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Physico-chemical and mechanical characterization of in-situ forming xyloglucan gels incorporating a growth factor to promote cartilage reconstruction

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ABSTRACT

The development of growth factors is very promising in the field of tissue regeneration but specifically designed formulations have to be developed in order to enable such new biological entities (NBEs). In particular, the range of therapeutic concentrations is usually very low compared to other active proteins and the confinement in the target site can be of crucial importance.
In-situ forming scaffolds are very promising solutions for minimally invasive intervention in cartilage reconstruction and targeting of NBEs. In this work injectable, in-situ forming gels of a temperature responsive partially degalactosylated xyloglucan (Deg-XG) incorporating the growth factor FGF-18 are formulated and characterized. In particular, injectability and shear viscosity at room temperature, time-to-gel at body temperature, morphology and mechanical properties of gels are investigated. The highly hydrophobic growth factor is favorably incorporated and retained by the gel. Gels undergo a slow erosion process when immersed in PBS at 37 °C that opens up their porous structure. The prolonged hydrothermal treatment leads to structural rearrangements towards tougher networks with increased dynamic shear modulus. Preliminary biological evaluations confirm absence of cytotoxicity and the ability of these scaffolds to host cells and promote their proliferation.

1. Introduction

Cartilage disorders are quite common, due to aging and obesity, and these diseases have a high impact on the quality of life [1]. The truncated form of Fibroblast Growth Factor 18 (tr-hFGF-18), a non-glycosylated protein member of the Fibroblast Growth Factor (FGF) family, has been shown to be a proliferative agent for chondrocytes and osteoblasts [2,3]. FGF-18 has been already proposed for the treatment of cartilage disorders such as osteoarthritis (OA) and cartilage injury (CI), either alone [4] or in combination with hyaluronic acid [5]. Indeed, its biological activity is related to chondrocytes proliferation, reduction of inflammation and of degenerative processes, leading to rapid functionality recovery and pain reduction.

OA is characterized by a progressive loss of cartilage at the level of articulations with consequent reduced motility, inflammation and pain while CI is typically induced by traumatic events leading to a damage of the cartilage in a well-defined and confined area.

In the case of CI, a traditional liquid formulation for intra-articular (i.a.) administration may induce uncontrolled cartilage growth also in healthy areas due to the presence of FGF-18 in the synovial fluid. This, in turn, can induce unwanted effects such as reduced joint mobility. The confinement of FGF-18 at the level of the target site would eventually promote the cartilage growth only in the damaged area.

In recent years, increasing attention has been paid to the preparation of either implantable or injectable scaffolds for cartilage regeneration. Clear advantages of the use of injectable scaffolds over implantable ones are minimal invasiveness (arthroscopy vs. traditional surgery) and targeted delivery at specific sites [6–9].

A tissue-engineered scaffold for the reconstruction of cartilage must possess specific features: adequate mechanical properties, porous structure, the ability to fill the defect and promote cell differentiation and the formation of new cartilage tissue, adhesion and compatibility with the surrounding cartilage, biocompatibility and bioresorption ability [6]. In particular, the joint environment requires very performing mechanical

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properties to withstand the joint loading. This does not mean that the artificial tissue should perfectly mimic the natural cartilage, because the scaffold could evolve and adapt to the surrounding environment post implantation/injection, synergically with the growing tissue [8].

Hydrogels are very promising candidates for cartilage reconstruction [10–14]. The highly interconnected, three-dimensional porous network promotes cell viability by migration, increases the transportation of nutrients, oxygen and metabolites and it adapts to the changing requirements of the growing colony [15]. It also ensures excellent patient compliance, due to the soft and rubbery consistency, and minimal invasiveness when in-situ forming.

Both synthetic and natural polymers can form hydrogels. Hydrogels from polysaccharides have distinct advantages, such as a variety of chemical functionalities, that can confer mucoadhesive or antibacterial properties, affinity towards other biomolecules, in particular proteins, inherent biodegradability and non-immunogenicity [16].

An ideal candidate for the preparation of a hydrogel-based scaffold is an injectable biopolymer aqueous solution that can be injected in the body and rapidly gel upon a temperature increase from room temperature to body temperature [17]. A few polysaccharides, such as agarose, amylose, amylopectin and carrageenan, show thermo-gelling properties but none of them gels at physiological temperature [18]. Xyloglucan is a structural polysaccharide found in the primary cell walls of higher plants, which backbone is formed by β-(1,4) D-glucan, partially substituted by α-(1->6)-linked xylose units. Some of the xylose residues are β-D-galactosylated at the O-2 [19]. It is FDA approved as food additive [20]. When xyloglucan is partially degalactosylated (Deg-XG), with a galactose removal ratio (GRR) between 35% and 50%, it forms gels at body temperature from aqueous solutions of concentration above 1–2 wt% [21]. Thanks to this temperature-responsive behavior, Deg-XG has been already evaluated up to some extent as in-situ forming drug delivery depot [22–26] or as scaffold for the reconstruction of soft tissues [27].

In this work, aqueous solutions of Deg-XG at various concentrations are prepared for the incorporation of FGF-18.

In order to define the range of concentrations of polymer solutions suitable for the application, i.e. with the ability to flow through a syringe needle and promptly gel afterwards, injectability as function of conditioning time at room temperature and time-to-gel at body temperature are evaluated. Mechanical and morphological properties of gels are assessed by dynamic mechanical stress rheometry and scanning electron microscopy, respectively. Their erosion process in physiological conditions is investigated, with a control on the weight loss of polymer, eventual release of growth factor, morphological and mechanical changes of the scaffold. Preliminary in-vitro biological evaluations to ascertain absence of cytotoxicity and chondrocytes colonization ability of the scaffolds are also discussed.

2. Materials and methods

2.1. Materials

Tamarind seeds xyloglucan was purchased from Megazyme International (Ireland) and degalactosylated according to an established protocol [28,29]. The variant with a GRR of about 45% was selected. Human serum albumin (HSA) and Dulbecco's modified Eagle's medium were obtained from Sigma Aldrich, while Penicillin Streptomycin (Pen-Strep) and Dulbecco PBS 10× were obtained from Gibco. FGF-18 solution at 5.41 mg/ml was kindly provided by Merck Serono. The protein was supplied as a stock solution in PBS at pH 7.3, divided in aliquots and stored at −80 °C.

2.2. Gel preparation

Deg-XG was dissolved in water or in Dulbecco PBS buffer at pH 7.3 at the desired concentration, by homogenization for 5 h at 5 °C and 13,500 rpm. Deg-XG solutions were then autoclaved at 121 °C for 20 min and stored at 5 °C. Deg-XG solutions were mixed with FGF-18 solutions at a weight ratio 9:1. FGF-18 solution was either directly added to the polymer solution (at 9:1 ratio between the polymer solution and FGF-18) to attain the target final concentration of 540 μg/ml or ten times diluted to reach the target final concentration of 54 μg/ml. The mixture was gently stirred for 5 min and incubated at 5 °C for at least 18 h before use.

2.3. Injectability and shear viscosity measurements

The solutions were injected through a syringe with a G25 needle, as a worst case, at room temperature. Injection time and the residual amount of material in the syringe were measured. Shear viscosity measurements were performed using a stress-controlled Rheometer Ar 1000 (TA Instruments) with an acrylic plate-cone geometry (diam. 4 cm) and a gap of 30 μm, at 25 °C. In order to evaluate the stability of selected systems upon conditioning at room temperature, measurements were carried out after 1, 2 and 4 h of conditioning at 25 °C.

2.4. Time-to-gel at 37 °C

A "tilting test" was performed to assess the time-to-gel after incubation at 37 °C. Deg-XG solutions (2–3 ml) at various polymer concentrations (1–5 wt%) were incubated in transparent cylindrical tubes (diameter = 12 mm) and observed in time. Tubes were tilted to assess if the material was liquid-like or gel-like.

2.5. Rheological measurements

Rheological measurements were performed with a stress-controlled Rheometer Ar 1000 (TA Instruments) equipped with an acrylic parallel plate (diam. 40 mm). Temperature control was provided by a Peltier plate in a closed chamber and a gap of 500 μm was set. The exposed edges of the hydrogels were coated with silicone oil to prevent water evaporation. Preliminary experiments showed that mechanical properties of the gels were unaffected by the silicone oil, whereas the absence of silicone oil led to progressive increments of both storage and loss modulus while the gel was drying out on the rheometer plates for prolonged tests. Preliminary strain-sweep tests were performed at 6.28 rad/s (1 Hz) frequency in the shear strain range 0.001–10 to identify the linear viscoelastic region of the gels, frequency-sweep tests in the frequency range 1–1000 rad/s were performed at 0.004 strain, i.e. within the linear viscoelastic region and time-sweep tests were carried out at 6.28 rad/s (1 Hz) and strain of 0.004.

2.6. Hydrogel morphology

Surface morphology was imaged by a field emission scanning electron microscopy (FESEM) system (JEOL) at an accelerating voltage of 10 kV. Freeze-dried samples were mounted on SEM aluminum stubs by means of a graphite adhesive layer and coated with a gold layer by JFC-1300 gold coater (JEOL).

2.7. Gel erosion and growth factor release studies

Gels samples were put onto the pre-weighed inserts of a multi-well plate with a porous membrane (0.4 μm) and kept immersed in a receiving phase made of pH 7.3 PBS (Dulbecco PBS 1× + HSA (1 wt/vol%) + Pen-strep (1 wt/vol%)) at 37 °C. Orbital shaking at 100 rpm was provided. Gel samples were weighed before incubation and after various times of incubation at 37 °C. W_t is the weight of the sample at the time t and W_0 is the initial weight of the sample. For each system, 8–10 samples were weighed and weight variations were always lower than 2%.

The receiving phase was changed every 2–3 days. At every change the collected receiving phase was analyzed by HPLC (LC-2010 AT Prominence instrument, Shimadzu, equipped with ZORBAX 300SB-C18 guard column and column) and Biacore measurements to estimate the