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Synthesis and characterization of elastic and macroporous chitosan–gelatin cryogels for tissue engineering

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Abstract

Elastic chitosan–gelatin cryogels of varying concentration of polymer precursors have been synthesized using glutaraldehyde as a crosslinking agent. The optimum co-polymer ratio of chitosan to gelatin was found to be 1:4 at the temperature of −12 °C for synthesis of chitosan–gelatin hybrid cryogels. chitosan–gelatin cryogels synthesized with low viscosity chitosan were morphologically better than those formed with medium and high viscosity chitosan. Pore diameters of chitosan–gelatin cryogels as measured by scanning electron microscopy (SEM) was in the range of 30–100 μm. While mercury porosimetry analysis revealed the majority of pores of the scaffold lying in the range of 30–50 μm. Porosity of chitosan–gelatin cryogels was found to be greater than 90% using Archimedes's principle. Unconfined compression tests showed significant elasticity of chitosan–gelatin cryogels and maintained their physical integrity even after compressing them up to 80% of their original length. The elastic modulus varied in the range of 36–39 kPa. Cyclic deformation analysis performed by compression of chitosan–gelatin cryogels with varying strains (10, 20 and 40%) showed no cracking or any significant deformation. The degradation of chitosan–gelatin cryogels was found up to 13.58 ± 1.52% at 37 °C within 8 weeks of incubation under sterile conditions and the cryogels swelled up to 90% of their capacity within two min. Efficient cell adherence, proliferation and extracellular matrix (ECM) secretion was observed by growing fibroblast (Cos-7) cell line on chitosan–gelatin hybrid cryogels which indicate potential of the material for tissue engineering applications.

Keywords: Chitosan–gelatin cryogel; Macroporosity; Biodegradable; Elastic; Cell adhesion

1. Introduction

Tissue engineering offers the potential to create functional and viable tissue constructs for patients requiring organ replacement [1,2]. One potential approach for creating tissue constructs is to isolate viable cells from the patient, expand the cell population, culture cells *in vitro* on a scaffold, and then implant the resulted tissue engineered construct back into the patient. In this approach, a three-dimensional (3-D) scaffold should be biodegradable and act like an artificial extra cellular matrix (ECM) so as to serve temporarily as a template to guide cell adhesion, unhindered proliferation and tissue development. The scaffold should also have an interconnected macroporous network to allow unobstructed cell penetration and transfer of nutrients, oxygen and waste products [3–5]. In addition to these essential properties, for engineering soft tissues like blood vessel, heart valves, cartilage, tendon, and bladder, which exhibit elastic properties, elasticity of scaffolds is being looked as a potential designing parameter [6].

Abbreviations: CG, chitosan–gelatin; 3-D, three-dimensional; DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; FBS, fetal bovine serum; HVC, high viscosity chitosan; kPa, kilo Pascal; LVC, low viscosity chitosan; MIP, mercury intrusion porosimetry; MVC, middle viscosity chitosan; OD, optical density; PBS, phosphate buffer saline; SEM, scanning electron microscopy; SMCs, smooth muscle cells; TNBS, 2,4,6-trinitro-benzenesulfonic acid.

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Several groups have reported that mechanical stimulation that involves cyclic strain of the cell-scaffold construct *in vitro* can influence the quality of the resulting tissue [7–11]. For example, cyclic mechanical strain during tissue development has shown the importance of mechanical signals in the development of smooth muscle cells (SMCs) containing vascular tissue engineered constructs [7–11]. Similarly, in cartilage tissue engineering, the importance of cyclic deformation in improvement of quality of tissue engineered construct either in terms of more ECM production or increase in mechanical strength of the constructs has been demonstrated [12–17]. Considering the importance of mechanical stimulus in tissue engineering, it becomes critically important to design scaffolds that must maintain their mechanical integrity during the mechanical strain application which might help to convey the mechanical signals to the cells adherent onto them. To achieve this, the designed scaffolds must be elastic and capable of withstanding cyclic mechanical strains without getting cracked or permanently deformed. As a result, there has been increasing interest in developing materials with good mechanical properties. The materials used for developing biodegradable scaffolds with elastic properties for tissue engineering applications include polyurethanes [18–22], poly(diol citrates) [6,23], polyhydroxyalkanoates [24–25], poly(ε-caprolactone) copolymers [26–29], poly(1,3-trimethylne carbonate) copolymers [30–32] and poly(glycerol sebacate) [33]. However, most of the materials used in these studies are synthetic and lack cell-recognition signals. Moreover, the synthesis of these materials requires complex procedures and is expensive in terms of commercial and clinical product development [6]. Freeze-drying (lyophilization) is one such method which is used for synthesizing porous polymeric scaffold/matrices, though it is time consuming and an expensive process. It involves two main steps freezing and drying. The principle in freeze-drying is sublimation, the conversion of a solid (ice) directly into its gaseous form (water vapor). Most lyophilization processes are completed by a period of desorption drying, which takes long time that confers difficulty in process handling. Matrices using natural polymers chitosan and gelatin by freeze-drying technique for their application as scaffolds in tissue engineering have been synthesized [34–36]. However, these studies suggested improvement in scaffold development technology with improved characteristics of polymer scaffolds for tissue engineering application.

In contrast, cryogels are gel matrices that are synthesized at subzero temperatures using monomeric or polymeric precursors [37]. These gels can be obtained through the formation of both physically and covalently crosslinked homogeneous or heterogeneous polymer networks. At subzero temperature, most of the solvent gets frozen while part of the solvent is left unfrozen (so-called *unfrozen liquid microphase*) where dissolved substances concentrate and undergo chemical reactions [38]. These chemical reactions in the liquid microphase lead to gel formation and the crystals of frozen solvent act as porogens. After thawing the ice crystals, a system of large interconnected pores is formed within the gel. Cryogels have some important characteristics which include interconnected macroporous structure, mechanical stability and elasticity [37]. Cryogel technology and freeze-drying are basically following same principle for the synthesis of porous scaffolds but unlike freeze-drying, cryogel technology is simple one step process. In cryogel technology the ice crystals serve as the porogens and makes pores on melting, which give the possibility of controlling the pore size. At present, cryogels are being used in various areas of biotechnology including use as chromatographic materials [39], carriers for the immobilization of molecules [40] and cells [41], matrices for cell separations [42,43], and cell culture [44]. Typical elastic nature of cryogels has been utilized to develop a unique method to detach affinity bound bioparticles and synthetic particles from the cryogel matrix through elastic deformation [45]. Elastic poly(vinyl alcohol) cryogel has been suggested for cartilage replacement in an arthritic shoulder replacement [46]. Characteristic interconnected pores, macroporous 3-D structure and elastic nature of cryogels prompted us to look for their application in synthesizing scaffolds for tissue engineering.

Chitosan is a linear polysaccharide consisting of $\beta(1 \rightarrow 4)$ linked D-glucosamine residues with a variable number of randomly located *N*-acetyl-glucosamine groups. Chitosan has been shown to have excellent biocompatibility, biodegradability, non-toxicity, adsorption properties and ability to be degraded by lysozyme, a naturally occurring enzyme. Chitosan is available in different degree of deacetylation, viscosity and molecular weight [47]. Gelatin is also a biodegradable polymer with many attractive properties, such as excellent biocompatibility, nonantigenicity, plasticity and adhesiveness, and it is widely used in biomedical and pharmaceutical fields.

The work focuses here to synthesize a macroporous, interconnected elastomeric scaffold from natural polymers like chitosan and gelatin which could withstand deformational loading without cracking and undergoing permanent deformation. We used the cryogel technology to synthesize chitosan–gelatin matrix and mechanically characterized them under static as well as dynamic deformational loading. We have also checked the cell-material interactions and biocompatibility of synthesized macroporous chitosan–gelatin scaffolds using fibroblast (Cos-7) cell line.

2. Materials and methods

2.1. Materials

Low viscosity chitosan (LVC) (viscosity: $\leqslant$200 mPa s and MW: 150,000), medium viscosity chitosan (MVC) (viscosity: 200–400 mPa s and MW: 400,000) and high viscosity chitosan (HVC) (viscosity: $\geqslant$400 mPa s and MW: 600,000) were purchased from Fluka (Buchs, Switzerland). Gelatin (from cold water fish skin; MW: ~60,000), Dul-

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