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In-situ birth of MSCs multicellular spheroids in poly(L-glutamic acid)/chitosan scaffold for hyaline-like cartilage regeneration

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ABSTRACT

The success of mesenchymal stem cells (MSCs) based articular cartilage tissue engineering is limited by the presence of fibrous tissue in generated cartilage, which is associated with the current scaffold strategy that promotes cellular adhesion and spreading. Here we design a non-fouling scaffold based on amide bonded poly(L-glutamic acid) (PLGA) and chitosan (CS) to drive adipose stem cells (ASCs) to aggregate to form multicellular spheroids with diameter of 80–110 μm in-situ. To illustrate the advantage of the present scaffolds, a cellular adhesive scaffold based on the same amide bonded PLGA and CS was created through a combination of air-drying and freeze-drying to limit the hydration effect while also achieving porous structure. Compared to ASCs spreading along the surface of pores within scaffold, the dense mass of aggregated ASCs in PLGA/CS scaffold exhibited enhanced chondrogenic differentiation capacity, as determined by up-regulated GAGs and COL II expression, and greatly decreased COL I deposition during *in vitro* chondrogenesis. Furthermore, after 12 weeks of implantation, neo-cartilages generated by ASCs adhered on scaffold significantly presented fibrous matrix which was characterized by high levels of COL I deposition. However, neo-cartilage at 12 weeks post-implantation generated by PLGA/CS scaffold carrying ASC spheroids possessed similar high level of GAGs and COL II and low level of COL I as that in normal cartilage. The *in vitro* and *in vivo* results indicated the present strategy could not only promote chondrogenesis of ASCs, but also facilitate hyaline-like cartilage regeneration with reduced fibrous tissue formation which may attenuate cartilage degradation in future long-term follow-up.

1. Introduction

As a typical hyaline cartilage, articular cartilage is constituted of a specialized extracellular matrix (ECM), including collagen type II (COL II) and proteoglycans, that contributes to the unique biomechanical properties of articular cartilage [1]. Natural articular cartilage lacks self-healing capacity due to poor blood supply and innervation, making it difficult to deal with damages [2]. Tissue engineering exhibits great promise in restoration of injured cartilage in both structure and function [3]. However, as demonstrated in numerous reports, most of the neo-generated cartilage exhibited fibrocartilage features, expressing more collagen type I (COL I) but less COL II, also showing inferior capacity to support high dynamic compressive loads. Thus, generating engineered cartilage with more hyaline cartilage features other than fibrocartilage still remains a challenge [4].

During chondrogenesis in the embryonic vertebrate limb, one critical event is the “condensation” of mesenchymal cells with rounded profile, which triggers the chondrogenic differentiation of progenitor cells [5,6]. Techniques currently used to start *in vitro* chondrogenesis of mesenchymal stem cells (MSCs) share a cell aggregation step as a prerequisite for commitment to the chondrogenic lineage, in order to duplicate “condensation” that takes place *in vivo* during limb development [7,8]. The dense mass of aggregated MSCs creates an environment with strong cell–cell interactions that promotes the immediate differentiation of MSCs into chondroblasts, and displays enhanced differentiation capacities upon induction [9]. Such cellular aggregates are achieved through the hand drop technique [10], centrifugation [11] and continuous agitation of a suspension culture [7,12]. As for materials, low dimensional materials including cell repulsive substrates such as chitosan (CS) film [9,13,14], magnetic nanoparticles [15] and biomimicking nanofilaments [16] were proposed to induce

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spherical aggregate formation for further research and application. Nevertheless, little attention has been focused on the in-situ formation of spherical aggregates inside of three dimensional (3D) porous scaffolds, especially for the application of aggregate/scaffold complexes in stem cell-based cartilage regeneration. Most scaffolds supported MSCs adhesion and spreading, possessing no mimic of "condensation", thus encouraging fibrous matrix production [4,17]. To mitigate the challenges associated with current design strategies for cartilage tissue engineered scaffolds, a new strategy was introduced in this paper to enhance the chondrogenesis of MSCs and further promote hyaline-like cartilage regeneration. This was achieved through constructing a kind of porous scaffold that could weaken cellular adhesion while supporting in-situ birth of multicellular spheroids.

Accordingly, when the adhesive force between cell and substrate is lower than that between cells, spontaneous cellular aggregation will be achieved [18]. Meanwhile, cellular adhesion was dominated by the protein adsorption of substrate under physiological conditions with presence of serum proteins [19]. Thus, protein repulsive technology could be employed to create a scaffold that encourages spheroid formation. Commonly used methods to resistant protein adsorption are to coat substrate with polyethyleneglycol (PEG) polymers, oligoethyleneglycol self-assembled monolayers (OEG-SAMs) and polyelectrolyte multilayer films, which share common structural and chemical characteristics: a hydrophilic nature, and being hydrogen bond acceptors/donors [20–22]. It was hypothesized that the non-fouling ability of these materials was attributed to the hydration layer, a tightly bound water layer formed via either hydrogen-bond or electrostatic induction [22]. Upon these materials, biodegradable polyelectrolytes with biomimetic characters and biodegradation are favorable candidates for scaffold construction [22].

Thus in the present study, two kinds of polyelectrolytes with opposite charges, poly(L-glutamic acid) (PLGA) and CS, were employed to develop the protein repulsive porous scaffold for cartilage tissue engineering. PLGA was cross-linked with CS through amide bonds to create a hydrophilic network, followed by freeze-drying to yield a porous scaffold. The formation of adipose derived stem cell (ASC) spheroids within pores of PLGA/CS scaffold was evaluated *in vitro*. The enhancement of ASCs chondrogenesis and hyaline-like cartilage regeneration, as well as limitation of fibrous matrix *in vivo* were detected in order to illustrate the advantage of the present cartilage scaffold design strategy (Fig. 1).

2. Materials and methods

2.1. Preparation of PLGA/CS 3D porous scaffolds

PLGA (viscosity-average molecular weight $Mv = 10 \times 10^4$, prepared from poly (γ-benzyl-L-glutamate) which was synthesized by the ring-opening polymerization of the N-carboxyanhydride of γ-benzyl-L-glutamate), was dissolved in a low concentration of sodium hydroxide solution to form homogeneous solution. Acetic acid was then used to adjust the pH of the solution to 6, followed by adding N-Hydroxysuccinimide(NHS) and by1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride(EDC) in turn to activate the γ-carboxyl of PLGA. After stirring for 8 h, the solution was mixed with chitosan solution (chitosan, $Mv = 4 \times 10^4$, DA ≤ 5%, purchased from Jinan Haidebei Marine Bioengineering Corp; dissolved by adding acetic acid until the pH reached at 4.5) to form a homogeneous and clear mixture. One minute later, the mixture was solidified to form a hydrogel. After dialysis and lyophilization, a porous scaffold with sponge-like structure was obtained. In this paper, the mole ratios of γ-carboxyl group and amino group were set as 1:1 to ensure the consumption of carboxyl and amino groups. The polymer concentration was 0.5% and 3%. The freezing temperatures were set as −20 °C and −80 °C.

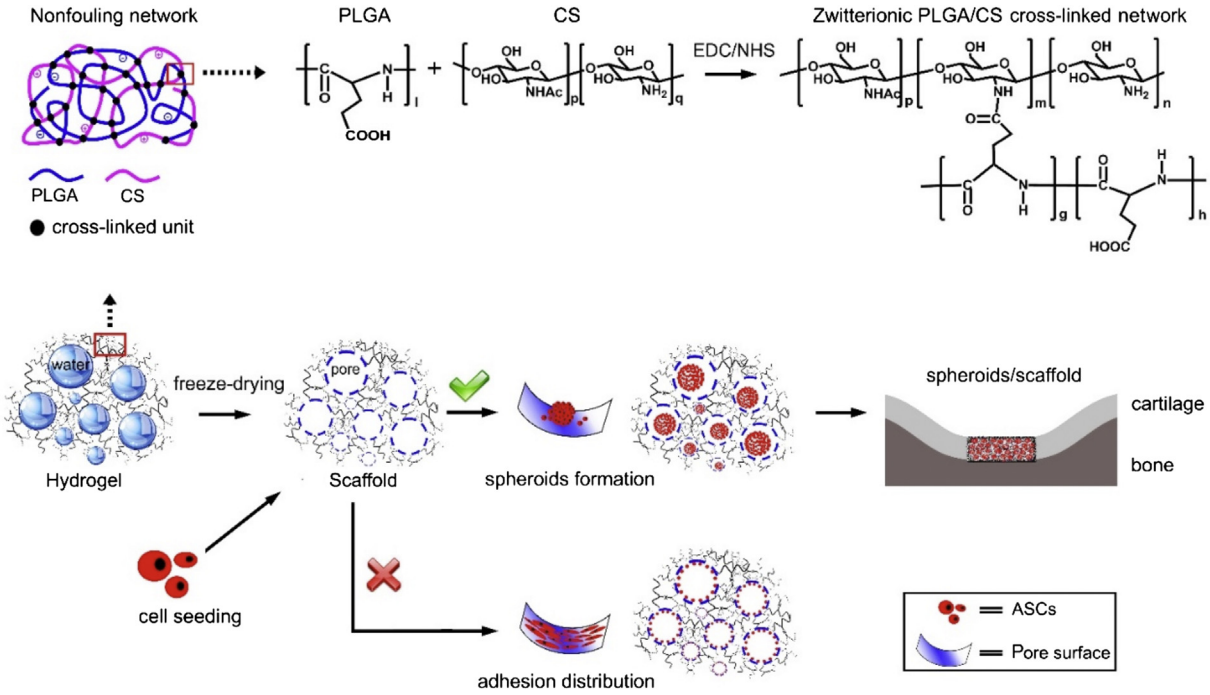

Fig. 1. A schematic of the present scaffold design strategy for cartilage regeneration. Non-fouling polyzwitterionic material was based on PLGA and CS. EDC associated with NHS possesses high reaction efficiency, but 100% of cross-linking efficiency is unable to achieve. Freeze-drying method was employed to create the porous structure and surfaces for ASCs contact. The new strategy proposed in this paper was to construct a scaffold that could encourage spontaneous multicellular spheroid formation to regenerate more hyaline-like cartilage.