Acta Biomaterialia 27 (2015) 66–76

Contents lists available at ScienceDirect

Acta Biomaterialia

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

Protective effects of reactive functional groups on chondrocytes in photocrosslinkable hydrogel systems

M. Bartnikowski, N.J. Bartnikowski, M.A. Woodruff, K. Schrobback, T.J. Klein *

Institute of Health and Biomedical Innovation (IHBI), QUT, 60 Musk Avenue, Kelvin Grove, Queensland 4059, Australia

ARTICLE INFO

Article history:
Received 4 May 2015
Received in revised form 4 August 2015
Accepted 25 August 2015
Available online 28 August 2015

Keywords:
Cartilage tissue engineering
Hydrogel
Gelatin
Photoinitiator
Cytotoxicity

ABSTRACT

Photocrosslinkable hydrogels are frequently used in cartilage tissue engineering, with crosslinking systems relying on cytotoxic photoinitiators and ultraviolet (UV) light to form permanent hydrogels. These systems are rarely assessed in terms of optimization of photoinitiator or UV dosage, with non-cytotoxic concentrations from literature deemed sufficient. We hypothesized that the number of reactive functional groups present within a hydrogel polymer is highly relevant when crosslinking, affording cytoprotection to chondrocytes by preferentially interacting with the highly reactive radicals that are formed during UV-mediated activation of a photoinitiator. This was tested using two photocrosslinkable hydrogel systems: gelatin methacrylamide (GelMA) and gellan gum methacrylate (GGMA). We further assessed the effects of two different UV dosages on chondrocyte differentiation while subject to a single photoinitiator dosage in the GGMA system. Most notably, we found that a higher ratio of reactive groups to photoinitiator molecules offers cytoprotective effects, and future developments in photocrosslinkable hydrogel technology may involve assessment of such ratios. In contrast, we found there to be no effect of UV on chondrocyte differentiation at the two chosen dosages. Overall the optimization of photocrosslinkable systems is of great value in cartilage tissue engineering and these data provide a groundwork for such concepts to be developed further.

Statement of Significance

Photocrosslinkable hydrogels, which use photoinitiators and predominantly ultraviolet light to form stable matrices for cell encapsulation and tissue development, are promising for cartilage tissue engineering. While both photoinitiators and ultraviolet light can damage cells, these systems have generally not been optimized. We propose that the ratio of reactive functional groups within a polymer to photoinitiator molecules is a critical parameter for optimization of photocrosslinkable hydrogels. Using photocrosslinkable gelatin and gellan gum, we found that a higher ratio of reactive groups to photoinitiator molecules protected chondrocytes, but did not affect chondrocyte differentiation. The principle of cytoprotection by functional groups developed in this work will be of great value in optimizing photocrosslinkable hydrogel systems for cartilage and other tissue engineering applications.

1. Introduction

Articular cartilage tissue engineering has a major focus around the development of hydrogel technologies. These materials offer desired characteristics, such as a high level of hydration and diffusive capacity, which positively influence chondrogenesis [1–3]. While hydrogels that crosslink through mechanisms such as ionic interactions avoid many issues of cytotoxicity that may be introduced during chemical crosslinking, photopolymerization offers advantages that are not mirrored in other crosslinking systems. Photopolymerization reactions are initiated when chemicals, photoinitiators (PIs), that are sensitive to a particular wavelength of light are exposed to radiation within their relevant excitation range and thus decompose into a high-energy radical state [4,5]. This produces an initiating species (a free radical) that attacks a first monomer unit, with other units adding further in a chain polymerization to form the final polymer macromolecule [5]. The carbon–carbon double bond found in vinyl monomers is the key linkage that reacts through radical-initiated chain polymerization [6]. The primary advantage of photocrosslinkable systems is that gelation only occurs during the exposure to the triggering stimulus

* Corresponding author.
E-mail address: t2.klein@qut.edu.au (T.J. Klein).

http://dx.doi.org/10.1016/j.actbio.2015.08.038
1742-7061/

and hence the start, duration and intensity of the reaction may be controlled as desired.

Photocrosslinkable hydrogels used in cartilage tissue engineering largely consist of biocompatible materials that have been chemically modified, attaching highly reactive vinyl groups to certain locations on the monomer and hence allowing easy reaction with PIs under ultraviolet (UV) curing conditions. Although vinyl groups react preferentially when radical polymerization is taking place, carbon–carbon double bonds are also present in other locations within the matrix, such as on other macromer amino acids (in the case of protein-based gels) or within cells themselves (when crosslinking cell-containing gels). As radical crosslinking is highly reactive and the cytotoxicity of PI compounds (when activated) is well known [4–7], it is debatable as to what extent the reaction prefers functional groups as opposed to non-targeted reactive species. Further, the concentrations of PIs such as the commonly explored 2-hydroxy-1-[4-(2-hydroxyethoxy)phenyl]-2-methyl-1-propanone (Irgacure 2959; IC), have been described in the literature as anywhere from 0.05% to 0.5% w/v [1,4,7]. In all of these cases however, there is a lack of detailed analysis on optimal PI concentration, and in no source is there an assessment of the relevance of PI concentration and the number of reactive groups present in the precursor solution with respect to survival of encapsulated cells. In particular, it remains unclear whether potential damage to cells is predominantly linked to the amount of UV exposure or the action of excessive free radicals during cross-linking, as UV exposure has been identified as a cause of cytotoxicity. While use of UV curing systems may be done without cytotoxic effects at low dosages, advantages in mechanical properties may be reached when higher concentrations of both PI and UV are used, and hence optimization of this system is desired in terms of a trade-off between cytotoxicity and mechanical properties. Further, as a comparison of multiple crosslinking technologies has led to the identification of differences between chondrocyte behavior [8,9], modification of UV dosage may have greater effects than simply those of cytotoxicity and mechanical properties; influencing cells long-term through damage to DNA and proteins and hence affecting their ability to proliferate or differentiate.

The work herein focuses on two photocrosslinkable systems, the biocompatible hydrogels gelatin methacrylamide (GelMA) and gellan gum methacrylate (GGMA). Gelatin is a product of collagen derived from bones, skin and connective tissues subject to hydrolysis [10]. It is hence a protein hydrogel, consisting of natural extracellular matrix (ECM) macromolecules that retain cell binding motifs such as Arg-Gly-Asp (RGD) as well as matrix metalloproteinase (MMP)-sensitive degradation sites [11]. The reaction of gelatin with methacrylic anhydride results in the formation of methacrylamide functional units which allow chemical photocrosslinking into a cytocompatible hydrogel [2,12,13].

Gellan gum (GG) is an FDA-approved food additive produced by the bacterium *Pseudomonas elodea*. The repeating unit of gellan gum consists of a tetrasaccharide of one α-L-rhamnose, one β-D-glucuronic acid and two β-D-glucoses [14–16]. Gellan gum hydrogel stability is largely influenced by manipulating the presence, concentration and valence of cations that are present in the gel solution [14,15,17,18]. Similar to gelatin, GG may be modified through reaction with methacrylic anhydride, yielding gellan gum methacrylate (GGMA), a cytocompatible and chemically crosslinkable hydrogel [1].

In this study we explored the cytocompatibility of IC at various concentrations coupled with various UV exposure times, within hydrogels that contain a varied number of reactive functional groups. We hypothesized that the reactive functional groups (RG) afford cytoprotection to chondrocytes by preferentially interacting with the highly reactive radicals formed during UV-mediated activation of IC. More specifically, higher amounts of RG may quench free radicals before they can react with and damage cellular molecules. Manipulation of the ratio of IC to RG was therefore expected to affect cell viability. We also investigated the effects of UV crosslinking time on chondrogenesis in a two week *in vitro* culture. We aimed to form a deeper understanding of the interplay between IC concentration, RG and UV exposure in chondrogenic hydrogel systems for tissue engineering.

2. Materials and methods

Gelatin (G2500), gellan gum (Gelzan™ CM G1910), and methacrylic anhydride (MAAh, 276685) were purchased from Sigma–Aldrich (St. Louis, MO). 2-Hydroxy-1-[4-(2-hydroxyethoxy)phenyl]-2-methyl-1-propanone (Irgacure 2959; IC) was purchased from BASF (Ludwigshafen, Germany). All quoted percentages are weight per volume (% w/v).

2.1. Macromer synthesis

Hydrogel groups comprised of methacrylate-functionalized gelatin in both high and low degrees of functionalization (gelatin methacrylamide; GelMA-H and GelMA-L) and gellan gum (gellan gum methacrylate; GGMA).

Functionalization of gelatin and gellan gum was performed as previously described [1,12]. Briefly, gelatin and gellan gum were dissolved in Milli-Q water (Merck Millipore, Billerica, MA) at 10% and 1%, respectively. In the case of gelatin, 0.6 g of MAAh per 1 g was added for GelMA-H while 0.06 g of MAAh per 1 g was added for GelMA-L, both were reacted for 1 h at 50 °C [12]. Gellan gum was reacted with 8 g of MAAh per 1 g for 6 h, with temperature maintained at 60 °C and pH monitored and adjusted to 8.0 with 5 m NaOH [1]. After reaction, all gel solutions were subject to centrifugation to phase separate and allow the removal of insoluble MAAh, after which they were dialyzed at 37 °C (gelatin) or 60 °C (gellan gum) against Milli-Q water to allow the diffusion of any remainder of MAAh or methacrylic acid. After dialysis, both GelMA solutions were filtered through 0.2 μm filters (Merck Millipore) under aseptic conditions, pH adjusted to 7.4, lyophilized with 0.2 μm filters exclusively providing air exchange then stored at −20 °C in sealed, sterile tubes. GGMA was subject to the same processing apart from initial filtration to avoid macromer loss due to long and variable chain lengths within batches (200–500 kDa).

2.2. Degree of functionalization

The degree of functionalization (DOF) of GelMA-H, GelMA-L and GGMA was assessed using proton nuclear magnetic resonance (^{1}H NMR) spectroscopy. A Varian Direct Drive NMR spectrometer (Agilent Technologies, CA) operating at 400 MHz for hydrogen, was used to record ^{1}H NMR spectra. All samples were dissolved at 1.0% in D_2O at a temperature of 50 °C. Spectra were recorded at an operational temperature of 50 °C, with 32 scans and a recycle delay of 30 s. For GelMA-H and -L, methacrylamide shifts were normalized against the aromatic signal of phenylalanine, at δ 7.4 ppm [19]. DOF was defined as the proportion of modified lysine groups of gelatin, as previously described for collagen I methacrylamide [19]. The area obtained from the integral of intensities of the protons present at the methacrylamide carbon–carbon double bond (I_{DB}) occurring at δ 5.6 and 5.8 ppm was normalized to proton number (nH_{DB}), and the area obtained from integrating the peak of the aromatic groups (I_A) was normalized to the protons interacting with the aromatic ring (nH_A). The prevalence of modified groups and aromatic residues were then normalized to their prevalence by number in porcine gelatin, averaged from numerous sources [20–24] in order to allow for the quantitation of total methacrylamide to total possible lysine groups (Eq. (1)):