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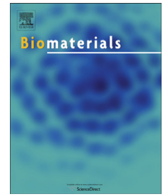

An interpenetrating network-strengthened and toughened hydrogel that supports cell-based nucleus pulposus regeneration

Yibo Gan [a], Pei Li [a], Liyuan Wang [a], Xiumei Mo [b], Lei Song [a], Yuan Xu [c], Chen Zhao [a], Bin Ouyang [a], Bing Tu [a], Lei Luo [a], Linyong Zhu [d], Shiwu Dong [e], Fuyou Li [f], Qiang Zhou [a, *]

[a] *National & Regional United Engineering Laboratory of Tissue Engineering, Department of Orthopedics, Southwest Hospital, Third Military Medical University, Chongqing 400038, PR China*
[b] *College of Chemistry and Chemical Engineering and Biological Engineering, Donghua University, Shanghai 201620, PR China*
[c] *Department of Orthopedics, Xinqiao Hospital, Third Military Medical University, Chongqing, PR China*
[d] *Key Laboratory for Advanced Materials, Institute of Fine Chemicals, East China University of Science and Technology, Shanghai 200237, PR China*
[e] *Department of Biomedical Materials Science, School of Biomedical Engineering, Third Military Medical University, Chongqing 400038, PR China*
[f] *Department of Chemistry & State Key Laboratory of Molecular Engineering of Polymers, Fudan University, Shanghai 200433, PR China*

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A B S T R A C T

Hydrogel is a suitable scaffold for the nucleus pulposus (NP) regeneration. However, its unmatched mechanical properties lead to implant failure in late-stage disc degeneration because of structural failure and implant extrusion after long-term compression. In this study, we evaluated an interpenetrating network (IPN)-strengthened and toughened hydrogel for NP regeneration, using dextran and gelatin as the primary network while poly (ethylene glycol) as the secondary network. The aim of this study was to realize the NP regeneration using the hydrogel. To achieve this, we optimized its properties by adjusting the mass ratios of the secondary/primary networks and determining the best preparation conditions for NP regeneration in a series of biomechanical, cytocompatibility, tissue engineering, and *in vivo* study. We found the optimal formulation of the IPN hydrogel, at a secondary/primary network ratio of 1:4, exhibited high toughness (the compressive strain reached 86%). The encapsulated NP cells showed increasing proliferation, cell clustering and matrix deposition. Furthermore, the hydrogel could support long-term cell retention and survival in the rat IVDs. It facilitated rehydration and regeneration of porcine degenerative NPs. In conclusion, this study demonstrates the tough IPN hydrogel could be a promising candidate for functional disc regeneration in future.

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1. Introduction

Degenerative disc disease (DDD) is closely related to the process of aging and occurs in high incidence. It is one of the main causes of neck pain, low back pain and even disability, and it seriously affects the quality of life and working ability of patients [1]. With a rapidly aging population, DDD leads to a large burden on the public health and severe socioeconomic problems [2]. The common treatments of DDD include bed rest, physiotherapy, pain relief and surgery. However, these treatments do not overcome the pathological causes [3] and even result in some side effects. Therefore, there is an urgent need to find treatment strategies for DDD that focus on its etiology.

The intervertebral disc (IVD) has a confined structure, which is divided into three distinct zones: an inner hydrated gel-like nucleus pulposus (NP), a peripheral lamellar annulus fibrosus (AF) and superior and inferior cartilaginous endplates (CEPs) [4]. Fluid pressurization of the NP plays a major role in supporting axial spinal loads [5]. However, in degenerative discs, the NP fails to perform fluid pressurization because the NP is dehydrated; this dehydration causes significantly more axial compression on the NP and AF than usual, leads to an imbalance of the stress distribution on the IVD, and subsequently results in damage to the confined structure when the AF ruptures. Several attempts have been made to suture the AF defect to restore the confined structure and prevent disc reherniation [6,7]. However, most outcomes remain undesirable because it is difficult to achieve a strong long-term AF closure. DDD is believed to originate in the NP [8,9], in which gelatinous mucoid materials are replaced by fibrocartilages. Thus,

* Corresponding author.
E-mail address: zq_tlh@163.com (Q. Zhou).

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the regeneration of the degenerative NP is the key to the treatment of DDD. Owing to the limited regenerative capacity of nucleus pulposus cells (NPCs) [10,11], it 's hard to realize the self-renewal of the native NP. Tissue engineering and regenerative medicine (TERM) strategies have emerged as a promising solution to this issue [12,13]. Hydrogels are considered as potential candidates for the tissue-engineered nucleus pulposus (TE-NP) due to their similarities to the natural NP tissue, such as their hydrophilic and rheological properties [14,15]. Numerous types of hydrogels have been investigated for NP regeneration, such as alginate [16,17], chitosan [18,19], collagen [20,21], gellan gum [22,23] and composite hydrogels [24–26]. It has been illustrated that the ideal hydrogel for NP regeneration should satisfy the following requirements: it should 1) be injectable; 2) have a proper gelation rate to prevent leakage of the cells or gel after implantation, 3) have a high mechanical strength and suitable degradation rate, 4) it should provide swelling pressure at different loadings, 5) support cell proliferation and matrix deposition of encapsulated cells, and 6) be biocompatible to prevent adverse biological effects after implantation [27]. The NP bears highly compressive axial loads that are up to 70% of the exerted load on the spine and, in particular, it is subjected to higher compressive strains due to greater motion and disc space collapse during late-stage DDD [28]. Therefore, the mechanical properties are assumed to be the key parameters of hydrogels [29]. To achieve long-term regeneration, the implant should be tough enough to bear the axial compression in the early phase of regeneration, thus providing mechanical support and maintaining a three-dimensional (3D) template in the early stage of NP regeneration. Therefore, the toughness is an important mechanical criterion for assessing scaffolds for NP regeneration. However, most applied hydrogels are mechanically weak [30], which results in the collapse or even the bulging out of the structure during long-term compression and an inability to spatially and temporally support the NP tissue formation.

Several methods have been used to enhance the mechanical properties of hydrogels, e.g., fiber reinforcement; however, some ideal properties of the original materials were modified. Thus, it is difficult to improve the mechanical strength while retaining all the ideal properties of hydrogels, including their bioactivity and injectability, which limits the use of hydrogels for NP tissue engineering applications. Interpenetrating polymer network (IPN) hydrogels could be an attractive solution to this issue. IPN hydrogels are a type of unique “alloys” of crosslinked polymers. The synergetic effect of the different networks enables the IPN hydrogels to combine various advantages of the composite materials. Meanwhile, the toughness of the hydrogels can be tuned if the IPN hydrogel contains two types of independent networks, a rigid network and a flexible network, which makes IPN hydrogels especially suitable for load-bearing tissues. Previously, we prepared an IPN hydrogel composed of oxidized dextran (Odex), amino-modified gelatin and 4-arm poly-(ethylene glycol)-acrylate (4A-PEG-acr) [30]. This IPN hydrogel exhibited favorable cytocompatibility and feasible injectability. The most significant advantage was that the IPN hydrogel consisted of rigid 4A-PEG-acr networks and soft dextran/gelatin (Dex/Gel) networks, which made it possible to toughen the hydrogel by optimizing the ratio of the two networks. Before exploiting these gels for NP regeneration, it is necessary to improve the toughness and maintain the cytocompatibility.

The overall objective of this study was to realize the regeneration of the NP using the IPN hydrogel with an effective cell-based strategy. To achieve this, we optimized the mechanical properties and cytocompatibility of the IPN hydrogel by adjusting the mass ratio of two types of networks and determined the best preparation conditions that were most suitable for NP regeneration. First, the hydrogels were evaluated in terms of their mechanical properties (compression and shear) to achieve optimal performance. Second, the microstructures and hydration of the IPN hydrogel were examined. Third, the *in vitro* viability, proliferation, and extracellular matrix (ECM) deposition of the NPCs-hydrogel hybrid were evaluated. Finally, we explored the repair of the IVD *in vivo* by injecting the NPCs-hydrogel hybrid into NP defects of rats and porcine.

2. Materials and methods

2.1. Sample preparation

2.1.1. Cell isolation and culture

The animal use followed the guidelines of the local animal ethics committee [SYXK (YU) 2012-0012]. Primary NPCs were isolated from the IVDs of porcine (n = 5, male, aged ~3 months) from the Experimental Animal Center of Third Military Medical University, as previously reported [31]. In summary, the IVDs were obtained from the pigs until euthanasia via an overdose of pentobarbital (Aituo Chemical, Shenzhen, China) by intravenous injection, and then, the NP tissues were separated within 1 h after the surgery. The tissues were minced as finely as possible and treated with collagenase II (Sigma-Aldrich, USA) for 30 min under gentle shaking. After washing and centrifuging, the isolated cells were cultured in complete cell culture media [a DMEM/F12-based culture medium containing 10% FBS and 1% penicillin/streptomycin] in 5% CO_2 at 37 °C. Only for the *in vivo* study of rats in section 2.5.2, NPCs were isolated from lumbar discs and tail discs of Sprague-Dawley rats during CO_2 euthanasia (n = 10, male, aged ~9–12 weeks) in a similar manner. The primary passage 2 (P2) NPCs of porcine and rat presented adherently oval or spindle appearance, and were collected for the following experiments.

2.1.2. Preparation of Odex, amino-modified gelatin, and 4A-PEG-acr

Dextran (M_w 100,000) and gelatin were purchased from Sigma-Aldrich. Odex and amino-modified gelatin were synthesized using previously described procedures [30,32]. 4A-PEG-acr (M_w 10,000) was purchased from Jemkem Technology, Beijing, China. The resulting mixture was dialyzed (MWCO 7000 for Odex and amino-modified gelatin; MWCO 3500 for 4A-PEG-acr, JingKeHongDa Biotechnology Co., Shanghai, China) against distilled water to remove unreacted materials and other by-products. Finally, the mixture was lyophilized to obtain the final products.

2.1.3. Hydrogel formation

Dex/Gel/PEG hydrogels are prepared by a modified method from a previously reported process [30]. Briefly, dextran, gelatin, and PEG solutions, and I2959 solution (final concentration of 0.1 wt %, Sigma-Aldrich) in PBS were mixed to achieve a final polymer concentration of 10% in the gel. Then the pH of the mixture was adjusted to ~ 7.3–7.4 by triethylamine (Aladdin, Shanghai, China) and HCl. Then, the mixture was injected into a cylindrical mold (Ø = 10 mm, height = 5 mm) and incubated at 37 °C for 1 min, followed by exposure to 365 nm UV light (5 W cm^{-2}, UVATA, Shanghai, China) for 1 min (Fig. 1C). Our preliminary results indicated that when the mass ratio of the secondary/primary networks reached 1:1, the ductility was elevated relative to the hydrogel with a lower primary network proportion [30]. In this study, we continued to increase the primary network proportion to improve the toughness of the IPN hydrogel. However, the toughness of the IPN hydrogel could not be improved continuously when the mass ratio of secondary/primary networks was more than 1:8. Thus, the mass ratio of secondary/primary networks was set to 1:1, 1:2, 1:4 and 1:8. For the control groups, a single-network hydrogel of PEG and a Dex/Gel hydrogel were also prepared at a concentration of 10 wt%.

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