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Novel non-cytotoxic alginate–lignin hybrid aerogels as scaffolds for tissue engineering

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ABSTRACT

This paper presents a novel approach toward the production of hybrid alginate–lignin aerogels. The key idea of the approach is to employ pressurized carbon dioxide for gelation. Exposure of alginate and lignin aqueous alkali solution containing calcium carbonate to CO_2 at 4.5 MPa resulted in a hydrogel formation. Various lignin and $CaCO_3$ concentrations were studied. Stable hydrogels could be formed up to 2:1 (w/w) alginate-to-lignin ratio (1.5 wt% overall biopolymer concentration). Upon substitution of water with ethanol, gels were dried in supercritical CO_2 to produce aerogels. Aerogels with bulk density in the range 0.03–0.07 g/cm^3, surface area up to 564 m^2/g and pore volume up to 7.2 cm^3/g were obtained. To introduce macroporosity, the CO_2 induced gelation was supplemented with rapid depressurization (foaming process). Macroporosity up to 31.3 ± 1.9% with interconnectivity up to 33.2 ± 8.3% could be achieved at depressurization rate of 3 MPa/min as assessed by micro-CT. Young's modulus of alginate–lignin aerogels was measured in both dry and wet states. Cell studies revealed that alginate–lignin aerogels are non-cytotoxic and feature good cell adhesion making them attractive candidates for a wide range of applications including tissue engineering and regenerative medicine.

1. Introduction

Since discovered in 1930s, aerogels, ultra-light open-porous materials, have been gaining a great deal of attention in the foreground of material science and emerging technology. Attempts have recently been made to address a variety of regenerative medicine problems using aerogels as scaffolds [1,2]. Several polymers have been used as precursors to produce aerogel-based tissue engineering scaffolds: PLA [3], chitosan [4–6], and polyurea crosslinked silica [7–9]. The latter material has been extensively assessed *in vivo*.

Alginate is a well-known biomaterial and is widely used for drug delivery [10] and in tissue engineering [11,12] due to its biocompatibility, low toxicity, relatively low cost and simple gelation mechanism [13]. It is a polysaccharide comprising of mannuronic (M) acid and guluronic (G) acid residues obtained either from brown algae or from bacterial sources [14]. Owing to its gelling, thickening, stabilizing and viscosifying properties, alginate is a prominent component for food [15], textile and paper industries [16,17] as well as in pharmaceutical and medical fields [10,18,19]. However, due to the hydrophilic nature of the alginate chains, the protein adsorption is discouraged leading to the hampered cell adhesion and thus limiting potential tissue engineering applications [20,21]. Attempts have been presented in the literature to overcome this limitation including chemical grafting with oligopeptides [20,22]; blending with other biopolymers [23,24] and addition of hydroxyapatite [25]. In this work it was attempted to exploit a major constituent of lignocellulosic biomass, namely lignin, to produce hybrid alginate–lignin aerogels with the prospect of biomedical relevance. As pointed out by Smetana [26],

Abbreviations: BET, Brunauer–Emmett–Teller model; BJH, Barrett–Joyner–Halenda model; *q*, crosslinking degree; DMEM, Dulbecco's modified Eagle's medium; G, guluronic acid; IC_{50}, 50% inhibitory concentration; M, mannuronic; Micro-CT, micro-computed tomography; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium; Na-Alg, sodium alginate; PBS, phosphate buffered saline; PEG, polyethylene glycol; PLA, poly-(L-lactic acid); PVA, polyvinyl alcohol; PVP, polyvinylpyrrolidone; TCP, tissue culture polystyrene; TRIS, tris(hydroxymethyl)aminomethane.

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the ratio between hydrophilicity and hydrophobicity of the surface is an important factor of cell adhesion. Lignin is expected to reduce hydrophilicity of alginate and hence provide more suitable environment for cells to adhere, grow and differentiate. Bearing in mind ultimate stability of lignin, it was also expected that the presence of lignin may abate the scaffold degradation rate and help to match it with the rate of new bone tissue regeneration.

Due to its abundance and low price, it is of definite interest to usher lignin into high-value products, *i.e.* biomaterials, adsorbents, thermal insulators. Several attempts have been reported in the literature on lignin as a part of biomaterials exemplified by composites with hydroxyapatite [27,28]; as a carrier in laxative formulations [29]; allergenicity reducer for latex rubber [30]. Potential applications in food industry are also reported [31]. For comprehensive overview on other application of lignin and lignin-based products readers are referred to recently published reviews [32–34].

One objection against lignin as a material for biomedical and pharmaceutical applications is its phenolic nature. Organosolv lignin has been reported to be slightly cytotoxic for peripheral blood mononuclear cells [28]. One lignin derivative, sulphonated lignin, when blended with fish gelatin, showed cytotoxicity only at very high concentrations (IC_{50} in the range 1500–1750 μg/ml) [31]. IC_{50} values in the range of 400–1200 μg/ml were found for lignins from different sources by Ugartondo et al. [35]. Microalgae (*Chlamydomonas reinhardtii*) and Backer's yeast (*Saccharomyces cerevisiae*) show indistinguishable loss of viability after incubation with lignin nanoparticles compare with a control sample [36]. From this data it can be surmised that generally lignin is not cytotoxic up to moderate concentration. One aim of this work is to prove whether Ca-crosslinked alginate–lignin aerogels are non-cytotoxic and to evaluate them as potential biomaterials.

Apart from lower hydrophilicity and higher stability another potential advantage of lignin is its antimicrobial activity. Although antimicrobial properties of the phenolic units of lignin are well documented [32], there has been some controversy in the literature whether lignin and lignin containing materials have antimicrobial activity. Erakovic et al. [28] have found no significant antimicrobial activity of films obtained by electrophoretic deposition from 1 wt% suspension of organosolv lignin in the presence of hydroxyapatite. Some antimicrobial activity was detected for sulphonated lignin [31]. However, no direct comparison of water insoluble lignin with sulphonated lignin is possible. Antimicrobial action of the latter may be ascribed to its surface active properties. Study of Dizhbite et al. [37] revealed antibacterial effect of kraft lignin and related it to the high activity as radical scavenger. Lignin-related compounds from pine cone are found to induce varieties of antiviral activity [38].

Composites and blends of lignin with cellulose [39], cellulose acetate [40], xanthan gum [41], PEG [42], PVA [43], PLA [44], PVP [45,46] are known from the literature. Even though there may be only weak interaction between lignin and principal constituent, addition of lignin may offer advantages such as more control over water uptake [41] and improved mechanical properties [31,45]. Importance of conjugating lignin with polysaccharides for *in vivo* expression of various kinds of immunopotentiating activity is also reported [38]. These features may also have a beneficial effect with respect to biomedical applications.

Gelation by a reaction with crosslinkers is a common technique to obtain lignin aerogels. Gelation with resorcinol formaldehyde [47], phenol formaldehyde [48], tannin formaldehyde systems [49] and α,ω-diglycidyl ethers [50] are reported. To the best of our knowledge, ionic crosslinking of pure lignin or polymer blends containing lignin has not been reported. In this work a goal was set to use alginate as a "glue" for lignin. Presence of alginate allows the use of ionotropic gelation instead of chemical crosslinking. Gelation of alginate induced by pressurized carbon dioxide was recently developed [51] and is used in this work to gel alginate–lignin mixtures. In processing of biomedical materials, CO_2 induced gelation have certain advantages over conventional internal and diffusion gelation methods: (i) carbon dioxide, being volatile acid in water media, can be recovered at post-processing stages; (ii) fast depressurization leads to macroporous foam-like hydrogels; (iii) bactericidal activity of pressurized CO_2 simplifies preparation of food and medical materials [52]; and (iv) the process potentially allows to avoid ambient pressure solvent exchange and can be directly combined with subsequent supercritical drying [51,53].

2. Materials and methods

2.1. Chemicals

Alginic acid sodium salt (suitable for immobilization of microorganisms grade, catalogue no. 71238) was obtained from Sigma Life science, Germany. Lignin was produced as described below (Section 2.2). Calcium carbonate (light, precipitated powder, particle size *ca.* 1 μm) was purchased from Magnesia GmbH, Germany. Sodium hydroxide (>99%) and anhydrous ethanol (99.9%) for the solvent exchange were purchased from Carl Roth GmbH and H. Möller GmbH & Co. KG, respectively. Carbon dioxide used for drying (99.9 mol% purity) was procured from AGA Gas GmbH (Hamburg, Germany). In case of *in vitro* cell culture studies, the chemicals used were of analytical reagent or tissue culture grade. Deionized water was used throughout the study.

2.2. Starting solutions

Lignin was obtained from wheat straw as described elsewhere [50,54]. This process was carried out by the biorefinery research group at the Institute of Thermal Separation Processes, Hamburg University of Technology (Germany). Briefly, wheat straw was fractioned by a hydrothermal pretreatment with liquid hot water at 473 K and 5 MPa followed by an enzymatic hydrolysis step (50 °C, pH 5, Novozymes CTec2, 72 h). Water insoluble lignin was collected after the enzymatic cleavage. Lignin was washed with water and dried at 70 °C for 50 h. 3 wt% solution of lignin was prepared by mixing a certain amount of dried lignin with 1 M NaOH and overnight stirring.

3 wt% sodium alginate solution was prepared by gentle overnight stirring of Na-Alg powder with water. After the preparation both solutions were bottled and stored at 5 °C.

Calcium carbonate powder was dispersed in Na-Alg solution with a high speed homogenizer Ultra-turrax (IKA, Staufen, Germany). Then lignin solution was added to obtain desired alginate-to-lignin ratio: 2:1, 3:1, 4:1 or 5:1 (w/w). Mixture was diluted with water to keep 1.5 wt% overall biopolymer concentration (alginate + lignin) and once again homogenized (Ultra-turrax) for 1 min. Two crosslinking degrees (q) were used: alginate-to-$CaCO_3$ of 1:0.1825 (w/w) is referred as $q = 1$. $q = 2$ corresponds to the doubled amount of $CaCO_3$. Resulting suspension was filled into a standard 48 multiwell plate (BD Biosciences, USA) and subjected to CO_2 induced gelation.

2.3. CO_2 induced gelation and hydrogel foaming

Multiwell plates with Na-Alg/lignin/$CaCO_3$ mixture were placed into an autoclave and exposed to gaseous carbon dioxide at 4.5 ± 0.5 MPa and room temperature for 24 h. The autoclave described elsewhere [55] was used for both gelation and supercritical drying. To study effect of the depressurization rate on macroporosity of the gels, pressure release was employed at 0.8 MPa/min and 3 MPa/min. The gels were left in the air till