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A novel gellan gel-based microcarrier for anchorage-dependent cell delivery

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Abstract

Competent vehicles are highly sought after as a means to transplant cells for tissue regeneration. In this study, novel hydrogel-based microspherical cell carriers are designed and developed with an FDA-approved natural polysaccharide, gellan gum. The bulk fabrication of these microspheres is performed via a water-in-oil (W/O) emulsion process followed by a series of redox (oxidation–reduction) cross-linking treatments; this enables the microspherical dimensions to be precisely manipulated in terms of injectability, and simultaneously ensures the structural stability. To acquire adhesion affinity with anchorage-dependent cells (ADCs), a covalent coating of gelatin is further applied on the microspherical surfaces. The final product is constructed as a variety of gelatin-grafted-gellan microspherical cell carriers, abbreviated as "TriG" microcarriers. The cell-loading tests are conducted, respectively, with human dermal fibroblasts (HDFs) and human fetal osteoblasts (hFOBs). Morphological observation from optical microscopy and field emission scanning electron microscopy indicates that the HDFs spread well and populate rapidly on surfaces of TriG microcarriers. Immunofluorescent staining reveals the activation of focal adhesion and subsequent organization of F-actin from the attached cell surfaces, which suggests the TriG microspherical substrate is favorable to ADC adhesion and therefore capable of promoting HDF proliferation to achieve confluence by turning over three times within 10 days. The hFOBs are also cultivated on the TriG carriers, where ideal viability and clear potentials for osteogenesis are demonstrated by fluorescent "Live/Dead" screening and specific histobiochemical indications. All these findings suggest that the TriG microcarriers are suitable to provide open platforms for therapeutic ADC proliferation and differentiation.

Keywords: Microspheres; Gellan gum; Polysaccharides; Hydrogel; Gelatin

1. Introduction

In recent decades, therapeutic cell transplantation has played in increasingly important role in regenerative medicine, for which, obviously, both the quality and quantity of the transplanted cells have a critical influence on the actual functionality of the overall treatment. As a major family of therapeutic cell species, mass transfer of anchorage-dependent cells (ADCs) must rely on appropriate cell-laden vehicles that function to maintain the cell population and also ensure the cell phenotype remains intact during delivery [1,2]. Among numerous strategies intended to enhance the attachment or encapsulation of living cells, the use of microspherical carriers is a promising new approach that originates from a setup for mass production of mammalian cell-derived vaccines. Given its superiority in both cell expansion and conveyance, this approach has latterly evolved and been adopted for tissue engineering use [3]. For this purpose, microspheres with diameters ranging from 100 μm to a few millimeters were fabricated with synthetic or natural polymers such as PLGA, dextran and collagens, enabling vast and conveyable cell-loading interfaces to be generated and built into the bulk of engineered tissues. As reported in the pioneer studies, preliminary but encouraging outcomes were particularly yielded from the trials with musculoskeletal cells or their progenitors [3–6]. Despite the transitional deficiencies in the existing models

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due to imperfect cell affinity, biocompatibility or other physical characteristics, extensive interest remains in these microcarriers due to their ability of injectable mass conveyance and the unique focal adhesional accommodation for ADCs. Efforts are being continuously devoted to the development of novel microspherical models in order to improve delivery efficacies.

In this study, gellan gum is innovatively employed as the matrix material of the newly developed microspherical cell carriers. Prior to this trial, gellan gum, as an US Food and Drug Administration (FDA)-approved food additive, had been exploited in pharmaceutical products acting as oral drug carriers or in situ ophthalmic gel materials [7–9], as well as potential tissue engineering scaffolds [10]. Gellan gum is a linear anionic extracellular polysaccharide secreted by *Sphingomonas paucimobilis* with repeating tetrasaccharide units comprising of one α-L-rhamnose, one β-D-glucuronic acid and two β-D-glucose residues. The commercially available gellan gum has been deacetylated from its native form and therefore contains more acetyl groups on the glucose residues. Being a ubiquitous natural polysaccharide, gellan gum is characterized by its unique gelling behavior that involves a dual mechanism: temperature-dependent hydrogen bonding and cation-induced electrical incorporation. Gelation proceeds through the formation of double helical chain complexes followed by an inter-chain crosslinking via hydrogen bonds synchronizing with cationic bridging between the polyanionic saccharide backbones [7,8]. It has been demonstrated that a gellan gel can be rapidly formed either with divalent cations (Ca^{2+}) or monovalent physiological saline (0.9% NaCl), while fewer raw materials are required to make it as strong as the widely used agarose or alginate gels, which is very likely attributable to the dual crosslinking ties in the gellan gels [9,10]. In addition, mechanical tests of gellan gel in physiological conditions have indicated that its elastic modulus and storage modulus fall into the range of 10^3 and 10^2 Pa, respectively [10,11], and studies in simulated physiological environment have proven the long-term fate of the gellan-based constructs to be compatible with the human body [12].

Given all the advantages of gellan gels, the final barrier to their use, which is also the most common hurdle for general (synthetic or polysaccharide) hydrogels serving as cell-loading substrates, is the lack of ADC affinity due to their extremely hydrophilic nature in bulk and particularly on the outer surfaces. Once they are seeded with cells in culture medium, their highly hydrated gel phase resists the adsorption of extracellular matrix (ECM) proteins (e.g. fibronectin, collagen and laminin) and consequently blocks the ADC attachment [13]. The intrinsic property of hydrogel, on the other hand, renders it a programmable platform with neutral background in terms of uncontrollable or non-specific adhesion so that intentionally rendered specific functionalities can be extracted and highlighted. Many ways to improve the cell adhesion on hydrogel surfaces have been tried, such as incorporation of Arg–Gly–Asp (RGD) peptides or immobilization of collagen/gelatin moieties [13,14], and several effective strategies have been developed to crosslink the proteins to polysaccharides or poly(ethylene glycol)-based polymers, such as enzymatic catalysis, end functionalization or ultraviolet (UV) activation [15,16]. In this study, the gellan microspheres are covalently coated with gelatin layers to create the cell binding ligands on which human ADCs, including fibroblasts and osteoblasts, are cultivated for appraisal of cell delivery and developmental efficacies.

2. Materials and methods

2.1. Fabrication of gellan microspheres

2.1.1. Fabrication

Phytagel®, one of the commercially available products of gellan gum, and all other chemicals and reagents were purchased from Sigma–Aldrich (St. Louis, MO), unless otherwise noted. The gellan microspheres were prepared using a water-in-oil (W/O) emulsion method. Briefly, gellan was completely dissolved in deionized water (2%, w/v) after being heated at 70–80 °C for 15 min. The hot solution was quickly poured into pre-heated 90 °C peanut oil under stirring at ~500 rpm for a few minutes. The mixture was finally transferred into large amount of 1% $CaCl_2$ solution for another 5 min to enhance the gel strength according to its gelling mechanism [7–9]. The spheres produced were collected by filtration through a sieve and extensively wash with deionized water before microscopic observation.

2.1.2. Characterization

The particle size distribution was analyzed with Image J Software (National Institutes of Health, USA) by randomly selecting 121 microspheres from a total of 19 visual fields that had been pre-equilibrated in phosphate-buffered saline (PBS) or cell culture medium for at least 24 h. The frequency (%) of diameters was counted and recorded in increments of 50 μm. To obtain the swelling ratio, the microspheres were first lyophilized for dry weight (W_d) measurement, then soaked in PBS again for 24 h; the wet weight at equilibrium (W_w) was also measured. The swelling ratio is calculated by $(W_w - W_d)/W_d$.

2.2. Gelatin grafting

2.2.1. Gellan oxidation

Sodium periodate was used to introduce aldehyde groups onto the gellan microspherical surfaces. The reaction was conducted by immersing the microspheres in sodium periodate solution with shaking and protection from light. In order to find the optimum process, various dosing ratios of gellan unit (GU, the repeating tetrasaccharide) to sodium periodate (6:1, 3:1, 1.5:1 and 1:1, mol/mol) and various reaction times (1, 2, 4, 6, 8 and 16 h) were used in all combinations. The hydrochloride hydroxylamine assay was, respectively, performed on the products collected from each

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