Biomaterials 33 (2012) 7478–7488

Contents lists available at SciVerse ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Iterative design of peptide-based hydrogels and the effect of network electrostatics on primary chondrocyte behavior

Chomdao Sinthuvanich [a,b], Lisa A. Haines-Butterick [b], Katelyn J. Nagy [a,b], Joel P. Schneider [a,*]

[a] Chemical Biology Laboratory, National Cancer Institute, Frederick National Laboratory for Cancer Research, Frederick, MD 21702, USA
[b] Department of Chemistry and Biochemistry, University of Delaware, Newark, DE 19716, USA

ARTICLE INFO

Article history:
Received 31 May 2012
Accepted 29 June 2012
Available online 28 July 2012

Keywords:
Peptide
Hydrogel
Cell delivery
Self-assembly
Chondrocyte
Tissue engineering

ABSTRACT

Iterative peptide design was used to generate two peptide-based hydrogels to study the effect of network electrostatics on primary chondrocyte behavior. MAX8 and HLT2 peptides have formal charge states of +7 and +5 per monomer, respectively. These peptides undergo triggered folding and self-assembly to afford hydrogel networks having similar rheological behavior and local network morphologies, yet different electrostatic character. Each gel can be used to directly encapsulate and syringe-deliver cells. The influence of network electrostatics on cell viability after encapsulation and delivery, extracellular matrix deposition, gene expression, and the bulk mechanical properties of the gel-cell constructs as a function of culture time was assessed. The less electropositive HLT2 gel provides a microenvironment more conducive to chondrocyte encapsulation, delivery, and phenotype maintenance. Cell viability was higher for this gel and although a moderate number of cells dedifferentiated to a fibroblast-like phenotype, many retained their chondrocytic behavior. As a result, gel-cell constructs prepared with HLT2, cultured under static *in vitro* conditions, contained more GAG and type II collagen resulting in mechanically superior constructs. Chondrocytes delivered in the more electropositive MAX8 gel experienced a greater degree of cell death during encapsulation and delivery and the remaining viable cells were less prone to maintain their phenotype. As a result, MAX8 gel-cell constructs had fewer cells, of which a limited number were capable of laying down cartilage-specific ECM.

Published by Elsevier Ltd.

1. Introduction

Cartilage is an avascular tissue essential for joint function. It provides a smooth and low-friction surface that cushions and protects the underlining bone from compressive loads and shear forces. Due to its avasculature nature, injured cartilage has a limited capacity to heal. Several clinical procedures have been developed to treat cartilage injuries such as arthroscopic debridement, marrow stimulation and osteochondral autografts [1,2]. In addition, cell-based therapies are being explored to regenerate native-like cartilage. For example, in Autologous Chondrocyte Implantation (ACI), chondrocytes are introduced to a defect site as a cellular suspension in media and a periosteal flap is used to confine the cells [3,4]. Alternatively, cells can be seeded onto a material scaffold and cultured *ex vivo*, affording cartilage-like tissue constructs that can be subsequently implanted [5].

Injectable hydrogels are now being explored to deliver cells in a minimally invasive manner for tissue engineering and cytomedical therapy [6]. Most materials coined as 'injectable gels' are actually delivered as liquids that undergo sol–gel transitions either during delivery or *in situ* after delivery [6]. For these systems, cells are suspended in a liquid precursor, syringe-delivered and ultimately encapsulated in the gel after the sol–gel transition takes place. The rate at which the sol–gel transition takes place is critical. If the rate of gelation is too fast, the material clogs the delivery device and if it is too slow, the cell-containing liquid precursor leaks to neighboring tissue resulting in low retention of cells at the implant site. Although there are some elegant examples of materials having appropriate rates of gelation [7,8], the design of injectable materials as cell delivery vehicles remains challenging. Not only must the material display proper rheological properties during delivery, but after the gel has formed, it's mechanical stiffness, which is known to influence cell phenotype, must be strictly defined [9]. In addition, the material's cytocompatibility and biocompatibility must be commensurate with cell type and the specific tissue to which the cells are being delivered.

* Corresponding author.
E-mail addresses: Joel.Schneider@nih.gov, schneiderjp@mail.nih.gov (J.P. Schneider).

0142-9612/$ – see front matter Published by Elsevier Ltd.
http://dx.doi.org/10.1016/j.biomaterials.2012.06.097

Our lab is developing a hydrogel system that allows cells to be syringe-delivered while encapsulated in a solid-like gel, eliminating the need to rely on a sol–gel phase transition to take place during or after the delivery event [10,11]. In this system, cells are encapsulated in a hydrogel network of self-assembled peptides, directly in a syringe. To encapsulate cells, unfolded peptides are first dissolved in aqueous buffer of low ionic strength. The subsequent addition of cell culture media triggers peptide folding into a β-hairpin conformation that undergoes rapid self-assembly, forming a fibrillar hydrogel network. When cells are present in the media, they are directly encapsulated in the hydrogel. The resulting solid-like gel-cell construct displays shear-thin/recovery mechanical properties. Thus, depressing the syringe plunger thins the gel, allowing it to flow through an attached narrow bore needle or catheter. On exiting the delivery device, the material immediately recovers its solid-like properties at the point of application. Pochan et al. have recently shown that during material-thinning through a narrow-bore capillary, the gel displays a flow profile characterized by a large central plug flow region and a very narrow shear zone close to the capillary wall. Thus, the vast majority of encapsulated cells are delivered in a plug of gel and do not experience significant shear rate during delivery [11]. The delivery mechanism afforded by these gels results in their acute retention at the implant site and allows the properties of the gel construct, such as its mechanical rigidity, microenvironment and cell density, to be strictly defined prior to cell delivery [10,12–14].

Although controlling the mode of delivery is important in developing a new vehicle, others factors that influence cell fate during and after their delivery must be considered. Several studies have demonstrated that the electrostatic nature of a material can play an important role in regulating cell behavior. Electrostatic interactions between cells and substratum or surrounding network have been shown to affect cell viability [15], cell adhesion [16–18], proliferative capacity [19], and differentiation potential [20,21]. Herein, an iterative peptide design approach is used to generate two hydrogels in order to determine if network electrostatics influences the ability of this class of peptide material to effectively encapsulate and deliver primary bovine chondrocytes, and to facilitate cartilage elaboration during tissue culture.

The two gels studied were prepared from MAX8 and HLT2, peptides that differ in their overall charge state at neutral pH, Table 1. MAX8, a previously published sequence, has an overall charge state of +7 per monomer and self-assembled networks of this peptide are highly electropositive. A systematic, iterative design approach was used to generate HLT2. This peptide carries a +5 charge per monomer and forms a gel network that is significantly less electropositive. The design of HLT2 was challenging in that the peptide needed for this study had to be substantially different in its charge state and thus primary sequence, but form a gel of similar mechanical properties to those formed by MAX8. This ensures that any observable difference in the phenotype of cells cultured within each gel would be primarily due to the electrostatic nature of the network and not other differences, such as the stiffness of the gel, that are known to influence cell behavior [9]. As will be discussed, the electrostatic character of these gels significantly influences the behavior of encapsulated chondrocytes, with the less electropositive HLT2 gel providing a better microenvironment for cell encapsulation, delivery and *in vitro* cartilage elaboration.

Table 1
Peptide sequences and respective formal charges at pH 7.4. Schematic of hairpin with associated amino acid positions. Bold positions are on the hydrophobic face of the hairpin. Non-bold positions are on the hydrophilic face.

Peptides	Sequences	Net charge
MAX8	VKVKVKVK^{D}P^{L}PTKVEVKVKV-NH_2	+7
K4E	VKVEVKVKV^{D}P^{L}PTKVEVKVKV-NH_2	+5
HIT1	VITKVKVKV^{D}P^{L}PTKVEVKVIV-NH_2	+5
HIT2	VITKVKTKV^{D}P^{L}PTKVEVKVIV-NH_2	+5
HLT2	VLTKVKTKV^{D}P^{L}PTKVEVKVLV-NH_2	+5

2. Materials and methods

2.1. Materials

Fmoc-protected amino acids were purchased from Novabiochem. PL-rink amide resin was purchased from Polymer Laboratories. 1-H-benzotriazolium-1-[*bis*(dimethylamino) methylene]-5-chloro-hexafluorophosphate-(1-),3-oxide (HCTU) was obtained from Peptide International. Trifluoroacetic acid, triisopropylsilane and 30% hydrogen peroxide were obtained from Acros organics. Diethyl ether, xylene, 10% neutral buffered formalin and Permount mounting medium were purchased from Fisher Scientific. Heat-inactivated fetal bovine serum (FBS), penicillin/streptomycin solution, 0.25% trypsin/EDTA solution and phosphate buffer saline (PBS) were obtained from Hyclone Laboratory Inc. Collagenase II was purchased from Worthington Biochemical Co. Dulbecco's Modified Eagle's Medium supplemented with 25 mM HEPES (DMEM/HEPES), live-dead cytotoxicity/viability kit, CellTracker™ green CMFDA and TRIZol were purchased from Invitrogen. Power SYBR Green PCR Mastermix, random hexamer and RNase inhibitor were obtained from Applied Biosystems. Unless otherwise stated, all other reagents were purchased from Sigma Aldrich.

2.2. Peptide synthesis and purification

Peptides were synthesized on PL-rink amide resin using an automated ABI 433A peptide synthesizer (Applied Biosystems, CA). Synthesis was carried out via solid-phase Fmoc chemistry with HCTU activation. Dried resin-bound peptides were cleaved from the resin and side chain deprotected by a trifluoroacetic acid: triisopropylsilane: deionized water (95:2.5:2.5) cocktail for 2 h under argon atmosphere. Crude peptides were precipitated using cold diethyl ether and then lyophilized. Reverse-phase-HPLC on semi-preparative Vydac C18 column was employed to purify the peptides. HPLC solvents consisted of Solvent A (0.1% TFA in water) and solvent B (0.1% TFA in 9:1 acetonitrile: water). Analytical HPLC and electrospray ionization (positive mode) mass spectrometry was performed to confirm the purity of the peptides. Lyophilized peptides were directly used for characterization and cell encapsulation with the exception of MAX8, which was dissolved in distilled water (1 mg/mL) and re-lyophilized twice before use.

2.3. Oscillatory rheology

Rheology experiments were performed on an AR-G2 rheometer (TA Instruments, DE) equipped with a 25-mm stainless steel parallel plate geometry with a 0.5 mm gap height. To investigate the rheological properties of the hydrogels at physiological condition, a 0.5 wt% peptide solution was prepared by first dissolving a 1 wt% stock of lyophilized peptide in a pH 7.4 buffer (25 mM HEPES). An equal volume of HEPES-supplemented DMEM (pre-incubated in CO_2) was subsequently added to peptide solution to initiate hydrogelation. 300 μL of resultant solution was immediately transferred to the rheometer plate equilibrated at 20 °C. A temperature ramp from 20 °C to 37 °C at a rate of 0.5 °C/s was then performed followed by an hour dynamic time sweep to monitor the evolution of the storage (G′) and loss (G″) moduli at an angular frequency of 6 rad/s and 0.2% strain. Oil was placed around the sample and on the plate to prevent evaporation.

To mimic syringe delivery, a similar procedure was followed to form the hydrogel. Here, after the temperature was ramped to 37 °C, the hydrogel was allowed to form for only 10 min at 6 rad/s and 0.2% strain. After which, 1000% strain was applied for 30 s at 1000% to disrupt the material. Subsequently, the ability of the hydrogel to reheal was monitored by measuring the recovery of G′ at 6 rad/s and 0.2% strain for additional 10 min.

2.4. Chondrocyte isolation

Knee joints of 24–30 month-old bovines were obtained on the day of slaughter. Cartilage was cut from the femoral condyles and minced into small pieces using a razor blade under sterile condition. Cartilage digestion was then carried out using a solution of 0.1% pronase in chondrogenic media at 37 °C, 5% CO_2 for 1 h.