Simple fabrication strategy toward mechanobiological double-network hydrogel for construction of tissue engineering scaffolds

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Abstract: *Since there are few effective treatments and a high likelihood of reoperation, treating articular disease clinically has long puzzled medical professors and researchers. Tissue engineering is an innovative approach to this problem that uses scaffolds with optimal mechanical properties to provide the appropriate microenvironment for rebuilding or replacing damaged areas. To stimulate the regeneration of tissues, the materials used to build the scaffolds must conform to the following traits: simple manipulation, great mechanical strength, incredible biocompatibility, and internal capacity. In view of the above, the researchers devised a mechanobiological double-network (DN) hydrogel (CS-PAA), using a straightforward ultraviolet radiation technique that involved short-chain chitosan (CS) covalently bonding with polyacrylic acid (PAA) in solutions. Due to the strong physical interaction between the carboxyl groups in PAA polymers and the CS chain-entanglement network, the CS-PAA DN hydrogel demonstrated remarkable mechanical properties, including high tensile strength, compressibility, and rheological properties. Furthermore, vitro biological research showed that the CS-PAA DN hydrogel may facilitate the activity and proliferation of bone mesenchymal stem cells (BMSCs'), and promote tissue healing. Consequently, the soaking technique described in this study can be used to create mechanical bioactive scaffolds that have the potential to increase their bio-applicability, while offering the benefits of long-lasting biological function and mechanical support. This will promote tissue-engineered hydrogel scaffolds to reach a higher level of development.*

Keywords: *DN hydrogel, chitosan, soaking strategy, biocompatible, mechanics*

1. Introduction

Pain, joint deterioration, and possible disability are caused by articular tissue damage, a common medical condition. Despite the long-standing use of surgical procedures and clinical strategies, including autologous implantation, treating articular tissue disease is still problematic due to the limited selection of implantation and inadequate long-term rehabilitation subsequently necessitating alternative designs and strategies. As typical scaffolds, hydrogels are appealing due to their superior biological compatibility and highly hydrated three-dimensional crosslinked soft-wet structure. Hydrogels' mechanical qualities are essential for their use as scaffolds for articular cartilage regeneration because they not only offer mechanical support but also foster the development of a microenvironment that supports the preservation of the phenotypic of cartilage-forming cells [1-3]. Nevertheless, most hydrogels are so brittle or mechanically soft that they aren't suitable for load-bearing scaffolds.

Two interpenetrating networks with distinct physical characteristics make up double-network (DN) hydrogels. One of these networks is flexible and elastic, which helps the hydrogel withstand tension, while the other is fragile and rigid, which allows energy to be released by breaking. DN hydrogels have the potential to be tissue-engineered scaffolds because of the cooperative interaction between these two networks, which allows them to achieve a well-balanced mechanical profile that offers the perfect balance between stiffness and flexibility. Nevertheless, the production of DN hydrogels frequently requires hazardous initiators, complex preparation procedures, and additional additives, which prevents their widespread application in the biosciences field [4-6]. Consequently, it is of utmost importance to develop various hydrogel scaffolds that possess excellent mechanical, biological, and applicationspecific characteristics. These include outstanding compatibility, mechanical robustness, and biomimetic microenvironmental features that closely mimic those found in actual tissues.

Chitosan (CS), which is a major polysaccharide featuring excellent biocompatibility and biodegradability, has the potential to create CS ionic networks via coordination interaction when it comes into contact with electronegative molecules or anions. As a result, CS has great promise as a crucial element in the production of hybrid DN hydrogels with mechanical properties and structures that are both very flexible and incredibly robust [7]. Besides being biocompatible, the scaffolds that can be designed should possess a porous network structure, mechanical strength, and a robust capacity to promote the development of stem cells into chondrocytes.

Using the aforementioned justification, here in this work, we proposed and prepared high-performance hybrid chitosan–polyacrylic acid (CS–PAA) DN hydrogels and modified the CS ionic networks to flexibly adjust their mechanics and topologies. A highly ductile chitosan-polyacrylic acid (CS–PAA) hydrogel was fabricated by incorporating short-chain chitosan, which had high solubility in neutral water, into the covalent network of polyacrylic acid (as shown in Fig. 1). Leveraging this dynamic coordination mechanism, the resulting double-network (DN) hydrogels exhibited remarkable mechanical properties. These properties included a high level of tensile strength, a robust elastic modulus, an extremely high degree of fracture toughness, and an outstanding antifatigue capacity. Moreover, it demonstrated excellent cytocompatibility, which allowed for cell activity and provided support for the adaptation of the scaffold. To facilitate tissue regeneration, our research illustrated the viability of utilizing the CS-PAA DN hydrogel as a mechanobiological hydrogel scaffold, which is expected to be applied in various biomedical fields.

2. Materials and Methods

2.1. Experimental materials

- Short-chain chitosan (CS, degree of deacetylation is greater than 90%, viscosity of 45 mPa s for 1% (w/v) solution)
- acroleic acid (98%)
- Manganese (II) chloride
- 2-Hydroxy-2-methyl-1-[4-(2-hydroxyethoxy) phenyl] propan-1-one
- Bone marrow mesenchymal stem cells (BMSCs).
- Methylenebisacrylamide (MBA, 98%)

2.2. Preparation of CS-PAA DN hydrogel

To generate CS-PAA hydrogel, the common approach is photoinitiated radical polymerization. Simply put, the process required 1.8 g AA, 1.0 g CS, 118 μL MBA solution (0.03 mol% of AA monomer, C_{MBA}=10 mg mL^{-1}) and 57.6 mg IR 2959 (1 mol% of AA monomer) to be dissolved in 10 mL of water (deionized). The subsequent step was putting previously-made solutions under ultraviolet light treatment. The ideal duration for hydrogel to form is four hours.

2.3. Characterizations

In order to obtain Fourier Transformed Infrared (FTIR) spectra, the TENSOR-27 spectrometer was utilized. The JSM-6700F microscope was applied to record field emission scanning electron microscopy (SEM) images. To ensure the conductivity of samples (previously free-dried), sputter-coating technology is used to apply a Pt layer for 90 seconds. Afterward, the hydrogels underwent mechanical tests at room temperature with an Instron 3365 apparatus. The mold that we used to test for tensile strength originated from plastic straws whose diameter was 6 mm, and that of checking compressing ability were glass sample vials whose diameter was 9 mm. Accurate measurement of the diameter was of paramount significance. The condition of tensile tests was a velocity of 50 mm per minute, while a lower velocity of 5 mm per minute was for testing compressibility.

2.4. Evaluating toxicity and cell proliferation

Cell counting Kit-8 assay was the way of assessing hydrogel extracts' cytotoxicity. Cells were directly exposed to the material we aim to test. BMSCs were cultured for 24 h in an environment of 5% CO2 at 37 °C before hydrogel extracts were applied. The initial medium was changed to a new one with the difference of additional 10 μL CCK-8 solution, and the time points were after 1 and 3 days. For the evaluation of cell viability, a microplate reader detected the solution's absorbance at the wavelength of 450 nanometers. Cells contained in the controlled samples were cultured in DMEM media at the presence of 10% fetal bovine serum (FBS). Outcomes ought to be same with the mean values calculated from three repeated measurements. The equation used for assessment is listed below.

$$\text{Cell viability (\%)} = (A_{\text{sample}}-A_{\text{blank}})/\ (A_{\text{control}}-A_{\text{blank}}) \times 100\% \tag{1}$$

2.5. Live and dead staining assessment

The last step is to conduct live/dead staining by applying a staining working solution, which is the product of mixing red-fluorescent propidium iodide (PI) stain and green fluorescence (AM) stain, to cell culture for 1 h at 37 °C. Then, confocal laser scanning microscopy is utilized for fluorescence emission analysis of BMSCs. The wavelengths are 568 nm and 488 nm, accordingly.

3. Results and Discussion

3.1. Preparation and structural characterization of PAA and CS-PAA hydroge

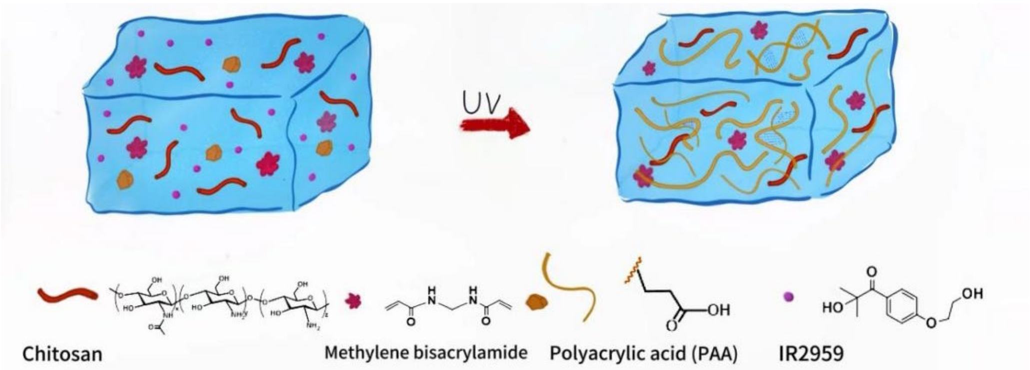

Figure 1. Schematic illustrations of fabricated CS-PAA DN hydrogel.

By using a simple adaptive soaking strategy, which can fairly control non-covalent bond interactions, CS-PAA DN hydrogel is prepared. Figure 1 clearly shows the schematic structure of the CS-PAA DN hydrogel. In order to prepare a highly ductile CS-PAA DN hydrogel, we added short-chain CS (highly soluble in water) into the PAA covalent network and used UV-initiated radical polymerization to obtain the targeted products. During this procedure, PAA underwent crosslinking via covalent bonds and double hydrogen bonds, and at the same time, CS was effectively dispersed within the hydrogel.

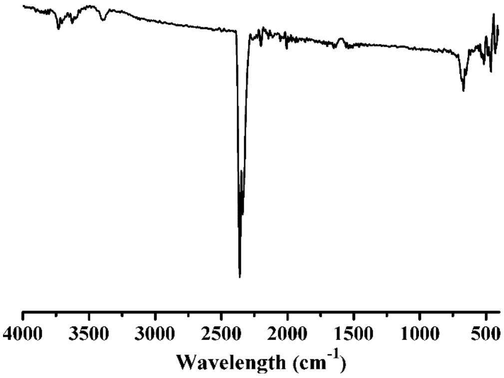

Figure 2. FTIR spectrum of CS-PAA hydrogel.

The strong evidence that could support hydrogel preparation shown in Figure 2 was afforded by The

FTIR spectrum. Due to the deacetylated CS polymer, the vibrational frequency recorded at approximately 3418 cm-1 (C-N) is around 1640 cm-1, and the distinctive absorption peaks in the amide I and II bands are around 1545 cm-1. The current obvious change in the distinct signal at 3418 cm^{-1} suggests that there is an interaction between the carboxyl group of the Polyacrylic Acid (PAA) and the amino group of the Chitosan (CS) polymer. This interaction can be attributed to the presence of positive and negative charge interactions.

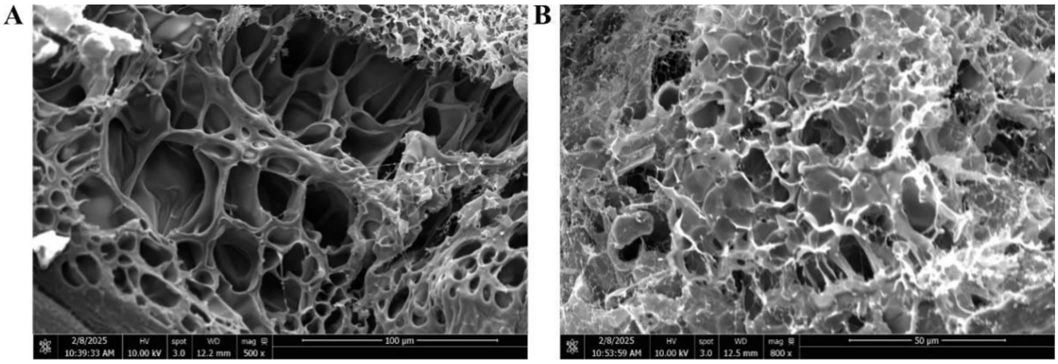

Figure 3. SEM images of (A) PAA hydrogel and (B) CS-PAA DN hydrogel.

Moreover, as shown in Figure 3, the PAA hydrogel exhibited a homogeneous honeycomb structure, while the CS-PAA DN hydrogel displayed a dense cluster morphology. This indicates the aggregation states of the DN hydrogels and highlights their remarkable stability.

3.2. Mechanics evaluation of PAA hydrogel and CS-PAA DN hydrogel

We typically regard the outstanding mechanical properties of CS-PAA DN hydrogel as led by its distinct network architecture.

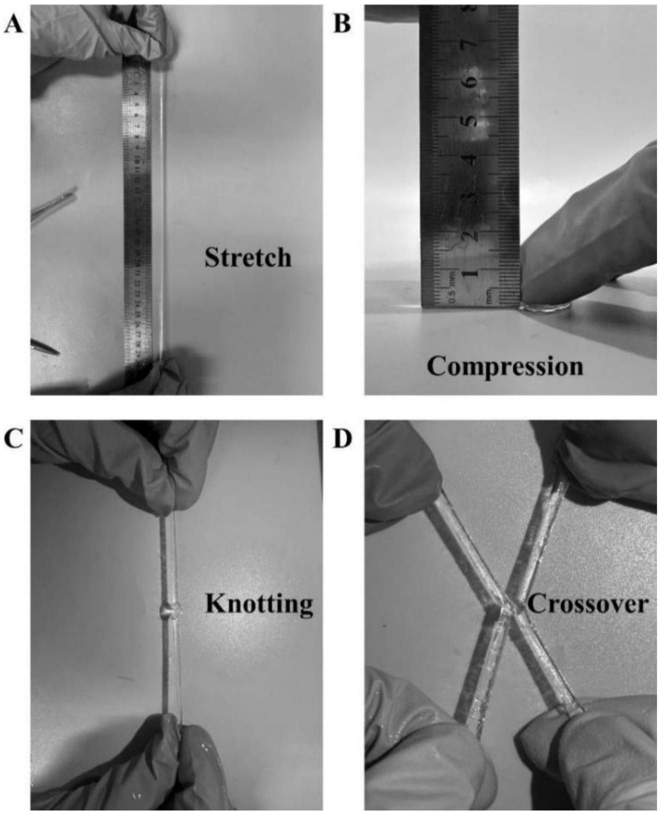

Figure 4. The extraordinary mechanical properties of the CS-PAA DN hydrogel: (A) stretching, (B) compression, (C) knotting and (D) crossover stretching.

As shown in Figure 4, the CS-PAA DN hydrogel was transparent and extremely resilient to various deformations such as stretching, compression, knotting, and crossover without sustaining significant damage. Once the applied pressure was removed, the hydrogel promptly reverted to its initial form. This effectively illustrated its remarkable elasticity and toughness, making it highly promising for various potential applications.

To conduct a quantitative assessment of the mechanical characteristics, it was of utmost importance to conduct further research on the typical stress-strain behaviors of not only the PAA hydrogel but also the CS-PAA DN hydrogel. When a 90% strain was applied, the compressing modulus of the CS - PAA DN hydrogel rose significantly to 0.09 MPa. As depicted in Figure 5, this value was sixfold greater than that of the initial PAA hydrogel. Similarly, both the recording value of new tensile strength and stiffness were higher than the composite hydrogel's value. Specifically, the tensile toughness of the CS-PAA DN hydrogel was 0.76 MPa, whereas the tensile toughness of the composite hydrogel was only 0.14 MPa. Due to the synergistic effect of several physical interactions, such as intricate chain entanglements, compact ionic networks, and robust hydrogen bonding, both the strength and the efficient energy dissipation are enhanced. This rise in energy dissipation guaranteed that the CS-PAA DN hydrogel can largely maintain its stability when subjected to external forces, which was an instrumental factor in the evaluation of tissue-engineered scaffolds.

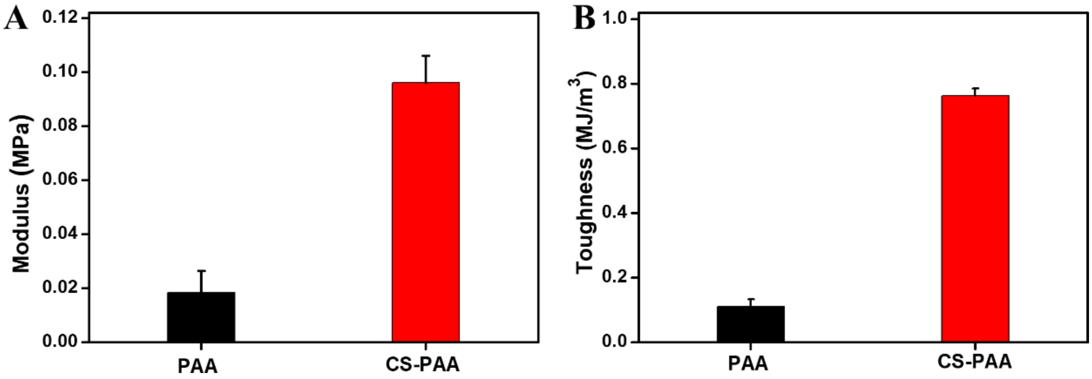

Figure 5. (A) The compressive modulus of PAA hydrogel and CS-PAA DN hydrogel. (B) The tensile toughness of PAA hydrogel and CS-PAA DN hydrogel.

Furthermore, in the range of tested rate, G' was greater than G'', indicating the formation of hydrogels. As shown in Figure 6, CS-PAA DN hydrogels' amount of G exceeded that of PAA hydrogels, further confirming that DN hydrogels had superior mechanical performance. Especially during the frequency increase from 0.1 to 100, the gap between these two widens. The above mechanical test results indicate that CS-PAA DN hydrogels have brilliant mechanical characteristics.

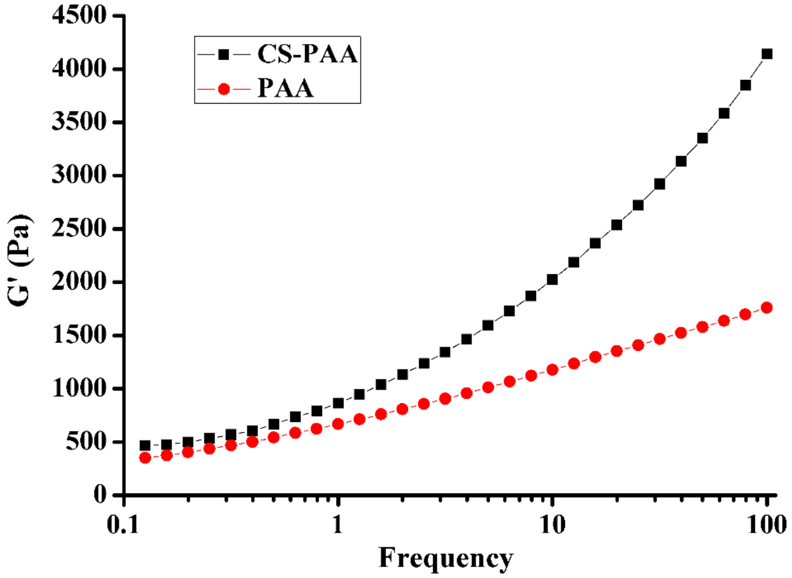

Figure 6. The storage modulus of PAA hydrogel and CS-PAA DN hydrogel.

3.3. Cell activity and proliferation

Considering the crucial properties of PAA and CS-PAA hydrogels in the biocompatibility of tissue engineering implants, cells' potential for growth and proliferation of PAA and CS-PAA hydrogels was assessed by the CCK-8 method. After developing in the medium containing hydrogels extracts for 24 h, Figure 7 shows that the cells' activity drops a bit in the acidic medium of the PAA portion, but the extension of developing time resulted in the increase of proliferating time for 3 days, illustrating that these hydrogels promoted the cells' ability of adhesion and growth.

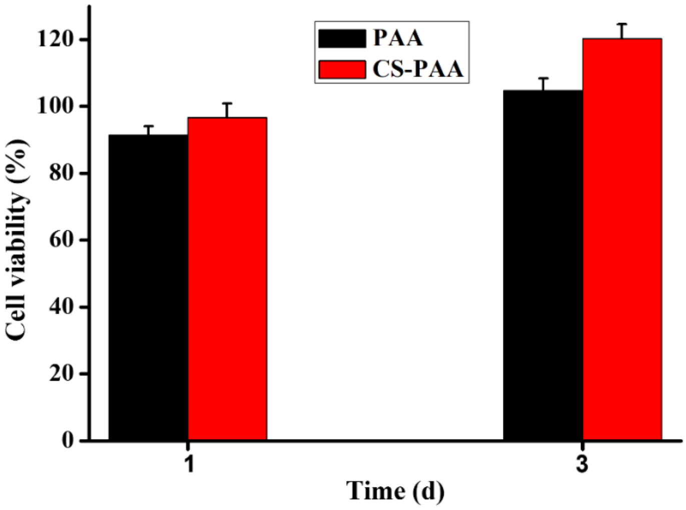

Figure 7. The cells' activity and proliferated ability after the cultivation of PAA and CS-PAA hydrogel extracts at different times.

The live/dead staining depicted in Figure 8 confirmed the outstanding results of these two types of hydrogels when illuminated by green fluorescent lamps. It demonstrated that the majority of cells were able to survive and grow well within the hydrogel extracts. Therefore, according to the simple one-step immersion method, these CS-PAA DN hydrogels with high adaptable glue behavior, brilliant mechanical performance, and excellent biological compatibility, can easily become the suitable bracket of cartilage tissue engineering.

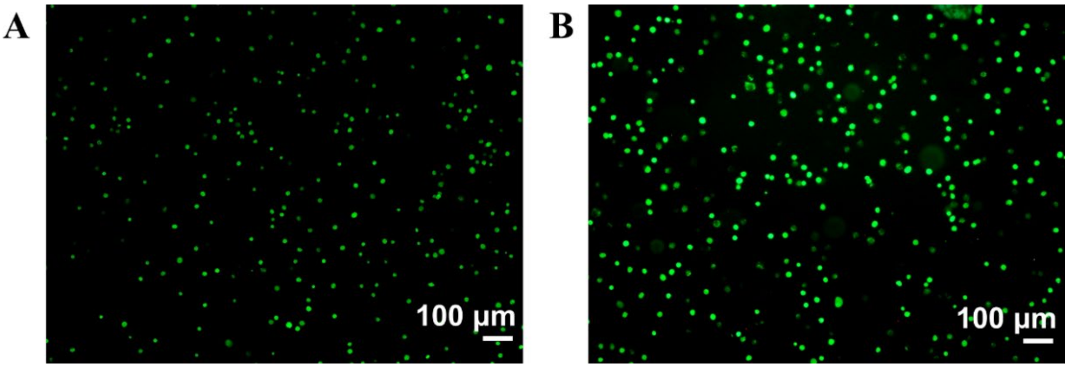

Figure 8. Live/dead staining showing the activity and growth of bone marrow mesenchymal stem cells after treatment of (A) PAA hydrogel and (B) CS-PAA DN hydrogel extracts on the first day. The green cells represent the cells that are alive, and the red cells represent the cells that are dead.

4. Conclusion

In conclusion, by using an ordinary and common soaking technique, a highly mechanobiological CS-PAA DN hydrogel was easily fabricated. This DN hydrogel demonstrated porous networks and exceptional mechanical properties with high strength and toughness to provide the appropriate mechanical support as an engineered scaffold. This was achieved by utilizing numerous physical interactions of PAA and/or CS chains entanglements, hydrogen-bond interactions, and positive and negative charged attraction. Given its favorable properties for cell growth and proliferation, this CS-PAA

DN hydrogel appeared advantageous for steady differentiation and maintainable cell viability, which can aid with tissue regeneration. Thus, this discovery has important implications for comprehending scaffold-induced long-term biological effects and aiding in the creation of tissue regeneration, in addition to providing a universal method for creating a broad variety of bioactive DN hydrogels for implantation use.

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