Biomaterials 35 (2014) 3558–3570

Contents lists available at ScienceDirect

Biomaterials

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

The bioactivity of agarose–PEGDA interpenetrating network hydrogels with covalently immobilized RGD peptides and physically entrapped aggrecan

Ganesh C. Ingavle [1], Stevin H. Gehrke [2], Michael S. Detamore*

Department of Chemical and Petroleum Engineering, University of Kansas, Lawrence, KS 66045-7609, USA

ARTICLE INFO

Article history:
Received 23 October 2013
Accepted 1 January 2014
Available online 24 January 2014

Keywords:
Interpenetrating networks (IPN)
RGD conjugation
PEGDA hydrogel
Tissue engineering
Cartilage regeneration

ABSTRACT

Our previous reports of interpenetrating networks (IPNs) have demonstrated drastic improvements in mechanical performance relative to individual constituent networks while maintaining viability of encapsulated cells. The current study investigated whether covalent linkage of RGD to the poly(ethylene glycol) diacrylate (PEGDA) network could improve upon cell viability and performance of agarose–PEGDA IPNs compared to unmodified IPNs (control) and to IPNs with different concentrations of physically entrapped aggrecan, providing a point of comparison to previous work. The inclusion of RGD or aggrecan generally did not adversely affect mechanical performance, and significantly improved chondrocyte viability and performance. Although both 4 and 100 μg/mL of aggrecan improved cell viability, only 100 μg/mL aggrecan was clearly beneficial to improving biosynthesis, whereas 100 μg/mL of RGD was beneficial to both chondrocyte viability and biosynthesis. Interestingly, clustering of cells within the IPNs with RGD and the higher aggrecan concentration were observed, likely indicating cell migration and/or preferred regional proliferation. This clustering resulted in a clearly visible enhancement of matrix production compared to the other IPNs. With this cell migration, we also observed significant cell proliferation and matrix synthesis beyond the periphery of the IPN, which could have important implications in facilitating integration with surrounding cartilage *in vivo*. With RGD and aggrecan (at its higher concentration) providing substantial and comparable improvements in cell performance, RGD would be the recommended bioactive signal for this particular IPN formulation and cell source given the significant cost savings and potentially more straightforward regulatory pathway in commercialization.

1. Introduction

Fabricating mechanically strong three-dimensional (3D) matrices that support cell growth and tissue formation is a prerequisite for many cell culture and tissue engineering applications [1,2]. Hydrogels are excellent scaffolding materials for repairing and regenerating a variety of tissues because they can provide a highly swollen 3D environment similar to soft tissues [3–9] and allow diffusion of nutrients and cellular waste through the elastic network. However, most synthetic hydrogels typically exhibit minimal biological activity [3,4] with a lack of desired mechanical integrity and may not provide an ideal environment for encapsulated cells. Mimicking the mechanical aspects of natural tissues can be used to enhance the functionality of engineered tissues and the development of hydrogels that are stronger mechanically may be beneficial for various biological and biomedical applications [10,11].

By generating composite hydrogels, it may be possible to reproduce the properties of a natural extracellular matrix (ECM). One approach to creating composite materials is the fabrication of an interpenetrating network (IPN) of polymers. An IPN consists of a polymer network containing molecularly entangled chains of a second polymer [12]. It has been demonstrated that synthetic and non-biological IPN hydrogels can achieve an increase in strength, failure stress, and stiffness while maintaining elasticity [13–15]. The research team of JP Gong and Y. Osada at Hokkaido University in Japan reported the synthesis of interpenetrating networks of various combinations of biological and synthetic polymers with dramatically improved mechanical properties [16–25]. Regarding biomedical applications, they recognized that the properties they measured with these IPNs compared favorably with biomaterials

* Corresponding author. Tel.: +1 785 864 4943; fax: +1 785 864 4967.
E-mail addresses: ganesh@ku.edu (G.C. Ingavle), shgehrke@ku.edu (S. H. Gehrke), detamore@ku.edu (M.S. Detamore).
[1] Tel.: +1 785 864 4965; fax: +1 785 864 4967.
[2] Tel.: +1 785 864 4956; fax: +1 785 864 4967.

0142-9612/$ – see front matter © 2014 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biomaterials.2014.01.002

such as cartilage. Recently, a new hydrogel consisting of an IPN of alginate and polyacrylamide (PAAM) was reported that displayed remarkable mechanical properties [26]. They explored the maintenance of mechanical properties of these extremely tough IPN hydrogels as a surface for mouse mesenchymal stem cell culture and as a material for *in vivo* acellular implantation [27]. These cells exposed to the IPN gel-conditioned media maintained high viability. Implantation of these IPN hydrogels into subcutaneous tissue of rats for 8 weeks led to mild fibrotic encapsulation and minimal inflammatory response, suggesting this as a possible biomaterial strategy in tissue engineering application. However, such IPNs are generally not applicable for cell encapsulation due to toxic materials, toxic photoinitiators, shorter photopolymerization wavelengths, and/or lengthy photopolymerization times.

Incorporation of various bioactive signals into biomaterials has enhanced the adhesion of cells [28,29] and may promote an enhanced biomimetic environment for encapsulated cells suspended in 3-dimensional hydrogels. Biological molecules can provide cues to stimulate cells to proliferate, migrate, differentiate, and produce ECM. To the best of our knowledge, aggrecan, a key structural component of cartilage [30], has not been used in its purified form by others as a bioactive signal in scaffolds for cartilage tissue engineering, although its demonstrated ability to promote chondrogenesis in monolayer culture [31,32], along with its ability to improve chondrocyte performance in IPNs in our previous work [33], together provide a strong rationale for evaluating aggrecan as a signal and/or "raw material" (i.e., building block) in cartilage tissue engineering [34]. The cell-adhesive peptide sequence Arg–Gly–Asp (RGD) has been shown to promote viability, proliferation, and biosynthesis with various cell types in tissue engineering applications [35–41]. As a result, RGD, which is found in cell-binding domains of extracellular matrix proteins, has been widely used in biomaterials where cell adhesion is desired. With the aim of providing a cellular microenvironment mimicking that found *in vivo*, recent efforts have been made to incorporate specific peptide sequences into the scaffold architecture, both to encourage bio-specific cell adhesion and to induce and control other cellular functions, such as migration, proliferation, ECM synthesis, and tissue remodeling [42].

To facilitate cell adhesion, small peptides derived from extracellular matrix (ECM) proteins have been important targets for the covalent coupling type of bio-specific modification [37,43–51]. For example, RGD-modified PEG monoacrylate was copolymerized with PEG diacrylate (PEGDA) to create cell-adhesive PEG hydrogels [37,50,51]. Different peptide sequences have been included successfully in photopolymerizable single network polyethylene glycol (PEG)-based hydrogels [50,52–55] and in poly(D,L-lactico-glycolic acid) (PLGA) scaffolds [56]. Recently, we introduced the approach of including a "raw material" from cartilage, namely aggrecan or chondroitin sulfate (CS), to serve as a bioactive signal to cells encapsulated in IPN based on agarose–PEGDA hydrogels for cartilage tissue engineering, which led to significantly improved *in vitro* performance of encapsulated chondrocytes [33,57].

Given our previous success with aggrecan, and the success of others with RGD in single-network PEGDA gels, our goal was to determine whether the more cost-effective RGD would provide comparable benefits to cell performance, or whether the more expensive aggrecan would provide significantly better results that would better justify its use. We further expanded on our previous work by investigating two different aggrecan concentrations (4 and 100 μg/mL), as opposed to our previous study that examined only a single concentration of 40 μg/mL [33]. This approach of encapsulating cells with bioactive signals is expected to be a valuable tool in investigating the cell–material interactions in a high toughness IPN hydrogel and to design a bio-functionalized material scaffold for cartilage tissue engineering applications.

2. Materials and methods

2.1. Materials

High purity PEG diacrylate (DA) (average molecular weight 2000 Da) was obtained from Sunbio Inc. (Anyang city, South Korea). The photoinitiator, 2-hydroxy-1-[4-(2-hydroxyethoxy) phenyl] 2-methyl-1-propanone (Irgacure 2959), was purchased from Ciba Specialty Chemicals Corp. (New York, NY). Acryloyl–PEG–N-hydroxysuccinimide (ACRL–PEG–NHS) (3400 Da, Laysan Bio Inc, AL, USA) was used as received. 2-hydroxyethyl agarose (Type VII, cell culture grade), aggrecan from bovine articular cartilage and arginine–glycine–aspartic acid (RGD) (≥97% purity) were obtained from Sigma–Aldrich (St. Louis, MO) and were used as received.

2.2. Arg–Gly–Asp (RGD)-modified PEG monoacrylate synthesis and immobilization of RGD on PEG network

Acrylate–PEG–RGDS was synthesized following previously described methods [37]. Briefly, Arg–Gly–Asp (RGD) was reacted with an equimolar amount of acrylate–PEG–N-hydroxysuccinimide in a sodium bicarbonate buffer (50 mM, pH 8.2) for 2 h at room temperature. The N terminal α-amine of the RGD peptide was coupled with active N-hydroxysuccinimide ester from acrylate–poly(ethylene glycol)–N-hydroxy succinimide (acrylate–PEG–NHS) to form RGD-modified PEG monoacrylate (acrylate–PEG–RGD) (Fig. 1). The product was lyophilized and stored at −20 °C until use. FTIR spectroscopy was performed to assess the RGD coupling with PEGDA in lyophilized solids. A PerkinElmer FTIR One spectrometer (PerkinElmer Life and Analytical Sciences, Waltham, MA) with a universal attenuated total reflectance (UATR) accessory was used to acquire the spectra. The solid sample was placed on a diamond crystal surface and covered with a stainless steel slide, and pressure (100 Torr) was applied to ensure good contact between the protein and the crystal. ^{1}H nuclear magnetic resonance (NMR) was performed on the dried sample to quantify complete RGD conjugation. High-resolution, ^{1}H NMR spectra were taken on a Bruker Advance DRX 500 spectrometer operating at 500 MHz. Deuterated water (90% DI + 10% D_2O) was used as a solvent, and the macromer concentrations were varied between 2.5 and 3 wt. %.

RGD was then immobilized within the PEGDA network upon copolymerization of acrylate–PEG–RGD with the appropriate concentration of PEG-DA monomer by photopolymerization in the presence of the photoinitiator Irgacure 2959 (0.1% w/v), as illustrated in Fig. 2.

2.3. Chondrocyte isolation and culture

Articular cartilage samples were dissected aseptically from the ankles of 8–9 month old male Duroc Hogs, which were obtained from a local butcher. Cells were harvested within 36 h after slaughter following aseptic procedures as described in our previous reports [58,59]. The articular cartilage samples were diced into approximate 1 mm^3 pieces using a scalpel in autoclaved PBS. After rinsing with PBS three times, the cartilage samples were digested in a sterile-filtered solution of 30 mL of 2 mg/mL type II collagenase (305 U/mg; Worthington Biochemical) for 18 h on an orbital shaker in a humidified 37 °C, 5% CO_2 incubator. The digested cartilage solution was filtered through a 100 μm filter cell strainer to remove undigested cartilage lumps. The filtrate was then centrifuged at 1500 rpm for 5 min, and then the cell pellet was resuspended in chondrocyte culture medium in a humidified incubator (37 °C, 5% CO_2). The culture medium consisted of Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L D-glucose supplemented with 10% fetal bovine serum (FBS), 1% nonessential amino acids, 1% sodium pyruvate, 50 μg/mL ascorbic acid and 0.25 mg/mL penicillin–streptomycin–fungicide. The DMEM and supplements were obtained from Invitrogen (Grass Islands, NY). The cell number was determined using a hemocytometer. The freshly isolated cells were grown to 90–95% confluence in T75 flasks and then retrieved by trypsin–ethylenediaminetetraacetic acid (EDTA) digestion. First passage chondrocytes were used in this study, and culture medium was changed every 2 or 3 days.

2.4. RGD and aggrecan incorporated agarose–PEGDA IPN synthesis and photoencapsulation

To study this incorporation effect, three different IPNs were synthesized and examined: (i) agarose–PEGDA IPN with no bioactive signals (IPN); (ii) agarose–PEGDA IPN containing 4 μg/mL aggrecan (IPN–agg 4); (iii) agarose–PEGDA IPN containing 100 μg/mL aggrecan (IPN–agg 100) and (iv) agarose–PEGDA IPN containing 100 μg/mL RGD (IPN–RGD 100).

Chondrocytes were encapsulated in the bioactive IPN hydrogel using our previously published methods [60]. The chondrocytes were detached from T75 flasks using trypsin-EDTA and resuspended in PBS at a high concentration, while an agarose solution was prepared by adding 0.3 g agarose powder (cell culture grade) to 10 mL PBS and autoclaving for 30 min. The agarose solution temperature was monitored under an aseptic environment until 39 °C was reached. Cells from the PBS suspension were counted using a hemocytometer. Once the agarose solution temperature reached 39 °C, the chondrocyte suspension was mixed with the liquid agarose solution in a 1:2 ratio for a final concentration of 25 million cells/mL in 2% agarose. The cell suspension was mixed thoroughly with the agarose solution and pipetted into sterilized cylindrical silicone molds measuring 5 mm in diameter and 1.9 mm in