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The effect of calcium chloride concentration on alginate/ Fmoc-diphenylalanine hydrogel networks

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ABSTRACT

Peptide based hydrogels gained a vast interest in the tissue engineering studies thanks to great superiorities such as biocompatibility, supramolecular organization without any need of additional crosslinker, injectability and tunable nature. Fmoc-diphenylalanine (FmocFF) is one of the earliest and widely used example of these small molecule gelators that have been utilized in biomedical studies. However, Fmoc-peptides are not feasible for long term use due to low stability and weak mechanical properties at neutral pH. In this study, Fmoc-FF dipeptides were mechanically enhanced by incorporation of alginate, a biocompatible and absorbable polysaccharide. The binary hydrogel is obtained via molecular self-assembly of FmocFF dipeptide in alginate solution followed by ionic crosslinking of alginate moieties with varying concentrations of calcium chloride. Hydrogel characterization was evaluated in terms of morphology, viscoelastic moduli and diffusional phenomena and the structures were tested as 3D scaffolds for bovine chondrocytes. In vitro evaluation of scaffolds lasted up to 14 days and cell viability, sulphated glycosaminoglycan (sGAG) levels, collagen type II synthesis were determined. Our results showed that alginate incorporation into FmocFF hydrogels leads to better mechanical properties and higher stability with good biocompatibility.

1. Introduction

In the last decade, small molecular weight hydrogelators consisting of short peptide sequences, which are capable of self-assembling into new supramolecular structures via non-covalent interactions gained a vast interest [1–3]. Self-assembling peptide nanostructures possess a wide array of potential applications including organoelectronics [4], nanoelectronics [5], biosensors [6], photonics [7], and tissue engineering [8] as well as biomaterials science [9–11]. The structures were reported as to be biocompatible several times, suitable for the protection of bioactive molecules like protein and enzymes, and also have a potential as matrices for cell development and differentiation [12,13].

The integrative forces of small gelators for the gelation process includes hydrogen bonding, hydrophobic and hydrophilic interactions due to amphiphilic nature, Van der Waals forces, electrostatic interactions and π-π stacking of aromatic groups and side chains [14–16]. Fluorenyl-9-methoxycarbonyl (Fmoc)-functionalized peptide family is one of the most widely studied low molecular weight gelators, thanks to the protective aromatic moiety it has. Fmoc-diphenylalanine (FmocFF) have the ability to form hydrogels after a sequential decrease in pH of the solution without any need for an additional crosslinking agent [17–19]. The resulting gel network does not only have a cytocompatible nature but also be able to mimic the extracellular matrix. This attribution promotes the structure as a promising three dimensional culture environment for many cell type, especially for the ones that are not suitable for monolayer culture, such as chondrocytes, since the optimal circumstances for their culture requires an infrequent density and relatively hypoxic conditions compared to cell types [20,21].

However, their endurance against the force load hinders the use of these peptide hydrogels as biomaterials. In addition to that, pristine FmocFF hydrogels was found to be unstable during incubation at physiological pH, which seriously limits the utilization as drug delivery or tissue engineering application [22]. One of the main approach to enhance the mechanical integrity of the structures is to blend them with other polymers. It is well known that the structures generated by the conjugation of polysaccharides and lower molecular weight molecules exhibit more feasible mechanical responses in drug delivery and cell adhesion studies. Additionally, the 3D network structure of the polysaccharides resembles to natural extracellular matrix organization [23]. A newly

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synthesized polysaccharide-peptide hybrid network by Huang et al, consist of FmocFF and konjac glucomannan, exhibited a higher fracture resistance than the both entity has [24].

Alginate hydrogels were previously reported as favourable scaffolds for chondrogenic cell transplantation [25–27]. Chondrocytes can be easily laden into alginate hydrogels prior to ionic crosslinking by divalent cations like calcium. Chondrocytes entrapped in alginate hydrogels substantially maintain their characteristic spherical morphology, which can be referred as a physical indication of chondrogenesis [28,29].

In the present study, we investigated the FmocFF-Alginate hybrid hydrogel structures in terms of crosslinker concentration. FmocFF peptides were dissolved in alkaline solutions of alginate and let to gelate by sequential pH decrease by adding glucuno-D-lactone (GdL). Dissolved alginate in obtained structures was further coagulated with calcium chloride in various concentrations, in order to complete gelling process. Physical characterization of hydrogels was carried out by morphologic evaluation, in vitro degradation, viscoelastic measurements and diffusional properties. Hydrogels were also assessed biologically with entrapped bovine chondrocytes and investigated for 3, 5, 7 and 14 days of cultivation in vitro.

2. Materials and methods

2.1. Materials

FmocFF peptide was purchased from Bachem (Bubendorf, Switzerland) in lyophilized form. Alginic acid sodium salt from brown algae, calcium chloride, glucono-D-Lactone (GdL) were purchased from Sigma (St. Louis, MO, USA). Cell culture media, Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Biological-Industries (Israel). Anti-collagen II antibody was purchased from Abcam (Cambridge, UK) and Blyscan sulphated glycosaminoglycan staining kit was purchased from Biocolor (Carrickfergus, UK). Vancomycin.HCl was purchased from Santa Cruz Biotechnology (Texas, USA). All chemicals and reagents were used as received without any analytical purification.

2.2. Hydrogel preparation

FmocFF dipeptide with varying concentrations were dissolved in Dulbecco's Modified Eagle Medium (DMEM), of which pH was adjusted to 10.5 with 0.1 M NaOH prior to dissolution. The final concentrations of FmocFF in solutions for the gel optimization study were between 10 and 30 mM (data not shown), 20 mM of FmocFF was chosen as the optimum concentration for this study. Solution process lasted for 4 h and was performed in a water bath, at 37 °C, with an orbital shaking rate of 125 rpm. Then GdL (glucono-D-lactone) was added to each specimen with a concentration of 20 mM. After 10 s of vortex, the solutions were stored at room temperature for 2 h [30]. Binary hydrogel structures (FmocFF-Alg) were prepared as follows. Sodium alginate was dissolved in in DMEM with a concentration of 4 mg mL^{-1} prior to adjusting its pH to 10.5 with 0.1 M NaOH. FmocFF dipeptide was added to alkaline alginate solution later on to obtain a 20 mM peptide solution with vigorous stirring at 37 °C. After complete dissolution of peptides, resulting mixture was separated into aliquots in tissue culture flasks and GdL was added to generate a sequential pH decrease with a concentration of 20 mM. After several seconds of mild agitation, the solution was left aside for 2 h in order to establish the self-assembled supramolecular arrangement of FmocFF nanofibers. The pH of the solution was monitored during the gelation and it dropped down to 7–7.4 after complete dissolution of GdL. Once the peptide gel network obtained, $CaCl_2$ solution was added to structures for the ionotropic gelation of alginate chains. Three concentrations of $CaCl_2$ were used as 0.1%, 0.25% and 0.5% (w/v) and the obtained samples will be denoted as FmocFF-Alg1, FmocFF-Alg2 and FmocFF-Alg3, respectively. FmocFF gel structures without addition of alginate was used as negative control group in every experiment and characterization step.

2.3. Hydrogel stability and in vitro degradation

Hydrogel structures were evaluated for their mechanical integrity and stability in physiologic pH and in vitro degradation profiles were obtained. Hydrogel pieces with a mass of approximately 500 mg were placed in pH 7.4 phosphate buffer saline (PBS) and kept in closed vessels at 37 °C in a water bath with a shaking rate of 50 rpm. In pre-determined time intervals, structures were removed from PBS, dehydrated with a gentle touch of tissue paper and mass of samples was weighed. The mechanical integrity and structural appearances were recorded.

2.4. Morphology

Morphologic characterization of the micro-nano structure of hydrogels were carried out by Scanning Electron Microscopy (Quanta 250 FEG, FEI, USA). Prior to analysis, samples were left to air dry at ambient conditions and gold coated with approximately 5 nm thickness.

2.5. Rheology

Rheological data of the hydrogel structures were obtained by a rheometer (ARES, TA Instruments, UK) with parallel plate geometry in 30 mm diameter and 110 μm gap. FmocFF and FmocFF-Alg hydrogels were synthesized in 30 mm diameter round moulds and directly analyzed at room temperature. Storage (G') and loss (G'') modulus of hydrogels were measured via frequency sweep test. The frequency was changed between 0.1 and 100 Hz at a constant strain %.

2.6. Diffusional properties and sustained drug release

Mass transport is an essential factor in tissue engineering and much more crucial in chondrocyte culture, because of the avascular nature of cartilage tissue. Another important issue, local active agent release from the scaffolds, was taken account in diffusional phenomena and for this reason vancomycin.HCl was chosen as a model small biomolecule, in order to investigate the release properties. Vancomycin.HCl was added to FmocFF and FmocFF-Alg solutions with 5 mg mL^{-1} concentration, prior to double crosslinking procedure and encapsulated by the hydrogel network. Drug entrapped hydrogels were placed in 2 mL PBS in capped vessels at 37 °C and the release of vancomycin was evaluated with microvolume UV spectrophotometer (Nanodrop 1000, Thermo Scientific, USA) at 280 nm wavelength. Released drug amounts were calculated with the calibration curve obtained by the known concentrations of vancomycin.HCl.

2.7. Encapsulation of chondrocytes in FmocFF and FmocFF-Alginate hydrogels

The FmocFF and FmocFF-Alg solutions were prepared according to the protocol stated above. Meanwhile, chondrocytes, which were isolated from articular cartilage of the knee joint of young calves formerly [31], were cultured in cell media (90% DMEM, 10% FBS with addition of a few drop of pen/strep solution) for 7 days. Then 1×10^6 cells per 1 mL was added into FmocFF and FmocFF-alginate solutions. Finally, 20 mM of GdL and varying amounts of $CaCl_2$ (0.1%, 0.25% and 0.5% (w/v) concentrations) were added into each solution and 1×10^5 cells (100 μL cell-gel solution) were transferred per well into 24 well plate for gelation. After gelation, 300 μL of culture media was added to each well and cells were cultured for 3, 5, 7 and 14 days. Media exchanged after 5th day of cell cultures with 72 h of intervals. Gelation and culture procedures were carried out in CO_2 incubator at 37 °C with humidified air, refreshing the culture media every day.