Biomaterials 120 (2017) 139–154

Contents lists available at ScienceDirect

Biomaterials

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

Sequentially-crosslinked bioactive hydrogels as nano-patterned substrates with customizable stiffness and degradation for corneal tissue engineering applications

Muhammad Rizwan [a, b], Gary S.L. Peh [c, d], Heng-Pei Ang [c], Nyein Chan Lwin [c], Khadijah Adnan [c], Jodhbir S. Mehta [d, e, ***], Wui Siew Tan [b, **], Evelyn K.F. Yim [a, f, g, h, *]

[a] Department of Biomedical Engineering, National University of Singapore, Singapore
[b] Institute of Materials Research and Engineering, Agency for Science, Technology and Research (A*STAR), Singapore
[c] Tissue Engineering and Stem Cell Group, Singapore Eye Research Institute, Singapore
[d] Duke-NUS Graduate Medical School, Singapore
[e] Singapore National Eye Centre, Singapore
[f] Department of Surgery, National University of Singapore, Singapore
[g] Mechanobiology Institute, National University of Singapore, Singapore
[h] Department of Chemical Engineering, University of Waterloo, Waterloo, Ontario, Canada

ARTICLE INFO

Article history:
Received 18 September 2016
Received in revised form
6 December 2016
Accepted 22 December 2016
Available online 23 December 2016

Keywords:
GelMA
Nano-patterning
Hydrogels
Human corneal endothelium
Hydrogel membrane
Photo-crosslinking

ABSTRACT

Naturally-bioactive hydrogels like gelatin provide favorable properties for tissue-engineering but lack sufficient mechanical strength for use as implantable tissue engineering substrates. Complex fabrication or multi-component additives can improve material strength, but often compromises other properties. Studies have shown gelatin methacrylate (GelMA) as a bioactive hydrogel with diverse tissue growth applications. We hypothesize that, with suitable material modifications, GelMA could be employed for growth and implantation of tissue-engineered human corneal endothelial cell (HCEC) monolayer. Tissue-engineered HCEC monolayer could potentially be used to treat corneal blindness due to corneal endothelium dysfunction. Here, we exploited a sequential hybrid (physical followed by UV) crosslinking to create an improved material, named as GelMA+, with over 8-fold increase in mechanical strength as compared to regular GelMA. The presence of physical associations increased the subsequent UV-crosslinking efficiency resulting in robust materials able to withstand standard endothelium insertion surgical device loading. Favorable biodegradation kinetics were also measured *in vitro* and *in vivo*. We achieved hydrogels patterning with nano-scale resolution by use of oxygen impermeable stamps that overcome the limitations of PDMS based molding processes. Primary HCEC monolayers grown on GelMA+ carrier patterned with pillars of optimal dimension demonstrated improved zona-occludin-1 expression, higher cell density and cell size homogeneity, which are indications of functionally-superior transplantable monolayers. The hybrid crosslinking and fabrication approach offers potential utility for development of implantable tissue-engineered cell-carrier constructs with enhanced bio-functional properties.

1. Introduction

The ability to create bioactive hydrogels with tunable stiffness and degradation is in great demand as material matrix degradation and stiffness have significant influences on cellular responses and tissue growth [1]. Gelatin methacrylate (GelMA), a bioactive hydrogel, is increasingly being used in tissue engineering and regenerative medicine. Applications include cell encapsulation,

* Corresponding author. Current address: Department of Chemical Engineering, University of Waterloo, Waterloo, Ontario, Canada.
** Corresponding author. Institute of Materials Research and Engineering, Agency for Science, Technology and Research (A*STAR), Singapore.
*** Corresponding author. Duke-NUS Graduate Medical School, Singapore.
E-mail address: eyim@uwaterloo.ca (E.K.F. Yim).

http://dx.doi.org/10.1016/j.biomaterials.2016.12.026
0142-9612/

cardiac and cardiovascular tissue engineering, bone, cartilage and muscle tissue engineering and bioink development for 3D printing [2]. The rise in popularity of GelMA is due to its proven biocompatibility, physico-chemical tailorability and the possibilities it offers for patterning with spatio-temporal control of UV-crosslinkable hydrogels [2]. Several strategies to improve the mechanical properties of gelatin methacrylate hydrogels have been reported. These include reinforcement with 3D printed microfibers [3], carbon nanotubes [4,5], graphene oxide [6], poly lactide-*co*-ethylene oxide-*co*-fumarate (PLEOF) [7], polyacrylamide [8] and polyethylene glycol [9,10]. Biopolymers such as gellan gum [11], hyaluronic acid [12], dextran [13] and silk fibroin [14] have also been incorporated to tune the mechanical properties of GelMA. The reported strategies have thus far focused on the addition of another component to the GelMA and involve complex and often expensive processes. Room temperature crosslinking of GelMA macromer solution has also been shown to improve the modulus of GelMA hydrogels [15]. To date, there lacks a proven method able to tune the strength and biodegradation properties of GelMA for tissue engineering application. Requirement to use biocompatible materials and preferably minimal chemical modifications and minimal synthetic additives in the material formulation remains an unmet challenge.

Similarly, the control of cellular fate by manipulating the cell behaviors with surface micro or nano-topographical cues is another key topic in biomaterials study. PDMS micro-molding and photolithography are the two most commonly used techniques for topographical patterning of hydrogels [16–18]. PDMS master molds have been used to pattern chemically crosslinked hydrogels such as alginate and PVA with nanoscale features [19–21]. However, PDMS micro-molding of UV-crosslinkable hydrogels including GelMA has only been able to achieve feature size on the order of only 10s of micrometers [22–25]. The reason PDMS molds cannot achieve high resolution pattering of UV-crosslinkable hydrogels, is likely due to the high oxygen diffusion coefficient of PDMS, which inhibits UV crosslinking at the hydrogel-PDMS mold interface [26,27]. UV-crosslinking with N_2 purging [28] or expensive quartz and silicon stamp-based nano-imprint lithography of hydrogels have been shown to enable high resolution patterning of UV-crosslinkable hydrogels [29,30]. The ability to pattern UV-cross-linkable GelMA with topographical cues in the range of 1 μm or lower with cost-effective and simple methods would benefit further translational use of the material to control cellular functions and stem cell differentiation [31]. The relevance of these sizes is related to the fact that the *in vivo* extracellular matrix also contains nanoscale topographical cues [32].

An impaired human corneal endothelial cell (HCEC) monolayer due to Fuchs' Endothelial Dystrophy (FED) or bullous keratopathy is one of the major causes of corneal blindness [33–35]. It is also the leading indication for corneal transplantations, which is the only solution for corneal blindness [36]. Due to a worldwide donor cornea shortage, tissue-engineered HCEC transplants are being investigated as an alternative tissue source [33,37]. HCECs may be implanted on a carrier or using a cell based approach [38]. The advantage of using a carrier is the ability to control cell density during the implantation process [39]. Ideally, the implantable film should be biocompatible and support HCEC adhesion, be optically transparent, permit diffusion of aqueous humor nutrient molecules and should be mechanically strong for easy handling by corneal surgeons [40]. Another distinct advantage of a synthetic carrier over a biological one would be the ability to slowly biodegrade after implantation. Previous research studies have utilized several synthetic and natural materials to fabricate a cell-carrier for HCEC monolayer transplantation [41–46]. While previous studies have laid the ground work and shown the potential of this method, the following important material properties for cell-carriers require further development: mechanical strength of the film in hydrated state, nutrient transport rates through the cell-carrier, biodegradation kinetics in relevant media, and effective support of the correct cell phenotype and relevant analysis [45]. Importantly, the majority of cell-carriers in use today are “passive”. They act primarily as a transfer support for transplantation of monolayer and do not participate in enhancing the functional markers and phenotype of the regenerated monolayer. Corneal transplants often fail due to loss of the donor HCECs from the transplanted monolayer. Studies show that 30% of HCECs are lost within the first 6 months of transplantation [47,48]. Designing implantable cell-carriers that not only serve as a transporter, but also enhance cell function and phenotype may improve the long-term success of corneal transplantation. Improving the cellular functions of the HCECs grown on the carrier would improve the cell-cell and cell-carrier adhesion, similar to native Descemet membrane (DM) [40]. In this study, we report on the development of highly tunable micro- and nano-patterned GelMA thin films and demonstrated its application in both tissue engineering and as cell-carrier support functions for HCEC monolayer culture and transplantation. We developed a simple method to improve GelMA mechanical strength over 8-folds and characterized our developed GelMA hydrogel in terms of compressive Young's modulus, rheology, extent of UV-crosslinking, hydration characteristics, biodegradability *in vitro* and *in vivo*, and permeability of the GelMA films to bovine serum albumin (BSA) and glucose with and without cell monolayers. Additionally, we developed a generic and simple nano-molding method to pattern micro- as well as nano-topographical cues on hydrogels films and demonstrated the ability of topographical cues to improve the cell functions and phenotype of HCEC monolayer.

2. Experimental section

2.1. Synthesis of gelatin methacrylate

Gelatin methacrylate (GelMA) was synthesized as described previously [49] with modifications. Briefly, 5 g gelatin (Type A, Sigma Aldrich) was dissolved in 50 ml Phosphate Buffer Saline (PBS). Once fully dissolved, 10 ml methacrylic anhydride (Sigma Aldrich) was added dropwise to the gelatin solution at 60 °C and the reaction was continued for 1 h under magnetic stirring. Subsequently, the mixture was diluted with deionized (DI) water, filled in 12–14 kDa cut-off dialysis tubes and dialyzed in DI water for 5 days at 37 °C. After dialysis, the GelMA solution was frozen at −80 °C and lyophilized to obtain white GelMA foam. The degree of methacrylation of gelatin was measured by using TNBSA assay, which was ≈89% in this work.

2.2. Preparation of GelMA hydrogels

Lyophilized GelMA was dissolved in PBS containing 0.5% w/v Irgacure 2959 (BASF) at 50 °C to prepare prepolymer solution. To prepare ‘regular’ GelMA hydrogel, the prepolymer solution at 37 °C was pipetted between glass slides separated by spacers (150–750 μm) and immediately crosslinked for 60 s by using UV irradiation (360–480 nm) at an intensity of 32 mW/cm^2 (UVACUBE 100, Honle) (Fig. 1). To prepare hybrid crosslinked GelMA hydrogel (GelMA+), the prepolymer solution was pipetted between glass slides and the glass slide chamber was incubated at 4 °C for 1 h. Immediately after incubation, the prepolymer was irradiated with UV light as described above (Fig. 1). After crosslinking, the GelMA and GelMA+ hydrogels were removed from glass slides.