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Bilayered HA/CS/PEGDA hydrogel with good biocompatibility and self-healing property for potential application in osteochondral defect repair

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ABSTRACT

The fabrication of osteochondral tissue engineering scaffolds comprised of different layers is a big challenge. Herein, bilayers comprised of double network hydrogels with or without nano hydroxyapatite (HAp) were developed by exploiting the radical reaction of poly(ethylene glycol) diacrylate (PEGDA) and the Schiff-base reaction of N-carboxyethyl chitosan (CEC) and oxidized hyaluronic acid sodium (OHA) for osteochondral tissue engineering. The bilayered osteochondral scaffold was successfully fabricated based on the superior self-healing property of both hydrogels and evaluated by scanning electron microscopy, macroscopic observation and mechanical measurements. In addition, the hydrogels exhibited good biocompatibility as demonstrated by the *in vitro* cytotoxicity and *in vivo* implantation tests. The results indicated that the bilayered hydrogel had great potential for application in osteochondral tissue engineering.

1. Introduction

For treating of osteochondral defects, arthroscopic debridement and microfracture are palliative clinical therapies, while osteochondral grafting and autologous chondrocyte implantation (ACI) are more effective treatments to restore hyaline cartilage [1,2]. However, problems such as limited donor source, two-step surgeries and immune response limit the use of these techniques. In recent years, tissue engineering has attracted considerable attention and is a promising alternative to osteochondral reconstruction [3,4]. For example, Brien and coworkers developed a multi-layered collagen-based scaffold according to the osteochondral structure, and the results showed that well-structured subchondral trabecular bone and hyaline-like cartilage were formed in the caprine joints after 12 months implantation[5]. The repair process of osteochondral tissue requires simultaneous regeneration of cartilage and subchondral bone because the defects always involving damages of both cartilage and subchondral bone [6,7]. Due to their dissimilar regeneration characteristics and abilities, it is still a big challenge in clinic [8,9].

The importance of subchondral bone in cartilage regeneration has been realized [10]. There has been increased evidence that subchondral bone plays a key role in the initiation and progression of osteoarthritis, and cartilage defects are difficult to repair without the support and fixation from subchondral bone [11–14]. Therefore, a lot of studies focus on the design of novel bilayered or multilayered osteochondral scaffolds containing both cartilage and subchondral bone structures. Due to heterogeneous layers in these scaffolds, the union of the chondral and subchondral bone layers is crucial to the construction of osteochondral scaffolds [15,16]. Currently, many strategies, such as 3D printing [17,18], freeze-drying [7,8,19], UV photo-crosslinking [4,20], stainless steel pin assem-

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bling [21], suturing [22] and fibrin bonding [23], were utilized to fabricate bilayered osteochondral scaffolds. However, there are also some drawbacks through these strategies, especially the integration, which may not be strong enough to withstand the shear stress from joint movement when the scaffold degraded quicker than tissue regeneration [24–26]. Hence, innovative fabrication techniques should be explored to improve the integration of chondral and subchondral bone layers.

Hydrogels, with the characteristics of high water content and three-dimensional network structure, are very similar to the extracellular matrix (ECM) of natural cartilage, which is conducive to cell growth [27–30]. Self-healing hydrogels based on dynamic covalent bonds (disulfide bonds [31], imine bonds [32,33], acylhydrazone bonds [34], phenylboronate ester complexations [35,36], Diels-Alder reactions [37]) and non-covalent bonds (hydrogen bonds [38–41], host-guest interactions [42,43], hydrophobic interactions [44], electrostatic interactions [45]) have attracted great attention because of their capabilities of maintaining the integrity of network structures, recovering the mechanical properties and extending service life. Among these interactions, hydrogels fabricated from dynamic imine bonds can self-heal automatically at physiological conditions without requiring additional stimuli, which are appropriate for tissue engineering [46–48]. However, rapid degradation resulted from the unstable dynamic bonds makes self-healing hydrogels difficult for osteochondral tissue engineering.

In this study, we develop a double network hydrogel for the chondral layer (designated as SC hydrogel) based on N-carboxyethyl chitosan (CEC), oxidized hyaluronic acid sodium (OHA) and poly (ethylene glycol) diacrylate (PEGDA). Meanwhile, hydroxyapatite (HAp) was introduced into the hydrogel to prepare the subchondral layer (designated as SS hydrogel). Due to the good self-healing property between these two hydrogels, we can easily fabricate the bilayered osteochondral scaffold (designated as SO hydrogel) by combining fresh surfaces of both hydrogels. To the best of our knowledge, this work is the first report to use intrinsic self-healing property of hydrogels to prepare osteochondral scaffold. Herein, this bilayered hydrogel suggests the potential to be applied in osteochondral defect repair.

2. Materials and methods

2.1. Materials

Hyaluronic acid sodium (HA) ($M_w = 1.1 \times 10^6$) was purchased from Shanghai Yuanye Biological Technology Co., Ltd. Chitosan (CS) (≥95% degree of deacetylation) was purchased from Shanghai Macklin Biochemical Co., Ltd. HAp (<100 nm particle size) was purchased from Shanghai Aladdin Industrial Corporation. PEGDA ($M_n = 700$), *N,N,N′,N′*-tetramethylethylenediamine (TEMED) and ammonium persulfate (APS) were purchased from Sigma-Aldrich. Live/dead cell viability kit was purchased from Life Technologies. Cell counting kit-8 (CCK-8 kit) was obtained from Boster Biological Technology. Rabbit bone mesenchymal stem cell (BMSC) growth media was purchased from Cyagen Biosciences Inc.

2.2. Synthesis of CEC

CEC was prepared according to the previous method [49]. Briefly, acrylic acid (1.46 mL) and chitosan (1.0 g) were added in 50 mL deionized water, and the mixture was magnetically stirred at 50 °C for 3 days. After adjusting the pH to 10–12 by NaOH solution (1 M), the solution was dialyzed (MWCO 8000) against deionized water for 3 days. Finally, the product CEC was obtained after lyophilizing.

2.3. Synthesis of OHA

HA (1.0 g) was dissolved in 100 mL distilled water, and then sodium periodate (0.535 g) was added. After magnetically stirred in the dark for 3 h, the reaction was terminated by adding ethylene glycol (1.5 mL) and stirred for additional 1 h. Then, the mixture was dialyzed (MWCO 3500) against deionized water for 3 days. The product OHA was obtained after lyophilizing. The degree of oxidation was quantified by the iodometry through determining the unconsumed periodate after the oxidized reaction according to a previous method [50]. Briefly, the reaction mixture (5 mL) was neutralized with 10% sodium bicarbonate solution (10 mL), followed by the addition of 20% potassium iodide solution (2 mL). The solution was kept under dark for 30 min, and then the liberated iodine was titrated with standardized sodium thiosulphate solution (0.01 M) using 1% starch (1 mL) as the indicator.

2.4. Characterization of CEC and OHA

^{1}H NMR (Bruker, Germany, 600 MHz, D_2O) was used to determine the grafting degree of CEC. Fourier transform infrared (FTIR) spectrum of CS, CEC, HA and OHA were recorded on a Bruker Vector 33 FTIR spectrometer using the KBr pellet method.

2.5. Preparation of SC and SS hydrogels

The precursor solutions were prepared by dissolving CEC (3%, w/v) and OHA (5%, w/v) in PBS buffer (pH = 7.4) separately. 200 μL PEGDA was added into 1 mL OHA solution, and 10 μL TEMED and 50 μL APS (10%, w/v) was added into 1 mL CEC solution. After dissolving, these two solutions were further mixed uniformly by vortex and homogeneous SC hydrogel was obtained. As for the SS hydrogel, HAp was added into the precursor solutions and then the mixture was treated with ultrasound to disperse HAp evenly to obtain HAp concentration of 1% (w/v). The process of SS hydrogel formation was the same to the preparation of the SC hydrogel.

2.6. Macroscopic self-healing property of PEGDA, SC and SS hydrogels

PEGDA hydrogel was obtained by mixing 2 mL PEGDA, 10 μL TEMED and 50 μL APS. Two pieces of PEGDA, SC and SS hydrogels were prepared, and one of each kind of hydrogels was stained with rhodamine B. All the hydrogels were cut into 4 pieces, respectively, and each kind of hydrogels was recombined with alternate color pieces together for 2 h at ambient temperature. Then all the healed hydrogels were immersed into PBS for 24 h for checking their self-healing performance.

2.7. Rheological measurements

The SS hydrogel disk (25 mm in diameter) was measured under strain amplitude sweep at a fixed frequency (1 Hz). The alternate step strain sweep of SC and SS hydrogel disks (25 mm in diameter) were measured at a fixed frequency (1 Hz). Amplitude oscillatory strains were switched from small strain (γ=1.0%) to subsequent large strain (γ=100%) with 120 s for every strain interval.

2.8. Preparation of the bilayered osteochondral scaffold

A bilayered osteochondral scaffold that mimicked the cartilage and subchondral bone was prepared by self-healing property of the SC and SS hydrogels in this study. SC and SS hydrogels were transversely cut and the fresh surfaces were immediately combined together to form the bilayered osteochondral scaffold. The

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