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A temperature-cured dissolvable gelatin microsphere-based cell carrier for chondrocyte delivery in a hydrogel scaffolding system ☆

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ABSTRACT

In this study, a novel therapeutic cell delivery methodology in the form of hydrogel encapsulating cell-laden microspheres was developed and investigated. As a model cell for cartilage tissue engineering, chondrocytes were successfully encapsulated in gelatin-based microspheres (mostly of diameter 50–100 μm, centred at 75–100 μm) with high cell viability during the formation of microspheres via a water-in-oil single emulsion process under a low temperature without any chemical treatment. These cell-laden microspheres were then encapsulated in alginate-based hydrogel constructs. By elevating the temperature to 37 °C, the cell-laden microspheres were completely dissolved within 2 days, resulting in the same number of same-sized spherical cavities in hydrogel bulk, along with which the encapsulated cells were released from the microspheres and suspended inside the cavities to be cultivated for further development. In this cell delivery system, the microspheres played a dual role as both removable cell vehicles and porogens for creation of the intra-hydrogel cavities, in which the delivered cells were provided with both free living spaces and a better permeable environment. This temperature-cured dissolvable gelatin microsphere-based cell carrier (tDGMC) associating with cell-laden hydrogel scaffold was attempted and evaluated through WST-1, quantitative polymerase chain reaction, biochemical assays and various immunohistochemistry and histology stains. The results indicate that tDGMC technology can facilitate the delivery of chondrocytes, as a non-anchorage-dependent therapeutic cell, with significantly greater efficiency.

1. Introduction

Microspheres have been used widely as delivery vehicles for drugs and cells, as they provide a minimally invasive means of transplantation [1–6]. In particular, many materials and fabrication methods in the delivery of cells for regenerative medicine purposes have been explored because of their advantages: the simplicity of large-scale culture of cells in microspheres of controlled sizes, the provision of a tunable three-dimensional (3-D) environment for cells, the ability to incorporate biochemical signals and biomechanical moieties as well as the simplicity of direct injection of cell-loaded microspheres into defect sites without trypsinization [5,7,8]. Studies were usually done through a two-step method of first fabricating microspheres, for example through single or double emulsion methods [9–11], electrospraying [12] and thermally induced phase separation [13], and subsequently seeding cells onto them. Although the above-mentioned methods were able to support cells, the microsphere fabrication techniques usually required specialized equipment or a significant amount of time, as thorough washing steps were necessary after chemical-based treatment. Furthermore, these techniques largely catered for anchorage-dependent cells such as fibroblasts [14]. Several other groups reported techniques of direct cell encapsulation into microspheres using either synthetic polyethylene glycol diacrylate [12,15], which requires surface modification and addition of enzyme-degradation sites, or natural biopolymers such as alginate [16], which possess batch-to-batch variation as well as uncontrollable degradation rates [17].

This study aimed to overcome the above-mentioned pitfalls of current technology through pioneering a one-step fabrication technique to encapsulate cells within microspheres. A simple and non-toxic water-in-oil single emulsion technique involving cell–gelatin suspension and soya oil was developed for manufacturing gelatin microspheres with non-anchorage-dependent cells: in this case chondrocytes. Gelatin microspheres would have then dissolved completely given a time window of 2 days at 37 °C incubation; the microspheres were therefore named temperature-cured dissolvable gelatin microsphere-based cell carriers (tDGMC).

tDGMC is designed as a versatile platform for the delivery of non-anchorage-dependent cells such as hepatocytes, pancreatic

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β-cells, chondrocytes and pluripotent stem cells, all of which are naturally cell aggregate-forming [18–23]. As chondrocytes were used in this experiment, tDGMC were used by combining with previously established phase transfer cell culture (PTCC) technology [24] to ultimately construct a 3-D scaffold-free living hyaline cartilage graft (LhCG) [25]. Briefly, blank gelatin microspheres and chondrocytes are co-encapsulated in an alginate gel phase, in which the gelatin microspheres act as porogens. Chondrocytes tended to proliferate into and fill up the cavities left behind by gelatin to form neotissue within, by means of PTCC phenomenon [24]. Given time, the neotissue islets further develop into the alginate gel bulk as well and merge with neighbouring islets to form a macroscopic cartilaginous construct whose structural integrity is no longer dependent on alginate. Alginate can be removed via chelation (immersion in sodium citrate (SC) solution) to obtain LhCG that is composed only of chondrocytes and their secreted extracellular matrix (ECM) [25]. Through implementation of tDGMC technology upon the PTCC system, the present authors aimed to accelerate the LhCG formation process to greatly convenience the clinical setting as a replacement for arthritic and damaged articular cartilage, in which tDGMC play two roles: creating cavities for better nutrient and waste diffusion and space for cell growth, as in the established PTCC system, while also delivering additional cells into the gel so as to accelerate development.

2. Materials and methods

2.1. Fabrication and size characterization of tDGMC

Gelatin solution (5% w/v) was prepared by dissolving 0.50 g gelatin (gelatin type A from porcine skin; Sigma) in 10 ml solution consisting of 5 ml phosphate buffered saline (PBS) and 5 ml chondrocyte medium (CC medium). The composition of CC medium is as follows: DMEM Glutamax (Gibco) with 20% v/v FBS Gold (Gibco), 0.4 mM proline, 0.01 M 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid, 0.1 mM non-essential amino acids, 0.05 mg ml^{-1} vitamin C, 100 mg ml^{-1} streptomycin and 100 units ml^{-1} penicillin. Passage 1 porcine chondrocytes were suspended in 37 °C gelatin solution at a concentration of 1×10^7 cells ml^{-1} gelatin. The suspension was added into a 50 ml beaker containing 15 ml soya oil (filtered and pre-warmed to 37 °C) and stirred for 2 min at 500 rpm at room temperature. The beaker was then transferred into an iced water bath and stirred for 10 min at 300 rpm. The emulsion was centrifuged at 700 rpm for 3 min. After removing the supernatant, the pellet of gelatin microspheres with chondrocytes encapsulated within, named tDGMC, was resuspended in 15 ml 4 °C 1 × PBS for washing. The suspension was centrifuged at 700 rpm for 3 min and washed again. A brief schematic of the procedures, which were done under sterile conditions, is shown in Fig. 1A. A list of abbreviations used in sample naming is given in Table 1.

For quantification of size distribution, tDGMC were suspended in a suitable amount of 1 × PBS in a 100 mm petri dish. 20 random images were taken under light microscopy (Carl-Zeiss) for determination of size distribution.

2.2. Fabrication of 3-D constructs

2.2.1. Fabrication of PTCC-tDGMC constructs

PTCC-tDGMC constructs were fabricated as shown in Fig. 1B. First, both 1×10^7 of passage 1 porcine chondrocytes and 0.30 g tDGMC were mixed into 1 ml alginate solution (1.5% w/v alginic acid dissolved in 0.15 M NaCl). Then, 80 μl of suspension was injected into each cylindrical silicon mould cavity, and the moulds were placed on a 100 mm petri dish pre-coated with $CaCl_2$-containing gelatin substrate (15% w/v gelatin and 102 mM $CaCl_2$ in distilled water). Each mould cavity was of 7 mm in diameter by 2 mm deep. For gelation of alginate to occur, the dish was placed in 4 °C for 4 min, after which 15 μl 102 mM $CaCl_2$ was gently added to the top surfaces of each construct and placed in 4 °C for 4 min again. Upon incubation at 37 °C, gelatin dissolved completely within 2 days, leaving behind chondrocytes suspended within the cavities, while chondrocytes were encapsulated within the alginate gel bulk. Suspended chondrocytes proceeded to proliferate into cell islets in the pores, while those in the alginate gel bulk proceeded to infiltrate the pores as well, forming neotissue consisting of chondrocytes and their secreted ECM. Given time, the neotissues continued developing and merged with each other.

2.2.2. Fabrication of PTCC-blkMC constructs

Blank (acellular) gelatin microspheres (blkMC) were prepared by an oil-in-water-in-oil double emulsion method as previously described [25]. Briefly, 30 ml 10% w/v gelatin solution (preheated to 70 °C) was added to a 100 ml beaker containing 10 ml ethyl acetate and stirred at 700 rpm for 1 min. The gelatin/ethyl acetate emulsion was transferred to another 100 ml beaker containing 60 ml soya oil and stirred at 350 rpm for 1.5 min. Dioxane and acetone were used to wash the suspension three times to remove the soya oil. Finally, the gelatin microspheres were dried in a 70 °C oven and sieved for size quantification.

PTCC-blkMC constructs were then fabricated using blank gelatin microspheres, as previously described [25]. Briefly, wetted blank microspheres of diameters <200 μm were cosuspended with 1×10^7 of passage 1 porcine chondrocytes in 1 ml alginate solution at a concentration of 0.3 g ml^{-1}, and gelled as above (Fig. 1C). Upon culture at 37 °C, the gelatin dissolved and left behind pores within the constructs. Chondrocytes within the alginate gel bulk naturally proliferated towards the cavities and proceeded to fill up the pores with neotissue.

2.2.3. Fabrication of blkGEL-tDGMC constructs

tDGMC was suspended in alginate solution (without mixing with cells) at a concentration of 0.30 g ml^{-1} alginate, injected into moulds and gelled as above. Blank alginate gel (blkGEL)-tDGMC constructs were used solely to observe the proliferation and viability of cells that had undergone the tDGMC technique.

2.2.4. Acquiring of scaffold-free constructs of LhCG

Each construct of PTCC-blkMC or PTCC-tDGMC was placed in a 15 ml tube containing 5 ml SC solution (55 mM in 0.15 M NaCl) for 10 min at room temperature to remove the alginate phase by dissolving and washing-off. This was done at weekly time points: days 14, 21, 28 and 35. After SC treatment, if the physical integrity was maintained from collapse, PTCC-blkMC and PTCC-tDGMC constructs were renamed LhCG-blkMC and LhCG-tDGMC, respectively.

All constructs were cultured in CC medium with gentle shaking (every alternate 12 h) on an orbital shaker at 50 rpm, 5% CO_2 and humidity at 37 °C.

2.3. Cell viability assays

The cell viability of the PTCC-blkMC and PTCC-tDGMC constructs was quantified by means of WST-1 assay (Roche, Switzerland). Briefly, each sample was incubated in 10% v/v WST-1 reagent in CC medium for 1.5 h in the dark at 37 °C, 5% CO_2. Medium was transferred to 96-well plate (Iwaki) and absorbance was measured at 450 nm against 690 nm reference absorbance by a microplate spectrum reader (Multiskan® spectrum, Thermo). Live/Dead staining (Invitrogen) was also used.