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A novel gelatin-based micro-cavitary hydrogel for potential application in delivery of anchorage dependent cells: A study with vasculogenesis model

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ABSTRACT

Hydrogels have been widely regarded as promising tissue engineering scaffolds and cell delivery vehicles, however, their inherent submicron- or nano-scale polymer networks severely inhibit the settlement of anchorage dependent cells (ADCs). Here, using endothelial progenitor outgrowth cells (EPOCs) as the typical ADCs, a gelatin-based micro-cavitary gel (namely Gel-MCG) is developed with gelatin-methacrylate and gelatin microspheres as precursor and porogens, respectively, to promote cellular focal adhesion and functions. The introduction of micro-cavitary structures within the Gel-MCG improves its physical properties as well as creates numerous gel-microcavity interfaces within gel-based matrices. Compared with conventional gelatin gel (Gel-G) scaffold, the Gel-MCG provides more suitable microenvironments for EPOCs' attachment, spreading, and proliferation, and then which leads to enhanced endothelial differentiation and vascularization as demonstrated by higher expressions of endothelial markers. The Gel-MCG system shows great potential as vehicle for the delivery of ADCs in tissue engineering.

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1. Introduction

Tissue engineering has been recognized as a promising technique to repair or regenerate tissues and organs de novo using a three-dimensional (3-D) scaffold and its resident cells [1]. The scaffold functions to deliver cells as well as supports and guides their growth and neo-tissue formation [2,3]. Hydrogel is an attractive type of scaffold due to its unique advantages, such as high water content, tissue-like elasticity, and injectability [4–6]. Compared to the prefabricated scaffold, the cells can be more abundantly and homogeneously encapsulated in hydrogel through rapidly curing the cell-suspended macromer solution [7].

However, the extensive applications of hydrogel in field of tissue engineering have been severely limited by its high spatial limitation for encapsulated cells [8]. The micron-scale cells are physically constrained within the submicron- or nano-scale hydrophilic polymer networks and thus restricted from stretching regardless of cell types; this phenomenon is even present in the interior of hydrogels containing cell-adhesive moieties such as RGD due to the unique 3-D physical binding mechanism for cell encapsulation [9]. The "trapped" cultivation of cells in hydrogel goes against their essential culture principles, especially for the anchorage-dependent (progenitor) cells (ADCs) such as fibroblasts, osteoblasts, and endothelial (progenitor) cells. The ADCs need to attach to interfaces and subsequently spread to maintain their normal morphology, otherwise they will lose the nature and undergo apoptosis gradually; the adhesion and spreading is obligatory for the survival of ADCs [10,11]. Therefore, the manipulation and optimization of hydrogel microenvironments should be devoted to create cell-affinitive interfaces and living spaces to promote ADC adhesion, spreading, and function.

Currently, two types of strategy have been proposed to improve cellular adhesion and spreading within hydrogel body. One approach is to fabricate hydrogel by concurrently introducing cell-adhesive domains and cell-degradable blocks into the networks, which facilitates cellular focal adhesion and endows ADCs with more spatial autonomy to remodel the gel environments for cell stretching to their natural morphology [12–14]. In addition, the composite hydrogel opens another way to achieve cellular adhesion and spreading within hydrogel body [15–18]. It is generally fabricated via two steps: (1) the ADCs are seeded and adhered onto the

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surface-functionalized organic or inorganic microspheres, and (2) those cell-laden microspheres are then encapsulated into hydrogel. The composite gel provides anchors for cellular adhesion as well as create living space for enhanced cell spreading and metabolism. Recently, a similar microcarrier-gel composite hydrogel has been developed by Zhong et al., which also facilitate cellular spreading and activities [19].

A micro-cavitary gel (MCG) system is devised in our previous study by creating micron-scale cavities using gelatin microspheres as the porogen, within non-adhesive gel body (e.g. alginate gel, agarose gel, hyaluronan gel), which has achieved promising outcomes in aiding proliferation and function of non-anchorage-dependent cells such as chondrocytes and induced pluripotent stem cells [20–23]. In this study, we aim to fabricated a MCG with typical cell-adhesive materials and explore the effect of micro-cavitary structure on cell fate of encapsulated typical ADCs. The model MCG, Gel-MCG, possessing hundreds of micron-scale cavities, is designed and fabricated with cell-adhesive gelatin-methacrylate (namely gelatin-MA) precursor and temperature-sensitive gelatin microspheres by the ultraviolet (UV) photocrosslinking method. The physical properties (such as swelling ratio, crosslink density) of the newly developed Gel-MCG are assessed, using conventional gelatin gel (named as Gel-G) as control. Endothelial progenitor outgrowth cells (EPOCs), served as a model ADC type, are photo-encapsulated into Gel-MCG and Gel-G constructs, respectively. The viability, proliferation, and differentiation of the EPOCs are evaluated in detail.

2. Materials and methods

2.1. Synthesis and characterization of gelatin-methacrylate (gelatin-MA)

The gelatin-MA precursor is synthesized by referring to previous work [24]. 6 g of gelatin (type A from porcine skin, Sigma-Aldrich) is dissolved in 150 mL of phosphate-buffered saline (PBS, pH 7.4) solution under stirring at 60 °C, followed by the addition of 40 mL of *N*,*N*-dimethylformamide (DMF, Sigma-Aldrich). After 2 h of stirring, 10 g of triethylamine (TEA, Sigma-Aldrich) and 20 g of glycidyl methacrylate (GMA, Sigma-Aldrich) is added dropwise into the solution, respectively, and then stirred for 5 days at 37 °C. Subsequently, the solution is concentrated using a rotary evaporator, dialyzed (molecular weight cut-off 14000) for 3 days against deionized (DI) water, and lyophilized to obtain gelatin-MA foam.

Freshly synthesized gelatin-MA precursor and the purchased raw gelatin, serving as control, is characterized with ^{1}H NMR spectra that are recorded on a Bruker Avance-300 spectrometer using deuterium oxide (D_2O) as the solvent. ^{1}H NMR spectroscopy is employed to determine the degree of methacrylation of gelatin-MA precursor.

2.2. Fabrication of hydrogel

Gelatin microspheres, based on physical crosslinking, are prepared using a double oil-in-water-in-oil emulsion, separated by sieving, and sterilized according to previously established strategy [20,25]. Briefly, 15 mL of ethyl acetate is added into 40 mL of gelatin solution (10%, g/mL) under stirring and then the emulsion is poured into 80 mL vegetable oil, followed by stirring for 10 min. Gelatin microspheres can be formed spontaneously in 300 mL cooled ethanol under stirring. The gelatin microspheres are dried and sieved via standard sieves.

The gelatin microspheres are temperature-sensitive; they can completely dissolve upon elevating the incubation temperature to 37 °C [20]. The gelatin microspheres with 150–200 μm diameters are used in this study.

For the fabrication of gelatin-based micro-cavitary gel (named as Gel-MCG), 0.3 g of gelatin microspheres are suspended in 1.0 mL of the solution of gelatin-MA precursor (12%, g/mL) in PBS containing photo-initiator Irgacure 2959 (0.1%, g/mL, Ciba Specialty Chemicals). Each 50 μL of the suspension is rapidly injected into a cylindrical mold (diameter 5.2 mm) with a pipette. Subsequently, they are exposed to UV light (365 nm, 30 mW/cm^2) for 4 min to gelation. The resultant covalently crosslinked gelatin composite hydrogels containing gelatin microspheres are carefully taken out from the molds and placed into PBS solution at 37 °C. The gelatin microspheres within the gelatin gels would gradually dissolve and leave behind cavities in situ, forming Gelatin-MCG. In addition, the conventional, non-cavitary gelatin hydrogels are fabricated from the same 12% (g/mL) of gelatin-MA precursor solution via the same procedure but without adding gelatin microspheres into the precursor solution, which are named as Gel-G, serving as control.

2.3. Physical properties of hydrogel

Freshly synthesized hydrogels are immersed in PBS (pH 7.4) at 37 °C. Samples in triplicate are taken out at predetermined time points, and the wet weight (W_w) of each sample is determined by gravimetric method after wiping off the liquid on the surface. Subsequently, the samples are freeze-dried to obtain the corresponding dry weigh (W_d). The mass-based equilibrium swelling ratio (Q_m) is calculated as follows [26,27]:

$$Q_m = \frac{W_w}{W_d}$$

The volume-based equilibrium swelling ratio (Q_v) is determined by the following equation:

$$Q_v = 1 + \frac{\rho_p}{\rho_s}(Q_m - 1)$$

wherein, ρ_p and ρ_s is the density of polymer (1.44 g/mL for gelatin) and solvent (1.0 g/mL), respectively. M_c, number-average molecular weight between cross-links, is estimated with a simplified Flory-Rehner equation [27,28]:

$$M_c = Q_v^{5/3}(0.5 - \chi)^{-1}\frac{V_1}{\upsilon}$$

wherein, υ and V_1 is specific volume of the polymer and molar volume of the solvent (18 mL/mol), respectively. χ is the polymer-solvent interaction parameter, and it is 0.49 for gelatin [29].

The effective crosslink density (ν_e) of hydrogel is determined by the following equation:

$$\nu_e = \frac{\rho_p}{M_c}$$

2.4. Cell culture

Mouse endothelial progenitor outgrowth cells (EPOCs) are purchased from Biochain Institute Inc. (Hayward, CA, USA, catalog No. Z7030031), and cultured according to manufacturer's instruction in EPOC growth medium (BioChain, catalog No. Z7030033) at 37 °C in 5% CO_2 atmosphere. The EPOCs at passages 14-17 are used in this study.

2.5. EPOCs encapsulation and culture

A 12% (g/mL) of gelatin-MA precursor solution in PBS solution containing 0.1% (g/mL) of Irgacure 2959 is sterilized via filtering through a 0.2 μm filter. Upon reaching 70–80% confluence, the

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