multilayers were used previously to generate blood compatible surfaces [19]. Also other studies have shown the usage of sulfated polysaccharides including cellulose sulfates in multilayers and investigated the influence of charge density on internal structure of multilayers and their biological interactions [20,21]. On the other hand, many of the natural polycations are weak polyelectrolytes with amino groups as the charged moieties. Such weak polycations used in the LbL technique are often polypeptides like poly-L-lysin [14] or polysaccharides like chitosan [22]. Chitosan is a co-polymer of *N*-acetylglucosamine and glucosamine linked via 1-4-beta glycosidic bonds produced by deacetylation of chitin, which is also available in huge quantities. However, the degree of deacetylation influences the various properties of chitosan like its solubility, biodegradability [23]. Chitosan possesses remarkable antimicrobial activity and promotes wound healing through a number of mechanisms [24,25]. Hence, it is not surprising that chitosan has been applied in different studies as polycation during multilayer formation [13,22].

Polysaccharide-based multilayer formation has been studied recently showing that the charge of GAGs and the deposition conditions like pH and ionic strength allow to control the multilayer composition and thickness [26,27]. It was shown that film thickness increases when the pH of the adsorbing polyelectrolyte is close to its pK_a value and the ionic strength increases within a narrow range [27,28], which affects also the hydration and swelling properties of the films [29]. It is also important to note that during the construction of multilayer films, the local interactions inside the multilayers are likely to depend on the nature of the polyelectrolytes that forms the outer layer. As shown by Xie and Granick, the ionization of weak polyelectrolytes inside multilayers, when interacting with strong polyelectrolytes, is dependent on the nature of the outermost layer of the film [30]. That means the ionization of weak polyelectrolytes changes during the subsequent addition of polyelectrolytes leading also to variations in corresponding film properties like thickness, hydration and mechanical properties [10], which in turn leads to differences in protein adsorption, controlled release of bioactive molecules like growth factors and cell responses [31]. Along with the presence of GAGs (e.g. heparin) in the outermost layers, adsorption of adhesive proteins like fibronectin or vitronectin may be affected [32,33], that is crucial for interactions with cells, which require specific adhesive ligands for communication with integrin cell receptors [34,35]. Hence, the use of natural or semi-synthetic GAGs bears great advantages for multilayer formation because of the change of multilayer properties due to the different charge density of molecules and dependence of charge and conformation on pH value and ionic strength [10,14]. In addition, the intrinsic bioactivity and biodegradability of these polyelectrolytes allows for highly biocompatible and bioresponsive surface coatings that are useful for a large variety of biomedical applications like blood compatible surface [36] or new approaches for making scaffolds and systems for different tissue engineering applications [37,38].

The current study investigates the construction of polyelectrolyte multilayers (PEM) assembled from semi-synthetic cellulose sulfates and heparin as polyanions in a comparative manner. Chitosan was used as polycation to pair with the polyanions. Additionally, poly(ethylene imine) (PEI) was applied to make the first polycation layer as a uniform anchoring layer to provide a better bonding to the underlying model glass or gold surfaces [39]. Two different cellulose sulfates (CS), CS2.6 and CS1.6 (where 2.6 and 1.6 shows the number of sulfate groups per repeating unit) with high and intermediate sulfation degree were used to study the multilayer formation process as well as their biological responses in comparison to heparin, that has only one sulfate group per repeating unit. The results of the study show significant differences in the multilayer properties from cellulose sulfates and heparin in relation to layer mass, amount of coupled water during layer growth, and other surface properties for the different polyanions, which in turn affected the adsorption of fibronectin and adhesion and growth of C2C12 myoblast cells.

2. Experimental

2.1. Materials

Microcrystalline cellulose (MCC) with an average DP of 276 was received from J. Rettenmaier & Söhne GmbH (Germany). Chlorosulfonic acid was purchased from Merck Schuchardt OHG (Germany) and sulfuric acid (98%) from Carl Roth GmbH (Germany). *N*,*N*-Dimethylformamide (DMF) was freshly distilled before synthesis. Deionized water was used in all experiments. Dialysis membrane with a molecular weight cut off of 500 Daltons was obtained from Spectrum Laboratories Inc (Rancho Dominquez, USA). Other chemicals were all of analysis grade and used as received.

Multilayers were fabricated on microscopy glass cover slips (Menzel, Germany). Prior to layer formation cover slips were cleaned for 2 h with 0.5 M NaOH (Roth, Germany) dissolved in 96% ethanol (Roth, Germany) followed by excessive rinsing with micropure water (10 × 5 min). New gold coated sensors for SPR (IBIS Technologies, Hengelo, The Netherlands) and AT-cut gold-coated quartz crystals for QCM-D (Q-sense, Gothenburg, Sweden) measurements were cleaned with 99.8% ethanol (Merck, Germany) and rinsed thoroughly with micropure water. After rinsing sensors were dried with nitrogen (1 bar) and placed immediately overnight in an ethanol (p.a.) solution of 2 mM mercaptoundecanoic acid (MUDA, 95%, Sigma, Germany) to obtain a negatively charged surface by the formation of a self-assembled monolayer exposing carboxyl groups [40].

For preparing polyelectrolyte solutions, poly(ethylene imine) (PEI) (MW 750,000 g/mol, Sigma, Germany), heparin (min 150 IU/mg, MW 8000–15,000 g/mol, Applichem, Germany), and two different cellulose sulfates synthesized (see below) CS1.6 and CS2.6 were dissolved under stirring at a concentration of 2 mg/ml in water containing 0.14 M NaCl. Chitosan solution was prepared from medical grade chitosan with a deacetylation degree of 85% (MW 500,000 g/mol, 85/500/A1, Heppe, Germany) in 0.14 M NaCl and 0.05 M acetic acid at 50 °C for 3 h.