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Injectable self-crosslinking HA-SH/Col I blend hydrogels for *in vitro* construction of engineered cartilage

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ABSTRACT

The injectable self-crosslinking blend hydrogel by combination of collagen I and thiolated hyaluronic acid could alleviate collagen I contraction *in vitro* and overcome weak cell adhesive sites of hyaluronic acid. Five groups of injectable hydrogels with different ratios were prepared to investigate their gelation time, injection force, mechanical properties, swelling capacity and disintegration performance. These results indicated that $Col_7HA\text{-}SH_3$ hydrogel achieved the optimal controlled and injectable effect, the gelation time was just ten seconds with injection force at 3.5 N, and the storage modulus of hydrogel could reach 11 kPa with frequency at 10 Hz. Furthermore, the phenotype maintaining, biocompatibility and chondrocytes proliferation were administrated by CLSM, SEM, histological staining, immuohistochemical staining, MTT test and glycoaminoglycans quantification. Similarly, the $Col_7HA\text{-}SH_3$ blend hydrogel encapsulated chondrocytes presented most excellent proliferation potential, phenotype maintaining, biocompatibility and convenient operational characteristics. These findings might approach the underlying clinical application of blend hydrogel in cartilage repair.

1. Introduction

Articular cartilage injury is an intractable clinical problem for a long time, which has low self-repair capability due to shortage of vascular tissue, innervation and lymphatics (Li, Teng et al., 2017; Sophia Fox, Bedi, & Rodeo, 2009). The current treatment relies mainly on autologous chondrocytes transplantation, which is limited by insufficient donor supply and easy dedifferentiation during culture (Huey, Hu, & Athanasiou, 2012). Tissue engineering, as one of the most front-line technologies, has been attracting significant attention for cartilage repair over the past 20 years (Boushell, Lu, Hung, Hunziker, & Strauss, 2016; Shafiee & Atala, 2017). The principle is that high concentrations of seeded chondrocytes obtained from *in vitro* expension are planted into scaffold materials with growth factor. They can form new cartilage tissue after implantation into human body so as to achieve the goal of cartilage repair and reconstruction (Chen et al., 2017). Therefore, an ideal scaffold is vital to successfully reconstruct defected cartilage (Yang, Chen, & Wang, 2009). Hydrogels help to maintain the round or oval phenotype of chondrocytes, which are similar to the morphology in the natural chondric extracellular matrix of chondrocytes (Wu, Dong, Li, Wang, & Cao, 2017; Xu, Yuan, Han, Lin, & Zhang, 2017). Therefore, they have been widely applied in cartilage tissue engineering and demonstrated magnificent prospects (Gorczyca et al., 2014; Karunanithi et al., 2016; Liu, Zeng et al., 2017; Liu, Zuo et al., 2017; Yang, Zhang, Yue, & Khademhosseini, 2017). Hydrogel is conducive to the exchange of nutrients and metabolic activity because of its good permeability (Guo, Lei, Li, & Ma, 2015). The cells can be 3D wrapped uniformly in the hydrogel scaffold environment (Zhang, Mujeeb, Du, Lin, & Ge, 2015). Herein, in the study of cartilage tissue engineering, the matrix components of chondrocytes are widely used.

Hyaluronic acid (HA) and collagen are key component of cartilage extracellular matrix (Kim, Mauck, & Burdick, 2011). Collagen, as an important component of cartilage tissue, is also a structural protein of extracellular matrix (ECM). Studies have shown that Col I not only has good biocompatibility and biodegradability, but also contain a large number of cell adhesion sites (Kleinman, Klebe, & Martin, 1981; Li, Chen, Kawazoe, & Chen, 2017). However, scaffolds fabricated from only Col I exist the shortcomings of poor mechanical strength, rapid degradation, and immunogenicity (Elisseeff et al., 2000; Mredha et al., 2017). In addition, Col I tends to severely contract after long time culture with cells *in vitro* (Palumbo, Pitarresi, Mandracchia, Tripodo, & Giammona, 2006). Similarly, HA is also an essential component of the

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ECM, in which its structural and biological properties mediate its activity in cellular signaling, wound repair, morphogenesis, and matrix organization (Collins & Birkinshaw, 2013; Toole, 2004). HA hydrogels have good biocompatibility and promote cartilage formation (Park et al., 2017; Zhu & Lou, 2006), but it inhibits cell attachment due to the lack of cell adhesion sites, as well as the hydrophilic and anionic properties. It is reported that cells prefer to selectively adhere to neutral or cationic interface and hydrophobic materials (Lam, Truong, & Segura, 2014; Zustiak, Wei, & Leach, 2013). These defects seriously restrict its clinical application in cartilage tissue engineering.

Therefore, in view of the respective advantages and disadvantages of Col I and HA, HA/Col I blend scaffolds have been extensively studied (Kim, Chung, & Park, 2008; Muzzarelli, Greco, Busilacchi, Sollazzo, & Gigante, 2012). Ideally, these blend scaffolds qualify the characteristics of better simulating native cartilage matrix, good biocompatibility, promoting cell proliferation and enhancing the mechanical properties of scaffolds (Wodewotzky et al., 2012). Bian et al. synthesized photo-cross-linkable hybrid hydrogels using methacrylated HA, chondroitin sulfate and type I Col, and studied the decoupled function of these cues in regulating the initial chondrogenesis, hypertrophy, and tissue mineralization by Human mesenchymal stem cells (hMSCs) (Zhu, Feng, Sun, Li, & Bian, 2017). It could be to upregulate mRNA expression of the chondrogenesis and decrease expression of the hypertrophic markers. Heilshorn and coworkers synthesized adaptable elastin-like protein-hyaluronic acid hydrogel platform by dynamic hydrazone bonds (Zhu, Wang, Trinh, Heilshorn, & Yang, 2017). The results indicated that the cartilage marker gene expression and sulfated-glycoaminoglycan (sGAG) deposition could be upregulated and enhanced with increasing HA concentration. Martin et al. reported that fibrin/hyaluronic acid hydrogel networks loaded recombinant human stromal cell–derived factor 1α to achieve functional repair of full-thickness bovine articular cartilage *via* homing of chondrogenic progenitor cells. The matrix formed by these cells was analogous in composition to native cartilage, and strongly adhered to surrounding tissues with possesses mechanical properties (Yu et al., 2015). However, there are still a lot of unsolved problems. For example, the longer gellation time does not meet the clinical requirements, blend hydrogel scaffold cannot gelled *in situ* (Dinescu et al., 2013; Zhang et al., 2011), relevant crosslinking agents increase the toxicity of scaffold materials (Koh, Jin, Kang, & Hwang, 2017; Li, Rodrigues, & Tomás, 2012; Omlor et al., 2012; Sacco et al., 2016), exogenous proteins produce immune risks.

In this study, we introduced thiolated hyaluronic acid (HA-SH) into Col I hydrogels to prepare five kinds of self-crosslinking HA-SH/Col blend hydrogels with different masses proportions (Bian et al., 2016; Oh et al., 2010). The HA-SH hydrogel was formed *via* the self-crosslinking process of disulfide bond derived from two free thiol groups. In the process of preparation, no chemical additives, cytotoxic crosslinking agent and byproduct were added or produced. When co-cultured with cells, the hydrogel could be disintegrated gradually by glutathione (GSH) produced by cells and provided enough space for cells proliferation. It was expected that the combination of HA-SH and Col might alleviate the problem of Col I contraction *in vitro* and overcome the lack of cell adhesive sites of hyaluronic acid. The gelation time, mechanical properties, swelling properties, disintegration properties and morphology of the blend hydrogels were investigated. Furthermore, the HA-SH/Col hydrogels encapsulated chondrocytes were fabricated as three dimensional (3D) constructures to simulate native extracellular matrix (ECM) for chondrocyte adhesion, spreading, proliferation and differentiation. The 3D constructures, after cultured *in vitro* for different time, were evaluated by confocal laser scanning microscopy (CLSM), histological staining, immuohistochemical staining and glycoaminoglycans (GAGs) quantification.

2. Materials and methods

2.1. Materials

Hyaluronic acid (HA, Cosmetic grade, Mw = 0.3 M Da) was purchased from Bloomage Freda Biopharm Corporation (Shandong, China), Type I Col I was purchased from Trauer company (Guangzhou, China), 1-Ethyl-3-(3-dimethyllaminopropyl) carbodiimide hydrochloride (EDCI, 99%), N-hydroxysuccinimide (NHS, 99%), cysteamie hydrochloride (CSA·HCl, 99%), and dithiothreitol (DTT, 99%) were purchased from Best-reagent Corporation (Chengdu, China), 5, 5′-Dithiobis-(2-nitrobenzoic acid) (DTNB, 99%) was purchased from Aladdin Corporation (Shanghai, China), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Sigma-Aldrich, α-Minimum Essential Medium (α-MEM, Hyclone), Fetal bovine serum (FBS, Gibco, Australia origin) was bought from Life Technologies Corporation (USA), Penicillin and streptomycin (Hyclone), Collagenase II (Gibco), Papain (sigma).

2.2. Fabrication of HA-SH/Col I blend hydrogel

2.2.1. Preparation and characterization of thioglycated hyaluronic acid (HA-SH)

Firstly, HA (2 g) and NHS (10 mmol, 1150 mg) were added to deionized water and stirred until formed transparent and homogeneous solution in 24 h at room temperature. Secondly, EDCI (25 mmol, 4810 mg) in solid form was added into the mixture solution and kept reacting for 2 h to activate the carboxyl group of hyaluronic acid. Thirdly, the reaction solution followed by the addition of CSA·HCl (25 mmol, 2840 mg) solution for 24 h and the value of pH was maintained at 4.75–5.0 during the reaction. Finally, the reaction solution was transferred into dialysis tube with a molecular weight cut-off of 8000–14,000 K Da and dialyzed against acidic deionized water (pH = 3.5) at room temperature for 72 h with frequent change of water. Eventually, the dialysate was freeze-dried to yield the solid HA-SH. HA-SH chemical structure was measured by ^{1}H NMR (400 MHz, Bruker AMX-400, USA), and the mercapto group content of HA-SH was determined by Ellman method (Ellman, 1959; Riddles, Blakeley, & Zerner, 1979).

2.2.2. The fabrication of HA-SH/Col I blend hydrogel

At first, the preparation of HA-SH solution and Col I solution: 15 mg HA-SH solid were weighed and dissolved in 1 mL α-MEM medium thoroughly. Similarly, pre-lyophilized Col I solid 15 mg was dissolved in 1 mL acetic acid solution (0.25 M) completely. Then, five experimental groups were prepared on the basis of different volume ratio: (1) Col I; (2) Col I: HA-SH = 7:3 (Col I_7HA-SH_3); (3) Col I: HA-SH = 5:5 (Col I_5 HA-SH_5); (4) Col I: HA-SH = 3:7 (Col I_3HA-SH_7); (5) HA-SH. Ultimately, five groups of liquid was shaken singly untill they became transparent. Finally, the blend hydrogel of five groups were adjusted to pH = 7.4 with 1 M NaOH, and immediately injected into homemade ring molds, which was made by polypropylene (diameter 8.5 mm, height 3.0 mm), and exposed to air for 30 min to form hydrogels (diameter 8.5 mm, height 3.0 mm) at 37 °C untill they could be detached from homemade ring molds.

2.3. Characterization of HA-SH/Col I blend hydrogel

2.3.1. The gelation time and morphology of HA-SH/Col I self-crosslinking hydrogel

The solution (flowable)-gel (non flowable) transition was determined by a flow test utilizing a test tube inverting method reported by Jeong et al. (Jeong, Bae, & Kim, 1999). Rheology measurements were performed to analyze accurate gelation time on a TA Discovery DHR-2 rheometer with a parallel-plate geometry (40 mm) and a 1.0 mm gap. The dynamic strain sweep was operated with constant ramp strain