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Cross-linkable alginate-graft-gelatin copolymers for tissue engineering applications

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ABSTRACT

When it comes to failing or injured tissues and organs, patients often end up on waiting lists for tissue or even organ transplantation negatively affecting the patient's quality of life. The multidisciplinary research field of tissue engineering may offer more innovative ways to replace or ideally regenerate failing tissues and organs. A widely used material in this research field is gelatin because of its biocompatibility and interesting hydrogel forming properties. However, at body temperature gelatin's mechanical properties are greatly reduced due to the dissolution of collagen-like triple helices. With the aim to obtain materials that retain their mechanical properties at body temperature, we propose to combine sodium alginate and methacrylamide-modified gelatin (Gel-MOD) in the form of a graft copolymer to obtain a material that closely resembles the extracellular matrix. The obtained materials can be cross-linked via three distinct pathways including cation mediated, temperature mediated or via covalent bond formation after UV irradiation in the presence of a photo-initiator. The current contribution covers the synthesis of the above mentioned alginate-graft-gelatin copolymers and the characterization of the resulting hydrogels. The materials developed are highly hydrophilic, showing high gel fractions and satisfactory mechanical properties. Moreover the attained storage moduli were tunable by divalent cation addition (72–275% increase at 21 °C, 42–405% increase at 40 °C). One formulation was found to outperform Gel-MOD in terms of mechanical properties at 40 °C, thus indicating the proposed strategy can be used to improve the mechanical properties of gelatin-based hydrogels. Moreover, *in vitro* biocompatibility assays indicated that cell adhesion and proliferation improves with increasing gelatin content. The present paper illustrates that the developed triple cross-linkable materials are suitable cell carriers, promising to be applied for biomedical purposes.

1. Introduction

Regenerative medicine is a growing interdisciplinary research field that may offer new treatments for patients with failing tissues due to disease or trauma. Regenerative medicine encompasses various strategies to repair or replace cells, tissues

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and organs to restore the impaired function of the affected body parts. Since regenerative medicine aims to bring the patient back to normal health, it can be distinguished from organ transplantation as the inherent need for immunosuppressant medication cannot be considered normal health [1,2]. Tissue engineering has been defined by Langer and Vacanti as an interdisciplinary field that applies principles from engineering and life sciences toward the development of biological substitutes that restore, maintain or improve tissue function [3]. Since cells are always embedded in a three-dimensional polymer network, the extracellular matrix (ECM), one method applies polymer constructs onto which cells can be seeded. These scaffolds should be designed in such a way that they mimic the ECM's mechanical and biological properties.

A material often applied in this regard is gelatin, a single stranded protein obtained from collagen by hydrolytic degradation [4]. Gelatin has already been used in a large variety of applications including food industry applications, pharmaceutical formulations, photographic and other technical products. Gelatin is an interesting biopolymer for tissue engineering applications, since gelatin solutions readily form gel-like structures upon cooling. This gelation is driven by hydrogen bonding and van der Waals interactions, resulting in the aggregation of certain gelatin domains into collagen-like triple helices separated by random coil peptide residues [5–7]. However, these junction zones, being physical in nature, melt at temperatures around 30 °C [4]. This implies that chemical cross-linking is required to avoid dissolution of the scaffolds at body temperature. Despite applying chemically cross-linked gelatin-based hydrogels, the mechanical properties remain lower at body temperature.

With the aim to overcome this limitation and develop scaffolds with mechanical properties that outperform modified gelatin (Gel-MOD), we propose the application of cross-linkable alginate-graft-gelatin copolymers for tissue engineering applications. Applying these systems also implies a better mimic of the aqueous environment cells naturally reside in, since the ECM is composed of both polysaccharides and proteins. To the best of our knowledge, the herein proposed triple cross-linkable alginate-graft-gelatin copolymers have not been applied to date.

Alginates are anionic polysaccharides derived from brown algae and consist of D-mannuronic acid (i.e. the M block) and L-guluronic acid (i.e. the G block) units arranged in an irregular, block wise pattern of varying proportions of GG, MM and MG blocks [8]. Mannuronic acid forms β (1 → 4) linkages, while guluronic acid forms α (1 → 4) bonds resulting in steric hindrance around the carboxylic acid groups. As a result, M blocks form linear domains while G blocks introduce folded regions responsible for a more rigid structure [8,9]. Alginate was selected in this study since it is an interesting biopolymer for biomedical applications because of its ability to rapidly form gels upon addition of multivalent ions [4,10]. Alginate's gelation mechanism is, however, hard to control and does not result in a uniform structure [11]. The formation of an ionotropic hydrogel starting from alginate upon Ca^{2+} addition mainly involves the GG blocks along the polymer backbone [9]. Grant et al. proposed a model in which the GG blocks were thought to combine with the Ca^{2+} ions forming structures resembling an egg-box [12]. In addition to its potential to form hydrogels in the presence of multivalent ions, alginate is mucoadhesive, biocompatible and non-immunogenic making it very suitable for tissue engineering applications [13].

In order to develop scaffold materials of which the mechanical properties remain unaffected at body temperature, gelatin type B was first modified with methacrylic anhydride in order to introduce methacrylamide pendant groups. Next, a fraction of alginate carboxylic acids was converted into reactive esters using carbodiimide chemistry in combination with N-Hydroxysuccinimide (NHS). Finally, unreacted amines present in the Gel-MOD were used for alginate conjugation. The obtained materials could be cross-linked via three distinct strategies including cation mediated, temperature mediated or via covalent bond formation after UV irradiation in the presence of a photo-initiator. The hydrogel materials were characterized via High Resolution-Magic Angle Spinning (HR-MAS) ^1H NMR spectroscopy, rheology, and swelling experiments. Finally, the *in vitro* cell viability and – proliferation behaviour was assessed.

2. Experimental details

2.1. Materials

Gelatin type B, isolated from bovine bone via an alkaline process, was obtained from Rousselot (Ghent, Belgium). Sodium alginate was purchased from Sigma–Aldrich Fine Chemicals (Bornem, Belgium). Potassium phosphate dibasic (K_2HPO_4), sodium phosphate monobasic (NaH_2PO_4), sodium borate ($Na_2B_4O_5$), sodium chloride (NaCl) and potassium chloride (KCl) used to prepare the buffer solutions, were obtained from Acros Organics (Geel, Belgium). Sodium azide was purchased from Avocado Research Chemicals Ltd. (Karlsruhe, Germany). Methacrylic anhydride, 2-mercaptoethanol, n-butylamine, 1-ethy l-3-(3-dimethylamino-propyl)carbodiimide (EDC), N-Hydroxysuccinimide (NHS), o-phthalaldehyde (OPA), hydrochloric acid (HCl) and sodium hydroxide (NaOH) and calcium chloride ($CaCl_2$) were acquired from Sigma–Aldrich (Bornem, Belgium) and were used as received. Ethanol was obtained from Chem-Lab (Zedelgem, Belgium). The photoinitiator Irgacure 2959 was received from BASF (Antwerp, Belgium). Dialysis membranes (MWCO 12,000–14,000 Da) were purchased from Polylab (Antwerp, Belgium). All NMR spectra were recorded in deuterated solvents obtained from Euriso-top (Saint-Aubin Cedex, France). Cell seeding experiments were conducted in cell culture media obtained from Gibco Invitrogen. Live/dead staining was performed using propidium iodide and calcein AM which were obtained from Sigma–Aldrich (Bornem, Belgium) and Anaspec (Fremont, USA) respectively. MTT (3-(4,5-dimethyldiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) obtained from Merck (Nottingham, UK), was applied to determine cell viability. Dulbecco's Modified Eagle Medium (DMEM) Glutamax medium, foetal bovine serum (FBS), penicillin–streptomycin (10 U/ml–10 mg/ml) and sodium-pyruvate were acquired from