Preparation and characterization of novel poly (vinyl alcohol)/collagen double-network hydrogels

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A B S T R A C T
Hydrogels prepared by conventional methods show poor performance in many aspects. Many double network (DN) hydrogels prepared by crosslinking agents have disadvantages such as toxicity. In this work, we prepared novel DN hydrogels with fish-originated collagen (Col) and poly (vinyl alcohol) (PVA), in which self-assembly of collagen and self-crosslinking of PVA were achieved. Infrared spectra indicated the existence of double network with chemical interactions between Col and PVA. X-ray diffraction (XRD) patterns showed that characteristic peak of freeze-thaw PVA in DN hydrogel was retained. Scanning electron microscope (SEM) images before and after degradation and swelling property tests indicated that the morphology of the hydrogel was a compact meshwork, which is consistent with the high water-retention rate. The degradation rate of the DN hydrogels was greatly enhanced from 6 to 33 kPa at a strain of 40%. This study indicates that DN hydrogels prepared by Col and PVA are an ideal biomaterial for tissue engineering.

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1. Introduction
Biomaterials with safety and biocompatibility are becoming popular in medical applications. Among biomaterials, collagen has gained much attention because of its extensive presence in the extracellular matrix of animals [1,2]. Collagen provides the main support function for cell attachment in vertebrates [3]. Collagen interacts with cells and regulates cell migration, proliferation, and survival [4,5]. Therefore, collagen has unique advantages over other polymer materials. As one application, hydrogels are a promising scaffold material because it has excellent properties of water retention and the ability to maintain a spherical morphology of encapsulated cells [6–8]. Hydrogels prepared by collagen are an ideal biomaterial for medical dressing, drug delivery, and tissue engineering [9–11]. Many researches on collagen hydrogels have been conducted. D. Choi et al. prepared layer-by-layer self-assembly collagen hydrogel using tannic acid and lignin. Doxorubicin was incorporated into the hydrogel membrane and sustained release was realized which demonstrated the potential feasibility of collagen-based hydrogels as a drug delivery system [12]. Nistor conducted research on crosslinked collagen/poly (N-isopropyl acrylamide) network which physically incorporated montmorillonite nanoparticles to adjust the properties of the stimuli-responsive hybrid systems [13]. Ma et al. prepared collagen/hydroxyapatite/alleondronate hybrid hydrogels as potential scaffolds for bone regeneration [14].

There are still challenges need to overcome for using collagen in medical applications. One challenge comes from the source of collagen. Most of collagens used in studies is extracted from terrestrial mammals, such as bovine and porcine collagens, which pose several problems such as the risk of zoonotic disease transmission [15,16] and religious restrictions [17,18]. Fish may be an alternative safer way to obtain collagen. In addition, collagen isolated from fish skin occupies 70%–80% of dry skin weight. Value-added collagen can be obtained from these low-cost sources [19,20]. Therefore, fish collagen (FC) is an excellent choice for collagen application in tissue engineering and it has exhibited similar characteristics to those of conventional collagens with a lower risk of disease transmission to humans [21–24].

Another main challenge, especially in composite collagen hydrogels, is the preparation method. There are many methods preparing composite hydrogels of collagen and PVA, including chemical and physical crosslinking. Formaldehyde, glutaraldehyde and genipin play essential roles in chemical crosslinking process. However, the toxicity of these crosslinkers will induce a toxic reaction when the hydrogel is applied on the body [25]. Compared with chemical crosslinking hydrogels, there is no such problem in hydrogels prepared by using physical crosslinking. Physical crosslinking methods, such as UV radiation and γ-radiation have been applied to prepare nontoxic hydrogels;
however, the hydrogel performances did not meet requirements when it was applied in tissue engineering.

In previous studies, DN hydrogels were prepared using double chemically crosslinking or hybrid physical/chemical crosslinking, in which covalent bonds of the first network were replaced with non-covalent bonds [26–30]. Designing a new generation of DN gels comprising two non-covalent associated networks is a promising technique. There are three advantages of DN collagen hydrogels: 1) double physical crosslinking is able to improve mechanical properties, degradability and swelling; 2) biological properties of collagen are preserved; and 3) the toxicity of chemical crosslinkers is avoided. PVA was chosen to prepare DN hydrogels with collagen because it is an excellent synthetic macromolecule, safe, and non-toxic. With good film-forming ability and mechanical properties, PVA is widely used in patches and scaffolds [31,32]. Although collagen/PVA (Col/PVA) composite hydrogels have been studied before [33–36], the DN hydrogels of collagen and PVA have not been investigated. DN hydrogels are prepared using the self-assembly and self-crosslinking capacity of collagen and PVA in our study, respectively.

2. Experimental

2.1. Materials

Pepsin-solubilized collagen (PSC) was isolated from Tilapia skin in our laboratory with general procedures established by Sun [37]. PVA (Mw 31,000–50,000 99% hydrolyzed) was purchased from Sigma (St. Louis, MO, USA). Acetic acid was purchased from Sinopharm Chemical Reagent Co., Ltd.

2.2. Preparation of DN hydrogels

The freeze-dried PSC was dissolved in acetic acid under magnetic stirring to prepare a collagen starting solution (10 mg/mL). The solution was diluted with an equal volume of PBS buffer (pH = 7.4) to obtain a final collagen-PBS blend solutions (5 mg/mL). Then, PVA solution was prepared via three steps: 1) add PVA in 100 mL of distilled water; 2) swell for 2 h in a 60 °C water bath; and 3) fully dissolved in a 90 °C water bath. The completely dissolved PVA solution was blended with the above solution by magnetic stirring for 2 h to make uniform solutions, and adjusted to pH 7.4 with 0.1 M NaOH or 0.1 M HCl at 4 °C before keeping at 30 °C in a water bath for 24 h to generate the first-layer network by self-assembly. The hydrogel was frozen at −20 °C for 8 h and thawed at room temperature. The freezing-thawing process was repeated three times to prepare the second-layer network.

2.3. FTIR characterization

Fourier-transform infrared (FTIR) analyses were performed to identify changes of collagen triple helix structure before and after gelling to understand detail structure of the links among collagen and PVA in DN hydrogels. The DN hydrogels, pure PVA solution and pure collagen solution were freeze-dried at −80 °C. The samples were ground with potassium bromide and characterized by using an ABB MB3000 MID-IR spectrometer equipped with a DTGS detector and Horizon software. Three substances in triplicate were scanned 32 times.

2.4. X-ray diffraction (XRD)

The X-ray diffraction (DMAX-2200) of DN and PVA after freeze-thaw samples were analyzed with CuKα radiation (λ = 0.154 nm). It scanned the 2θ = 5–30° an angular speed of 4° min⁻¹ to collect data.

2.5. Morphological observation

The morphology of samples was characterized using a scanning electron microscope (SEM; S-800, HITACHI, Tokyo, Japan). Samples of pure collagen hydrogels and DN hydrogels were prepared and freeze-dried at −80 °C to volatilize any acetic acid. These samples were cut into 1 × 1 cm square pieces and then coated with gold. The morphology of the samples was detected at 3.0 kV.

2.6. Permeability rate

The exact total weight of 10 mL water and centrifuge tubes was weighed accurately to four decimal places. Pure collagen hydrogels, pure PVA hydrogels and DN hydrogels with different Col:PVA ratios were prepared and air-dried at 37 °C for 48 h to obtain membranes. The pipe orifice of the centrifuge tube was sealed with the above membranes and the sealed area was measured. The centrifuge tubes were

Fig. 1. The mechanism of DN hydrogels preparation.
placed in a 37 °C incubator (LT-IBX60F, LEAD-TECH, Shanghai, China) for 24 h. The test duration and the total weight of tube with water were recorded and the permeability rate was calculated according to the following formula:

\[
\text{MVTR} = \frac{(M_1 - M_2) \times 1000 \times 24}{C_1 - C_2}
\]

\[
M_1, M_2 \text{ is the total weight (g) of the centrifuge tube and water before and after experiment, respectively; } S \text{ is the area of pipe orifice (cm}^2) \text{ while } T \text{ is the experimental duration (h).}
\]

2.7. Swelling performances

In order to investigate the swelling performances of the DN hydrogels, water content, water retention rate and swelling rate were measured, respectively.

2.7.1. Water content

Pure collagen hydrogels and DN hydrogels (diameter = 3.2 mm, thickness = 10 mm) with Col:PVA ratios of 25:75, 50:50 and 75:25 were prepared. Surface water was wiped off the hydrogels carefully. Hydrogels were freeze-dried at −80 °C for 48 h. Hydrogels before and after freeze-drying were accurately weighed and marked as M₁ and M₂, respectively. The process was repeated three times and water content was calculated according to the following formula:

\[
\text{Water content (\%) = } \frac{M_1 - M_2}{M_2}
\]

2.7.2. Water retention rate

DN hydrogels were prepared as described above. Surface water was wiped off the hydrogels carefully. Hydrogels were centrifuged at speed of 5000 RCF for 3 min. Hydrogels before and after centrifugation were accurately weighed and marked as M₁ and M₂. The process was repeated three times and the water retention rate was calculated according to the following formula:

\[
\text{Water retention rate (\%) = } \frac{M_1 - M_2}{M_1}
\]

2.7.3. Swelling rate

Pure collagen hydrogels and DN hydrogels with a Col:PVA ratio of 25:75 was prepared. The DN hydrogels were kept at 37 °C and air-dried for 48 h. The air-dried samples were immersed in PBS solution at pH 4.5, pH 7.4 and pH 9.8. One sample was removed from the solutions after a certain period time and the surface water was carefully wiped off. The hydrogels before and after swelling were accurately weighed and marked as M₁ and M₂. The total swelling rate was calculated according to the following formula:

\[
\text{Swelling rate (\%) = } \frac{M_2 - M_1}{M_1}
\]

2.8. Mechanical properties

2.8.1. Compression performance

DN hydrogels and pure collagen hydrogels were prepared with a diameter of 25 mm and a thickness of 15 mm. Hydrogels were compressed at a loading rate of 1 mm/min with a universal tensile testing machine (CMT4104, Shenzhen SANS Testing Machine Co. Ltd). Because DN hydrogels have a rubber-like high elasticity, the sample was difficult to crush. Therefore, the test was stopped when the sample was compressed to at a strain of 40%. Measurements were performed on six replicate samples.

2.8.2. Tensile performance

DN hydrogels and pure collagen hydrogels were prepared and air-dried for 48 h at 37 °C to obtain hydrogel membranes. The membranes were cut into pieces of the same size, 15 mm for length, 10 mm for...
width and 0.5 mm for thickness. The strain at 50% was set as an end-point to perform the tensile tests. Hydrogels were stretched at a loading rate of 10 mm/min with a universal tensile testing machine. Measurements were performed on six replicate samples.

2.9. Degradation test

2.9.1. Degradation rate

The effect of different collagen/PVA ratio and different pH environments on the degradation rate of hydrogels was investigated with the following experiments. The DN hydrogels with Col:PVA ratios of 25:75, 50:50 and 75:25 were prepared. Hydrogels were air-dried for 48 h at 37 °C. Air-dried samples were immersed in PBS (pH = 7.4) containing 200 μg/mL pepsin. A sample was removed after a certain period time and air-dried to a constant weight. These air-dried samples before and after immersing were accurately weighed and marked as M1 and M2. Calculate the degradation rate according to the following formula:

\[
\text{Degradation rate} \% = \left( \frac{M_1 - M_2}{M_1} \right) \times 100
\]

2.9.2. SEM observation after degradation

SEM images after biodegradation of collagen might give out some hint for investigating the micro/nano structure of the hydrogel. Therefore, DN (Col: PVA = 60:40) hydrogel samples were obtained after immersion in 0.02 M PBS buffer containing 200 mg/L for 24, 48, 72 and 96 h. SEM observation was conducted as the procedure above.

2.10. Statistical analysis

All experimental measurements were performed in triplicate and the results were showed as the mean values. The difference in permeability rate, swelling performances, mechanical properties and degradation rate were analyzed using a one-way analyses of variance. The significance level was set to be \( p < 0.05 \).

3. Results and discussion

3.1. Preparation of DN hydrogels

Gong et al. have proposed a two-step cross-linking method for the preparation of DN hydrogels [38,39]. They reported that high-strength hydrogels should meet the following three conditions: 1) the two substances of DN hydrogels should have their own properties. The first network should be a rigid polyelectrolyte network, and the second network should be a soft one. 2) Compared with the first network, the polymer generating the second network should have larger molecular weight 20–50 times. 3) The first network should play a major role in the cross-linked DN, and the polymer generating the second network should have weaker effect on the DN structure or only provide some additional mesh support with the first network. Our preparation of Col/PVA DN hydrogel was consistent with the above theory. PVA is a synthetic polymer with stable properties, and collagen is a natural polymer with good biocompatibility. Both of them are rich in hydrophilic groups, which provide the starting point to prepare homogeneous DN hydrogels. The molecular weight of PVA was much higher than that of collagen. In addition, the first-layer network formed by collagen played a major role in the DN hydrogels, whereas the second-layer network generated by PVA showed a weaker effect on the DN structure. The mechanism for the formation and structure of the DN hydrogels is illustrated in Fig. 1.

The DN hydrogels were prepared via successive processes including collagen self-assembly and PVA self-crosslinking. Collagen self-assembly is a complex process of overall interaction synergies, including interactions of non-covalent bonds, hydrogen bonds, hydrophobic forces, electrostatic forces, van der Waals forces, \( \pi \)-stacking forces. Because of the synergistic effect of weak interactions of noncovalent bonds, the structural stability and integrity of the self-assembled system can be maintained normally. However, in the self-crosslinking process of PVA, its molecular chains in the aqueous solution interact with each other and generate new entanglement points under low temperatures, and this process is mainly driven by the van der Waals forces, hydrogen

\[\text{Fig. 4. XRD patterns of samples. Neat PVA hydrogel after freeze-thaw cycling (a). DN hydrogel of Col: PVA = 80:20 (b), 70:30 (c) and 60:40 (d).}\]
bonding and other physical effects. The preparation procedure of DN hydrogels is facile and easy to employ in industry or the laboratory.

Photographs of samples show that pure collagen self-assembly hydrogel is stable to maintain its hydrogel state after repeated freezing and thawing cycles. With Col:PVA ratio decreasing, opaque milky white DN hydrogel gradually became transparent and clear. No delamination occurred in all DN hydrogel groups. This indicates that a homogeneous solution is obtained after two substance mixed well. However, interestingly, Col:PVA ratio of 50:50 group (Fig. 2d) are failed to form semi-solid gels. This indicated the ratio of collagen and PVA is a critical factor for DN hydrogel synthesis.

3.2. FTIR characterization

FTIR spectroscopy can be used to confirm the chemical structure of polymer as well as to identify interaction between functional groups of polymers [40]. The DN hydrogels are composed of two independent cross-linked networks, and the result of FTIR can help us further determine details of DN hydrogels composition. Fig. 3a shows the FTIR spectrum of pure collagen hydrogels, and the bands at 1650, 1547 and 1312 cm\(^{-1}\) were the C—N stretching vibration or C—H bending vibration of Amide I, Amide II and Amide III bands, respectively. These infrared characteristic peaks in Fig. 3a are consistent with the spectra of type I collagen of fish reported in the literatures, indicating that the triple helix structure was retained [41,42]. Fig. 3c shows that the DN hydrogels still maintain the Amide I, Amide II and Amide III bands at 1722, 1532 and 1420 cm\(^{-1}\), respectively. The DN hydrogels also showed an infrared characteristic peak at 1675 cm\(^{-1}\), corresponding to C=O stretching of the ether groups in PVA. In addition, there was a broad and strong absorption at 3100–3700 cm\(^{-1}\) which is attributed to the symmetrical stretching of —OH groups in PVA and collagen [43]. At the same time, the intermolecular hydrogen bond interactions between PVA and collagen molecular chains caused the —OH stretching peak of PVA shifted to a lower wavenumber after incorporating collagen. Furthermore, the absorption peak at 1140 cm\(^{-1}\) in DN hydrogels was the C—C symmetric stretching or C=O stretching vibration, which originated from collagen and the crystalline regions of PVA [44]. Infrared spectra indicated the existence of double network with hydrogen bonds between Col and PVA.

3.3. X-ray diffraction (XRD)

The XRD patterns of DN hydrogels with different Col:PVA ratios shows similar diffraction peaks around 20°, which is one of characteristic peaks [45]. PVA is derived from Polyvinyl acetate by hydrolysis. With small hydroxyl groups, crystallization ability is retained because geometric structure of the molecular chain is rarely disrupted. PVA crystallization was decreased because steric hindrance caused by group interactions of PVA and collagen in DN hydrogel; furthermore, the degree of physical cross-linking is weak with relative low ratio. Collagen does not form crystal structures and therefore does not affect X-ray diffraction (Fig. 4).

3.4. Morphological observation

Pore wall morphology and interconnectivity between pores are important for cell seeding, migration, mass transport, growth, gene expression and new tissue formation in three dimensions [46]. SEM images of DN hydrogels with different Col:PVA ratios were obtained (Fig. 5). The interior of DN hydrogels (Col:PVA = 90:10) were relatively sparse membrane-like meshes, and meshes get tighter and more regular after incorporation of PVA until ratio up to Col:PVA = 60:40. However, the internal structure of DN hydrogel of ratio 50:50 is disorganized and irregular. This corresponds to the failure of gelling of DN hydrogel with same ratio in above photographs. A porous interpenetrating mesh structure ensures a good permeability, which would promote cell proliferation and wound healing.

3.5. Permeability rate

The gas exchange property of tissue engineering materials, especially for wound dressing, is one of the essential factors [47]. The permeability rate in Fig. 6 showed ladder-like variation among hydrogels prepared by different materials. The permeability rate of the blank control group was 2805 cm\(^{-2}\) h\(^{-1}\), followed by that of collagen hydrogels. The permeability rate of DN hydrogels (Col:PVA = 75:25) was in second place among experimental groups, which still could meets the requirements of tissue engineering and medical dressings. The permeability differences among hydrogels were caused by its microstructure. Pure collagen hydrogels had large mesh size, and the permeability rate was high. However, there is a solid skeleton structure in DN hydrogels, and the mesh size was small. Therefore, the permeability rate was relatively lower than that of other groups.

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3.6. Swelling performance

3.6.1. Water content and water retention rate

The ability of a scaffold to preserve water is an essential index to evaluate its property for tissue engineering [48]. The ideal wound dressing should keep the wound environment moist [49]. As shown in Fig. 7, there was no significant difference in water content rates of the DN hydrogels with different Col:PVA ratios. However, the water-retention rates reduced as the Col:PVA ratios increased because the density of meshes increased in the DN hydrogel. Studies have shown that the higher the PVA content, the lower the degree of swelling [50,51]. PVA is highly hydrophilic, easily combining with the hydrogen bonds in water molecules to form non-frozen bound water, and easily interacting with hydrophilic groups in the nearby collagen molecules. Therefore, water was locked in the sturdy structure and hardly got out of meshes with smaller sizes even under a certain centrifugal force. Other studies have reported that the water-retention rate of hydrogel affects drug release performance. A high water retention rate is not always the ideal rate because it depends on its practical application.

3.6.2. Swelling rate

The water absorbing capacity of hydrogel is a significant factor for retaining a moist environment over the wound bed [52]. Fig. 8 shows the swelling rates of DN hydrogels in PBS at pH. There were significant differences in the swelling rate of DN hydrogels at different pH environments. Within the first 8 h, the hydrogels swelled rapidly, nearly approaching the maximum swelling rate. The three different pH environments used in this study simulated the environment of gastric acid, traumatic exudate and enteric fluid, respectively. DN hydrogels were sensitive to the condition of pH 1.5, i.e., gastric acid with a 95.9% maximum swelling. Collagen is a kind of amphoteric electrolyte with many acidic or alkaline pendant groups on the peptide chain. The swelling rate in a neutral solution is lower than that in an acidic solution. Under acidic conditions, hydrogels easily absorb aqueous solution, and the hydroxyl and amino groups in hydrogels become highly protonated. The molecular chain was stretches with charge repulsion, and the capacity of swelling increase. The possible reason that the lowest swelling rate occurred at pH 4.5 might be that the amount of positive ions was nearly equal with the negative ions in the hydrogels at this time, and no charge repulsion existed, so the internal mesh spaces were shrinking. DN hydrogels are sensitive to pH indicates that ionic groups in hydrogels have an important role in absorbing water inside the gel. This action suggests that the DN hydrogels are suitable for application in acidic environments, where they can make good use of its absorption property.
3.7. Mechanical properties

3.7.1. Compression performance

For medical materials, mechanical properties are of great importance because they affect their application in tissue engineering as scaffolds. Collagen hydrogels have poor mechanical performance; however, they can be improved by crosslinking reactions [53]. DN hydrogels were prepared by incorporating double physical crosslinks. The strain at 40% was chosen as the test endpoint because DN hydrogels prepared in this paper were difficult to reach mechanical failure under the certain compressive-stressed studied. Fig. 9 shows that the compressive modulus of the hydrogels varied significantly with different Col:PVA ratios. The compressive modulus of all DN hydrogels was higher than that of pure collagen hydrogels, and the modulus increased as the Col:PVA ratio decreased. The compressive modulus differences among hydrogels with collagen content of 90%, 80% and 70% were not significant, whereas the compressive modulus of 60% DN hydrogel was much higher than that of the other groups. Therefore, the compressive stress could be adjusted for a specific application, such as cartilage, bone, tissue or skin scaffolds.

3.7.2. Tensile performance

The tensile property in this paper is designed to test the mechanical stability of DN hydrogels after film-forming. Under the same conditions, there was no significant difference in the stresses of DN hydrogels as shown in Figs. 9 and 10. The anti-tensile mechanical property of DN hydrogels was enhanced as collagen concentration decreased (Fig. 10. A) and PVA content increased (Fig. 10.B). However, the results do not indicate an equal effect of collagen and PVA on the mechanical property changes. PVA content showed a more significant effect on the mechanical properties than collagen, which supports PVA plays a major role in the mechanical properties of DN hydrogels. DN hydrogels exhibited relatively high mechanical properties, which has been previously reported in several studies [54]. The excellent mechanical properties of DN hydrogels were mainly controlled by PVA because they generate strong network entanglements [55,56].

3.8. Degradation rate

3.8.1. Hydrogels with different Col:PVA ratios

Degradability is an important indicator for medical application, especially for implantable scaffolds and materials used in engineering, because it is related to safety for use in the human body. In addition, the release rate of the drug and bioactive molecules can be controlled by the hydrogel degradation process [57]. In this paper, the degradation experiments of DN hydrogels prepared by collagen and PVA were conducted in vitro under the action of protease. The results in Fig. 11 showed that all DN hydrogels degraded rapidly in the first 12 h because of enzymatic hydrolysis. After 12 h, the degradation rate approached a plateau, but continued to degrade slowly. The substrate was sufficient at the beginning and became insufficient after a certain period; therefore, the degradation rate decreased. The degradation rate of pure collagen hydrogels was the fastest, but it was decreased after PVA added into DN hydrogels. The higher proportion of PVA in DN hydrogels, the lower the degradation rate. This is because PVA cannot degrade by protease, and the degradation was mainly caused by collagen enzymolysis. In addition, the addition of PVA in DN hydrogel increased steric hindrance of enzymolysis for collagen and reduced degradation rate. Therefore, the degradation rate of DN hydrogels can be controlled by the Col:PVA ratios and the DN hydrogels can be widely applied.

3.8.2. SEM observation after degradation

Fig. 12 shows the internal micro-morphology of DN hydrogel changes with enzymolysis time increasing. It varies from dense pores to sparsely thin-line structures. This is respond to that the main collagen scaffold was gradually degraded, and the loose skeleton of the remaining PVA network is exposed. Therefore, it is achievable for collagen and PVA to generate double network hydrogel.

4. Conclusions

In this study, we prepared a novel DN hydrogels with fish-originated collagen self-assembly and PVA self-crosslinking. Results of FTIR showed that no new covalent bonds were generated, indicating that two substances were double physically crosslinked and the toxicity of crosslinking agents can be avoided. Results of swelling properties showed that DN hydrogels can absorb much more fluid in acidic environments. The permeability, SEM and water-retention experiments showed that the microstructure of DN was solid with tight meshes, which was consistent with the results of the mechanical properties. The DN hydrogels prepared with 40% PVA show the maximum compressive modulus of 33 kPa and maximum stress of 30.2 kPa, indicating that PVA has an important role in mechanical properties of DN hydrogels. The degradation rate of DN hydrogels can be controlled by the ratio of collagen and PVA, and the degradation rate is obviously decreased when the proportion of PVA is increased. The DN hydrogels prepared with the method described in this paper exhibit excellent performance, providing a potential feasible biomaterial for tissue engineering.

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