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A spatial patternable macroporous hydrogel with cell-affinity domains to enhance cell spreading and differentiation

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ABSTRACT

Cell adhesion and spreading are two essential factors for anchorage-dependent cells such as osteocytes. An adhesive macroporous hydrogel system, in which cell-affinitive domains and sufficient cytoskeleton reorganization space were simultaneously constructed, was proposed in this report to support cell adhesion and spreading, respectively, and facilitate cell differentiation and function establishment eventually. The adhesive macroporous alginate hydrogel was developed by RGD peptide graft and gelatin microspheres hybridization to generate cellular adhesion sites and highly interconnected macropores. The successful stretched morphology and enhanced osteogenic differentiation of MG-63 cells in this modified alginate hydrogel showed clearly the feasibility that cell function may be effectively facilitated. Besides, this hydrogel model can be further applied to construct complex micropatterned structure, such as individual microgels in shapes of circle, square, cross and ring, and osteon-like structure containing both osteogenic and vascularized area generated by a double-ring assembly. These results should provide this adhesive macroporous photocrosslinkable hydrogel system as potential three-dimensional scaffolds for guiding tissue formation, especially for the bioengineering of tissues that have multiple cell types and require precisely defined cell–cell and cell–substrate interactions.

1. Introduction

The three-dimensional (3D) interaction between cells and extracellular matrix (ECM) constitutes a dynamic regulatory system for directing tissue formation and regeneration [1–3]. As such, significant progress has been made in engineering 3D hydrogels capable of promoting cell function and many of these important ECM interactions [4–6]. However, the inability to precisely control cell behavior has often resulted in poor cell and ECM organization within engineered constructs that had limited ability to recreate complex tissues [7,8]. Another important challenge is to control the spatial organization of cells in their synthetic microenvironments which is especially vital in the bioengineering of tissues that have multiple cell types and require precisely defined cell–cell and cell–substrate interactions [9,10]. Therefore, the spatial patterning of hydrogels in structure or chemical component, motivated by the microscale heterogeneity of native tissue architectures, has recently been the focus and nodus of advanced tissue engineering constructs to provide better control of cellular behavior [7]. Microgels fabricated by photomask-based stereolithography have been successfully integrated into the generation of complex structures with well-defined microarchitecture and enhanced control of cell behavior and function [11–13]. While a major challenge in the use of micro/macro-scaled hydrogel scaffolds is the settlement of cell spreading, migration and differentiation in 3D gel matrices [14].

Cell adhesion and spreading are two essential factors for anchorage-dependent cells such as osteocytes. The survival of these cells first requires attachment to a substrate, which relies on the interactions between receptors on cell surface and substrates, and determines the proliferation, differentiation and many other important cell behaviors [15]. As such cells require a stretched morphology to maintain their normal phenotype and function, the spreading is another indispensable factor for their subsequent settlement and commitment in 3D microenvironments [16,17]. Cell spreading is based on the accomplishment cell adhesion, successful cytoskeleton reorganization and, more importantly, it cannot do without the availability of space in 3D scaffolds [18]. Evidently, cell function cannot be performed without the successful cell adhesion and spreading. Unfortunately, both these cell behaviors could scarcely be achieved in common gel matrices due to the low cell

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affinity and high cell constraint [14]. Hence, much work has been focused on functionalizing 3D hydrogels to spatially and temporally control their internal cell adhesion and spreading.

One of the most common strategies is chemical modification with cell-affinitive domains such as Arg-Gly-Asp (RGD) or hybridization with cell-adhesive materials to provide integrin-binding sites to cells in the inert bulk of the hydrogel [6,19,20]. Another strategy, of which the central idea still focuses on the provision of cell-affinitive interfaces, is to physically hybridize functionalized microspheres or nanoaggregates into hydrogel bulk to support cell adhesion and stretching on their spherical surfaces [21–23]. Cells could survive and spread well within the composite, yet should be pre-seeded and cultured on the surface of the microspheres for several days to make a sufficient cell adhesion and spreading before encapsulating into the hydrogels. This increased the cumbersome nature of the process inevitably but, more important, the cell-laden microspheres can not be uniformly encapsulated into the hydrogel since vigorous mixing may cause cell shedding from the surface of the microspheres. Some other studies have mainly focused on the creation of space for cell proliferation and migration by relying on specific enzymatic digestion or hybridization of micro-porogens produced by the self-degradation of the microspheres as well as the photodegradable hydrogel which is another ingenious strategy [24–27]. But few can reach balance between enhanced cell–matrix interaction and the creation of spatial freedom for cell spreading, which accordingly resulted in the unfavorable functional expression and tissue ingrowth although cells encapsulated in the 3D hydrogels can spread to some extent. A typical example is the living hyaline cartilage graft developed by Dong-An Wang group via the strategy of phase transfer cell culture [28,29]. The chondrocytes encapsulated in alginate constructs were indicated to grow into the cavities created by the degrading gelatin microspheres, proliferate into colonies, and ultimately fill up the cavities. However, because of the intrinsic non-cell-adhesive property, this hybrid hydrogel system could not support anchorage-dependent cells stretching into their favorable morphology but only suit such spherical cells like chondrocytes, and accordingly cannot be successfully applied on bone tissue construct.

In this work, the simultaneous construction of both cell-affinitive domains and sufficient spreading space is emphasized in the design and modification of 3D hydrogel materials for the regeneration of tissues such as bone. We investigate the hypothesis that the incorporation of RGD peptide and macropores into inert hydrogel substrate, namely adhesive macroporous hydrogel system, which provide cellular adhesion sites and cytoskeleton reorganization space, will support cell adhesion and spreading, respectively, and facilitate cell differentiation and function establishment eventually. Here, the inert alginate, inherently non-adhesive, exhibited as the model hydrogel material; MG-63 was represented to investigate the proliferation, spreading and functional expression of such anchorage-dependent cells encapsulated in 3D hydrogel matrix. To produce the desirable adhesive macroporous alginate hydrogel, cellular adhesion peptide RGD was first grafted onto the alginate molecule chain to provide integrin-binding sites to MG-63 cells. Simultaneously, the macropores were created by the rapid degradation of the incorporated gelatin microspheres at 37 °C to generate space for cell spreading. Based on the adhesion ability and spreading space construct, we expected that anchorage-dependent cells such as MG-63 cells around spheres could spread and migrate gradually to the space left by the degraded microspheres on the premise of RGD involvement, and thus the prolonged cell survival and tissue growth in the artificial microenvironment were further facilitated. A schematic demonstration of cell spreading and proliferation in the construction of adhesive macroporous hydrogel is shown in Fig. 1A.

On the basis of the photocrosslinkable ability, microgels with different shapes, such as circle, square, cross and ring, were fabricated by this adhesive macroporous hydrogel model respectively. Moreover, by a two-step photolithography method, double-ring microgels with human umbilical cord vein endothelial cells (HUVECs) laded in the inner layer and MG-63 cells laded in the outer layer were assembled to generate osteon-like structure imitating Haversian system for bone-restructuring tissue engineering. Predictably, this adhesive macroporous hydrogel system, possessing spatial patterned ability and simultaneously enabling prolonged cell survival and differentiation, would have great potential applications for tissue engineering, especially for bottom-up macroscopic tissue engineering, realizing functional and spatially structured, complex, large-scale tissues.

2. Materials and methods

2.1. Materials

Sodium alginate was supplied by Fluka (item no. 71238) with a G/M ratio of 70/30. The cell adhesive oligopeptide GCGYGRGDSPG (MW: 1025.1Da) with >95% purity (per manufacturer HPLC analysis) were obtained from ChinaPeptides Co., Ltd. The UV light source (Omnicure S1500) was purchased from EXFO Photonic Solutions Inc. Gelatin, methacrylic anhydride, Irgacure 2959, Alexa Fluor-594 phalloidin, DAPI (4′,6-diamidino-2-phenylindole), FDA (fluorescein diacetate) and PI (propidium iodide) were obtained from Sigma–Aldrich. MTT (methyl thiazolyl tetrazolium) was obtained from Amreso (USA). All other chemicals were acquired from Chengdu Kelong Chem Co. unless otherwise specified.

2.2. Gelatin microspheres fabrication

Gelatin microspheres were prepared by an oil in water in oil (o/w/o) double emulsion method [21]. An aqueous solution (20 ml) of 10 wt% gelatin preheated at 70 °C was added to 10 ml of ethyl acetate. After vigorous stirring for 2 min with mechanical stirrer at 700 rpm, the mixture was quickly poured into 60 mL edible oil under agitation at the rate of 300 rpm for 1.5 min. The mixture was then transferred to a cool water bath and maintained at the same stirring rate for 15 min before pouring into ice-cold ethanol for 10 min. Gelatin microspheres were collected by washing with acetone to remove the residual edible oil. The washed gelatin spheres were air-dried and separated by standard sieve. Gelatin spheres with diameters of 100–150 μm were separated and utilized for preparation of microspheres hybrid alginate hydrogel.

2.3. Hydrogel preparation

The adhesive alginate hydrogels encapsulating gelatin microspheres were formed based on a previously described methacrylated alginate (MAA) hydrogel system with modifications. Briefly, MAA was synthesized through esterification of sodium alginate and methacrylic anhydride based on previously described protocols [30]. The methacrylation percentage of the MAA used in these experiments was roughly 50%. MAA was dissolved in a triethanolamine-buffered saline (TEOA buffer: 0.2 M TEOA, 0.3 M total osmolarity, pH 8.0) containing Irgacure 2959 photoinitiator (final concentration of 0.5 wt %). The cell adhesive peptide RGD dissolved in TEOA buffer was added to the MAA solution at a concentration consuming 10% acrylate groups on MAA based on the Michael-type addition chemistry, and allowed to react for 1 h at 37 °C before the hydrated gelatin microspheres (100–150 μm) were mixed. Following re-suspension of cells in this hybrid solution, the resultant homogenous blend was subsequently poured into molds and then exposed to 7.9 mW/cm^2 UV light (360–480 nm) for 20 s to allow free radical polymerization of MAA chains by photocrosslinking. Four microspheres containing hydrogels (AG0.5, AG0.75, AG1 and AG1.25) were prepared with an initial MAA concentration of 30 mg/ml and final volume ratio of gelatin microspheres/MAA = 0.5, 0.75, 1 and 1.25. The pure MAA hydrogel without gelatin microspheres (AG0) was prepared as control.

2.4. Hydrogel characterization

The mechanical properties of the hydrogels were characterized by compressive stress–strain measurements using an electromechanical universal testing machine (Model: CMT 4104) with a 200 N load cell (Shenzhen SANS Testing Machine co., LTD.). The cylindrical gel sample, 8 mm in diameter and 2 mm in thickness, was tested at a strain rate of 0.3 mm/min. The elastic modulus was determined by the average slope of the stress–strain curve over the strain range 0–25%. Three parallel samples per measurement were performed, and the obtained values were averaged.

The effect of gelatin microsphere incorporation on hydrogel swelling and rate of dissolution was investigated through swelling studies. Hydrogels without gelatin (AG0) and encapsulating gelatin microspheres (AG0.5 ~ 1.25) were formed as described above and the initial wet weight (Wo) was obtained after 24 h gelation. Hydrogels were then incubated in PBS at 4 °C for 24 h, and then half of the hydrogels