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**Alginate/cartilage extracellular matrix-based injectable interpenetrating polymer network
hydrogel for cartilage tissue engineering**

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Abstract

In the present study, alginate/cartilage extracellular matrix (ECM)-based injectable hydrogel was developed incorporated with silk fibroin nanofibers (SFN) for cartilage tissue engineering. The *in situ* forming hydrogels were composed of different ionic crosslinked alginate concentrations with 1% w/v enzymatically crosslinked phenolized cartilage ECM, resulting in an interpenetrating polymer network (IPN). The response surface methodology (RSM) approach was applied to optimize IPN hydrogel's mechanical properties by varying alginate and SFN concentrations. The results demonstrated that upon increasing the alginate concentration, the compression modulus improved. The SFN concentration was optimized to reach a desired mechanical stiffness. Accordingly, the concentrations of alginate and SFN to have an optimum compression modulus in the hydrogel were found to be 1.685 and 1.724 % w/v, respectively. The gelation time was found to be about 10 s for all the samples. Scanning electron microscope (SEM) images showed homogeneous dispersion of the SFN in the hydrogel, mimicking the natural cartilage environment. Furthermore, water uptake capacity, degradation rate, cell cytotoxicity, and glycosaminoglycan and collagen II secretions were determined for the optimum hydrogel to support its potential as an injectable scaffold for articular cartilage defects.

Keywords: Injectable hydrogels, Alginate, Interpenetrating polymer network, Cartilage tissue engineering

1
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3 **1. Introduction**
4

5 Articular cartilage is an avascular tissue, allowing joint movement due to its no-friction, wear-
6 resistant, and load-bearing characteristics [1]. The avascular nature of cartilage makes it degrade
7 progressively with no self-repair. Furthermore, diseases like osteoarthritis are observed in all
8 ages, mostly in people over 65 [2,3]. In addition, accidents in driving and sports can cause
9 cartilage injuries, which shows the importance of the local treatment methods [4]. Although there
10 have been many attempts during recent decades to find suitable solutions for repairing the
11 cartilage tissue, this subject is still an unsolved challenge and requires novel clinical methods [5].
12 Due to the low self-regeneration ability, the natural recovery process of the cartilage tissue faces
13 several restrictions. As a result, several treatment methods, vary from heavy operations to
14 prosthetic implants based on the type and the intensity of the injury, are being used [6]. Despite
15 their effectiveness, current clinical methods for treating cartilage injuries and defects have
16 significant application limits, highlighting the urge for a new approach. Cartilage tissue
17 engineering can fill this gap by offering an effective recovery method for cartilage injuries
18 combined with biomaterials, proper cells, and growth factors [7].
19 Injectable *in situ* forming hydrogels have gained increased attention as carriers for drug delivery
20 or scaffolds for tissue engineering applications [8-10]. Due to their ease of use in medical
21 applications and need a minimally invasive procedure for implantation, these hydrogels can be
22 used as valuable assets in cartilage tissue engineering [11]. Some tissue-engineered treatment
23 methods attempt to culture the extracted chondrocyte cells from the patient in a three-
24 dimensional matrix-cell culture system. Consequently, articular chondrocytes secrete
25 extracellular matrix (ECM) materials and produce a natural implant that mimics healthy articular
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3 cartilage; meanwhile, the biomaterial scaffold degrades over time, allowing regeneration of new
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5 cartilage [12].
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8 Several studies use ECM-derived materials to introduce different biological cues such as
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10 proteins, glycosaminoglycans, and cytokines, resulting in better cell attachment, proliferation,
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12 and differentiation [13-14]. Nasiri et al. [15] prepared a porous scaffold composed of
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14 decellularized cartilage ECM and chitosan. Various chitosan/ECM scaffolds properties were
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16 investigated to confirm improved structural and biological characteristics of chitosan scaffolds in
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18 ECM presence. As a result, they observed improvement in their sample's mechanical strength
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20 and biocompatibility compared to the scaffold made of pure chitosan. In a similar study,
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22 Sivandzadeh et al. [16] fabricated porous injectable microcarriers (MCs) using acellular cartilage
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24 ECM and chitosan by electrospraying technique. They revealed that increasing the ECM content
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26 causes a decrease in the mechanical strength of the prepared MCs. They also evaluated the
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28 ability of porous MCs to support the chondrocyte cell growth without inducing cytotoxicity.
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31 Furthermore, Teng et al. [17] utilized a combination of hanging drop and suspension cultures in
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33 the presence of decellularized matrix powder (DEMP) for the development of injectable cell
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35 carriers for cartilage tissue engineering. After two weeks of culture, high secreted
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37 glycosaminoglycans (GAGs), collagen II, BMP-2, and TGF- β 1 growth factors were detected.
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39 They furthermore found desirable differentiation of mesenchymal stem cells (MSCs) cultured on
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41 DEMP to chondrocytes, indicating the decellularized cartilage tissue's potential as a
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43 chondroinductive material for cartilage regeneration. However, it is worth mentioning that
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45 different ECM-based studies have shown relatively low mechanical strength than natural
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47 cartilage. For instance, the hybrid porous scaffold fabricated by Nasiri et al. achieved a
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49 maximum mechanical strength of about 20 kPa [15]. Similarly, Bordbar et al. produced an
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injectable hydrogel derived from sheep's extracellular matrix, and the hydrogel's storage modulus was reported to be 20 KPa [18]. Accordingly, further modifications are needed to reach the desirable mechanical properties in hydrogels using ECM as their main constituent for cartilage tissue engineering. Employing an interpenetrating polymer network (IPN) based on two or more polymers is an efficient method to improve the mechanical properties of hydrogels. As a result, this hybrid product possesses the properties of its components. The most obvious advantage of this method is providing a reinforced structure due to the complexity of its network [19]. Also, IPN systems increase phase stability in the final product [20]. On the other side, ECM-derived materials can be non-crosslinked particles as cartilage scaffolds [21-22] or thermally crosslinked solutions at 37 °C [23]. The crosslinker should provide an appropriate gelation time for injectable hydrogels with no cytotoxicity for the encapsulated cells. It has been previously reported that HRP and H₂O₂ with low concentrations are efficient for fast gelation while showing good biocompatibility [24].

Alginate is an ionic polysaccharide with several practical features, including moderate gelation conditions, low toxicity, and availability, which allows it to be used for various medical purposes such as drug delivery, tissue engineering, and cell encapsulation. Alginate has been used extensively in cartilage tissue engineering because of its suitable scaffold-forming property, non-immunogenicity, and non-toxicity [25,26]. On the other hand, as a water and oxygen permeable compound, silk fibroin is a natural polymer produced by the silkworm, and its degradation can be controlled by protease enzyme. Moreover, biocompatibility and desirable mechanical properties make silk an interesting choice as a biomaterial for cartilage tissue engineering [27]. Singh et al. [28] synthesized a scaffold containing silk fibers and illustrated that the presence of these fibers improved the porosity, biodegradability, and mechanical properties of the scaffold.

Another study also extracted silk fibroin to produce a biphasic scaffold in osteochondral tissue engineering and investigated the interconnected pores, differential swelling, and tunable degradation [29]. Mirahmadi et al. used the silk fibroin nanofibers (SFN) in a thermosensitive chitosan scaffold to enhance the mechanical properties of the construct. Their results showed that besides mechanical improvement, more secretion of GAG and collagen type II was detected in the presence of silk fibers [27]. Furthermore, the improving effects of silk fibers on the mechanical properties of hydrogels were investigated by Yodmuang et al. [30]. All in all, SFN may be proposed as an attractive compound for cartilage tissue engineering applications since it can increase the mechanical strength of artificial cartilage and can also mimic the natural cartilage environment for the cells.

In this study, an injectable IPN hydrogel based on modified cartilage ECM and alginate was prepared and characterized for articular cartilage tissue engineering applications. Horseradish peroxidase (HRP) in the presence of hydrogen peroxide was used as a crosslinker after functionalizing the ECM with phenol groups. To the best of the authors' knowledge, it is the first study to report modifying the ECM with phenolic groups and using it in an IPN system. To reinforce the scaffold and mimic the nanostructured collagen fibers in the natural cartilage, powdered SFN has been used. Making an IPN hydrogel composed of cartilage ECM and alginate incorporated with SFN was the main goal of this study to enhance the mechanical properties of the construct. The design of experiments using response surface methodology (RSM) was employed to optimize and predict the compressive modulus of the prepared scaffolds by varying alginate and SFN concentration. Furthermore, the fabricated hydrogels were characterized, envisaging a material with appropriate physical and chemical properties. The ability of the

fabricated hydrogel to support the attachment and proliferation of human chondrocyte cells and glycosaminoglycan (GAG) and collagen II secretions were then examined.

2. Materials and methods

2.1. Materials

Medium molecular weight alginate from brown algae (medium viscosity), Tris(Hydroxymethyl) aminomethane, Triton X-100, pepsin (from porcine gastric mucosa, 0.7 FIP-U/mg), 1-Ethyl-3-(3- dimethylaminopropyl) carbodiimide (EDC, hydrochloride form), N-hydroxy-succinimide (NHS), tyramine hydrochloride, 2-(N-morpholino) ethanesulfonic acid (MES), horseradish peroxidase (HRP), dialysis bags (MWCO:12400), chondroitin sulfate C (shark cartilage extract), dimethylmethylene blue (DMMB), papain, and tetrazolium dye MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) were supplied by Sigma-Aldrich. Dimethyl sulfoxide (DMSO), sodium carbonate (Na₂CO₃), glucono delta-lactone (GDL), calcium carbonate (CaCO₃), formic acid (98-100% v/v), methanol (99% v/v), and hydrochloric acid (HCl) were purchased from Merck (Darmstadt, Germany). Dulbecco's Modified Eagle's medium (DMEM, high glucose), fetal bovine serum (FBS), trypsin/EDTA (0.25% w/v), penicillin/streptomycin, trypan blue stain (0.4 % w/v), and phosphate buffer saline (PBS) were provided by Bio-Idea group. Hydrogen peroxide (H₂O₂) was supplied by Dr. Mojallali Co. (Tehran Iran), and lithium bromide (LiBr) was purchased from Riedel-de Haën.

2.2. Decellularization of the cartilage tissue

The decellularization method was done according to the method reported in the literature [15-16]. The required human cartilage was obtained from multiple patients (average age of 54 years) during knee surgeries. These cartilages were sliced into small pieces and were freeze-dried at -56 °C. Dried samples were grounded into a fine powder in the presence of liquid nitrogen using a

ball mill (Amin Asia Fanavar Pars, Iran). The obtained powder was added to a solution of Trypsin-EDTA and stirred (150 rpm) for 24 h at 37°C in a shaker-incubator, while the solution was changed every 12 h. The solution was then decanted, and the separated powder was washed by PBS solution for 30 min followed by 24 h gentle mixing in the 10 mM solution of Tris-HCl at 37°C. Afterward, it was centrifuged for 5 min at 5000 rpm, and the solid part was added to a solution of 1%v/v TritonX-100. The mixture was mixed for 24 h followed by incubation in PBS solution. The obtained powder was washed several times with PBS solution to remove all the excess detergents. Finally, the decellularized cartilage powder was lyophilized for 24 h, milled again in the presence of liquid nitrogen, and kept in a -80°C freezer for further use.

2.3. Preparation of silk fibroin

In order to perform the degumming process to separate sericin proteins from the cocoons, 5 g of the cocoons were added to 2 lit of boiling 0.02 M Na_2CO_3 solution and kept for 30 min. The cocoons were then separated, washed several times with cold deionized water, and dried overnight at ambient temperature. Afterward, 9.3 M LiBr solution was added to dried silk, and the mixture was put in an oven at 60°C for 4 to 6 h to completely dissolve silk and obtain 20% w/v fibroin solution. The solution was then dialyzed against deionized water for two days while the dialysis water was changed 6 times. Finally, the silk solution was centrifuged twice at 7000 rpm and 4 °C for 20 minutes to remove all precipitated impurities [31]. The obtained solution was freeze-dried for two days and kept at room temperature prior to use.

2.4. Electrospinning and fiber characterization

Formic acid 98-100% was used to prepare 10%w/v fibroin solution. This solution was loaded into a 5 ml syringe (22-gauge needle). The voltage, injection flow rate, the distance between the cathode and anode, and collector's rotation speed were 30 kV, 0.4 ml.hr⁻¹, 14 cm, and 400 rpm,

respectively. The values were selected based on the literature with certain modifications [32]. The electrospun nanofibers were floated in methanol 90% for 20 min. The obtained SFN were dried overnight at room temperature and then grounded using a ball mill in the presence of liquid nitrogen.

2.5. Modification of ECM to incorporate phenolic hydroxyl groups

The ECM possessing phenolic hydroxyl (Ph) moieties (denoted as ECM-Ph) was prepared by the following developed method through conjugation with tyramine via carbodiimide-mediated condensation in an aqueous solution. First, the decellularized ECM powder (0.1 g) was added to 10 ml of 0.1 N HCl solution containing 0.1% w/v pepsin enzyme and kept under mild stirring for 72 h at room temperature. The solution was then filtered and dialyzed against deionized water for two days to remove the unreacted ECM. Afterward, MES powder was added to the solution to reach a concentration of 50 mM. The phenolization reaction was then started by adding 0.012, 0.0014, and 0.017 g tyramine, EDC, and NHS, respectively. It was stirred for 24 h at room temperature. The obtained solution was dialyzed against deionized water until the absorbance value at a wavelength of 275 nm, monitored by spectrophotometry (WPA Biowave II, Biochrom, UK), reached lower than 0.05. It guaranteed the removal of the unreacted tyramine from the solution. The first dialysis caused a rise in the pH of the ECM solution facilitating the pH adjustment by 50 mM MES solution to the desired pH value of 6. Ultimately, after the second dialysis, the solution was freeze-dried for 24 h, and the obtained ECM-Ph powder was kept in a sealed and opaque container at -20°C for further use [33-38].

2.7. Hydrogel preparation and gelation time

ECM-Ph powder (1% w/v) was added to deionized water, and this mixture was kept under stirring for 8 h. The alginate was then added to the solution with three different concentrations,

including 1%, 1.5%, and 2% w/v. The stirring was continued to obtain a homogenous solution. CaCO_3 and horseradish peroxidase (HRP) were then added to the solution to obtain 2% w/v and 1.25 U.mL⁻¹ concentrations, respectively. Afterward, three different weight ratios of SFN, including 0%, 1.5%, and 3% (w/v) was added to the mixture. In order to inhibit the agglomeration of SFN in the solution, samples were vigorously mixed before adding crosslinker components. Finally, GDL (4% w/v) and H_2O_2 (1 mM) were added. The solutions were injected into PDMS (polydimethylsiloxane) molds using an 18-gauge dual syringe needle. Samples with the different concentrations of alginate without adding SFN were also prepared to determine the effect of SFN on the hydrogel characteristics.

The phenoxy radicals of ECM-Ph reacted with each other by a radical coupling reaction, generating a C–C or C–O bond as a stable crosslink in the presence of HRP and H_2O_2 . Besides, alginate was crosslinked by Ca^{2+} cations forming an egg-box structure. The chemical structure of the IPN hydrogel composed of ECM-Ph and alginate and the schematic illustration of the approaches to make chondrocytes-encapsulated *in situ* forming SFN-reinforced IPN hydrogel has been shown in **Figure 1**.

Figure 1. The overall chemistry and the fabrication of the *in situ* forming hydrogel process. a) Enzymic HRP-induced crosslinking of ECM-ph. b) Schematic presentation of the ECM-Ph-alginate IPN system. c) Egg-shaped crosslinking between alginate and Ca^{2+} ions. d) Hydrogel with SFN nanofibers, e) Hydrogel without SFN nanofibers, f) the PDMS mold, and g) the schematic illustration of approaches to make chondrocytes-encapsulated *in situ* forming SFN-reinforced IPN hydrogel.

2.8. Experimental design and data analysis

The impacts of changing the alginate and SFN concentrations were analyzed through response surface methodology (RSM) using the Design Expert 10.0 software. The intended response for

this analysis was the compression modulus of the samples. A central composite design of experiments using each of these parameters at three levels, including alginate concentration of 1, 1.5, and 3 %w/v, and SFN content of 0, 1.5, and 3 %w/v, was used to determine the optimal condition for preparation of the injectable hydrogel. It is worth noting that the higher concentrations of SFN and alginate were tested, and the hydrogels were not injectable due to the high viscosity. All the input data regarding the independent variables for this model are listed in Table 1. The designed model proposed 15 experimental runs, of which 5 of those were the exact facsimile of the center point (A=B=1.5 %w/v). The acquired model was a quadratic polynomial (Equation 1).

$$Y = \alpha_0 + \alpha_1 A + \alpha_2 B + \alpha_{11} A^2 + \alpha_{12} AB + \alpha_{22} B^2 \quad (1)$$

where Y is the inverse square root value of the dependent variable, α_0 is a constant, α_1 and α_2 are linear coefficients, α_{11} and α_{22} are squared factors, and α_{12} is a cross-product factor. A and B are the independent variables, which were previously defined as concentrations of alginate and SFN, respectively. Design Expert uses curve fitting principles, including regression, p-value, and F-values, to find the best-aliased equations. These principles are applied for checking the compatibility of the input data with linear, cubic, quadratic, square root, etc. correlations. Analysis of variance (ANOVA) was used to check how the independent variables might influence the response. The predicted values by the model and the real values were investigated for fitting accuracy using R^2 and adjusted- R^2 . Ultimately, the optimum hydrogel was selected according to the model-predicted values offering the maximum compressive modulus.

Table 1. Experimental range and levels of independent variables

Factor	Name	Units	Levels		
A	Alginate	%w/v	1	1.5	2
	concentration				
B	SFN	%w/v	0	1.5	3
	concentration				

2.9. Chondrocyte isolation and culturing

Human primary chondrocyte cells were derived from the adult human articular cartilage. They were removed during a total knee replacement operation, similar to the method previously reported by Giannini et al. with some modifications [39]. The cartilage parts were separated from the received tissue using dissection. All the obtained pieces were then washed using a PBS solution containing 3%w/v penicillin-streptomycin to ensure the samples were sterile. They were then enzymatically digested for 12 h using a 4 mg/mL solution of type II collagenase. The prepared cell suspension was filtered using a nylon cell strainer with a mesh size of 70 μm and was centrifuged for 12 min at 2000 rpm to obtain isolated chondrocytes. The isolated cells were cultured inside 60 mm Petri dishes using a DMEM-F12 medium containing 20% w/v FBS and 1% w/v penicillin-streptomycin. Finally, DMEM supplemented with 10% v/v FBS was used to obtain a monolayer chondrocyte culture after the first passage.

2.10. Cell encapsulation and MTT assay

Preparation of the cell encapsulated hydrogels was similar to the method previously described for the cell-free hydrogels, with a difference that the seeded cells were added to the sterilized solution prior to adding H₂O₂. Human chondrocyte cells were encapsulated into the samples with

a density of 2×10^6 cells ml^{-1} . The samples were transferred into a 24 well plate containing DMEM supplemented with 20% FBS. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was conducted on the 1st, 3rd, 5th, and 7th days of culture to assess the viability of the cells within the hydrogels. The culture medium was removed in the mentioned time intervals, and the hydrogels were immersed in 0.5 mM sodium citrate for 90 s to facilitate penetration of MTT solution. Then, serum-free culture medium and MTT solution (9:1) were added, and the samples were incubated at 37°C for 12 h to improve precipitation of the purple formazan crystals. The solution was then removed, and DMSO was added to dissolve the precipitated formazan crystals. Subsequently, the absorbance of the samples was measured at the wavelength of 490 nm using an ELISA reader (BA3200, GARNI Medical Engineering Co.) [40].

2.11. Glycosaminoglycan (GAGs) and collagen II content determination

Encapsulated chondrocyte cells in the optimum hydrogels with a density of 2×10^6 cells ml^{-1} were cultured for 21 d. DMMB was used as an indicator to measure released sulfated GAGs to determine the proteoglycan content of the samples. 500 μL of the medium solution was extracted from the samples at specified time intervals and then added to a 2 mL vial containing 1.5 mL of acetone. The vial was tilted every time and kept at -20 °C for 24 h. Then the samples were centrifuged for 10 min at 8000 rpm and 4 °C. The solutions were decanted, and the remaining pellets were added to a mixture of 1 mL of papain digestive solution in PBS. The mixture contained 5 mM L-cysteine and 20 $\mu\text{g} \cdot \text{ml}^{-1}$ papain. The final solution was incubated for 16 h at 60 °C followed by boiling for 15 min. The ELISA plate reader was used at the wavelength of 525 nm to determine the GAG content of the sample. Solutions with specified concentrations of chondroitin sulfate were used to make the standard curve [27].

To quantify collagen II, first, sulfuric acid (7.0 N) was used to digest 1 ml of solution extracted from the samples. This process was continued for 16 h at 120 °C. Then, 2 ml of the obtained solution was transferred to a test tube where 1 ml of chloramine-T (%1.4) was added. The resulting solution was incubated for 20 min at room temperature. Next, 1 ml of 4-dimethylbenzaldehyde reagent was added, and then incubation was continued for 15 min at 60 °C. Absorbance measurements were conducted at the wavelength of 550 nm. The blank sample in these tests was 2 ml of deionized water. Hydroxyproline solution with different concentrations (0, 2, 4, 6, and 8 µg.ml⁻¹) was added to the standard samples to provide the calibration curve. Finally, the collagen II content of the samples was determined using the following correlation:

$$\text{Collagen II } (\mu\text{g/ml}) = \text{Hydroxyproline } (\mu\text{g/ml}) \times 8 \tag{2}$$

2.12. Characterization

2.12.1. Gelation time

The gelation time of the different samples was determined using the vial tilting method. No flow within 1 min upon inverting the vial was considered as the gel state. After gelation, 1 µl of 10% w/v CaCl₂ solution was added to every 1 µl of the hydrogel solution to increase the sturdiness of the samples.

2.12.2. Proton Nuclear Magnetic Resonance (H-NMR)

¹H-NMR spectroscopy was used to investigate and validate the phenolization of the cartilage ECM. D₂O was used as a solvent. Pristine ECM powder was added to 0.1% w/v pepsin in 0.1 N deuterium chloride solution (DCl) to prepare the control sample. Solutions with a concentration of 25 mg.ml⁻¹ were provided in NMR sample tubes (d=5 mm) using a Spectra Spin 400 Ultrashield (Bruker). Finally, the obtained spectra were evaluated and interpreted using MestReNova software.

2.12.3. Mechanical test

The compressive strength of the samples was measured using the Zwick-Roell materials testing system. The compression of the hydrogels was evaluated up to 50% strain at room temperature. The load cell and the loading rate were 2 kN and 0.1 mm.min⁻¹, respectively. The samples were freshly tested after being fabricated in a PDMS mold. The compression modulus of the samples was determined by calculating the slope of the first linear section of the stress-strain curves (approximately 10-20% strain). The results were then imported to the Design Expert to determine the optimum sample.

2.12.4. Morphological characterization

The morphology of the synthesized hydrogel was investigated by scanning electron microscopy (SEM, Seron Technology, AIS-2100, Korea). In order to investigate the effect of SFN on the pore size, the optimized hydrogels with and without SFN were subjected to SEM imaging. The samples were lyophilized and sliced to reach cross-sectional specimens and were sputter-coated with gold prior to observation. The ImageJ analysis software was used to determine the pore size of the samples.

2.12.5. Swelling ratio

In order to investigate the effect of the SFN on the swelling ratio, the optimized hydrogels with and without SFN were applied. The swelling ratio of the sample was determined by measuring the water uptake of the hydrogel incubated in 100 mM HEPES solution [37]. First, the sample was freeze-dried and weighted. Then, it was incubated in 100 mM HEPES solution at 37 °C for two days. The samples were weighed at specific time intervals, including 1, 2, 4, 6, 24, and 48 h, after removing the surface water with tissue paper. The swelling ratio was determined as below:

$$Q_m = \frac{W_w - W_d}{W_d} \times 100\% \quad (3)$$

where W_w and W_d are weights of the hydrogel in wet and dry conditions, respectively.

2.12.6. In-vitro biodegradation

In order to investigate the effect of SFN on hydrogel biodegradation, the weight loss of the optimized hydrogels with and without SFN was measured. The hydrogel was dried in a vacuum oven for 24 h and then was weighted. It was then incubated in 6 ml of 100 mM HEPES solution at 37°C for 4 weeks while the solution was changed every day [41]. The sample was removed from the medium after 7, 14, 21, and 28 d, vacuum dried, and weighted. The weight loss percentage was calculated using the following equation:

$$WL = \frac{W_0 - W_t}{W_0} \times 100\% \tag{4}$$

where W_0 is the initial weight of the sample and W_t is the sample weight at the aforementioned time intervals.

2.13. Statistical analysis

All the data were obtained at least in triplicate ($n = 3$), averaged, and expressed as mean $\pm$ standard deviation (SD). *P*-values less than 0.05 were considered statistically significant.

3. Results and Discussion

3.1. Proton nuclear magnetic resonance (H-NMR)

Several research works have reported the functionalization of collagen [33], gelatin [34-36], chondroitin sulfate [37], and hyaluronic acid [34,36,38] with phenol groups; nevertheless, but to the best of our knowledge, there is no report on ECM incorporated with phenol groups. To verify the phenol group grafting, ^1H -NMR spectroscopy was used. The ECM and ECM-Ph samples were tested, and the results are illustrated in **Figure 2**. The differences between the spectra of **Figure 2a** and **Figure 2b** are due to the presence of aromatic ring protons of grafted tyramine to ECM that appeared at 6.9 and 7.3 ppm [34], denoting as I and II in the ECM-Ph spectrum. Moreover, the

other differences between the two spectra were due to two times the dialysis of the samples during phenol grafting, which inevitably caused the removal of some of the ECM materials.

Figure 2. ^1H -NMR spectroscopy of a) ECM and b) ECM-Ph. The aromatic protons of the tyramine molecule are denoted in the spectrum as I and II.

3.2. SFN morphology

The morphology of SFN nanostructure was studied using SEM imaging. **Figure 3** demonstrates the SEM images of SFN with different orders of magnification. As can be seen, even after being grounded for 4 min, SFN still retained its nanostructure. The electrospun SFN prepared from the silk fibroin solution in formic acid (10 % w/v) had a mean diameter of 350 ± 45 nm. It was confirmed that the nano-sized structure of the silk nanofibers plays a vital role in mimicking the

extracellular matrix structure. They also provide strengthened mechanical features [42-44]. Accordingly, the prepared SFN nanostructure could cover the size limitations adequately.

Figure 3. The SEM images of grounded SFN with different magnifications. The mean diameter of the nanofibers was measured as 350 ± 45 nm.

3.3. Hydrogel mechanical properties and gelation time

The compression modulus of the samples derived from the stress/strain curves slope is summarized in **Table 2**. It can be comprehended that the concentration of alginate has a direct impact on the compression modulus of the samples, so increasing the alginate content in samples 1, 4, and 7 increased the modulus. On the other side, when the alginate concentration was low (1% w/v), the addition of SFN caused an improvement in compression modulus, which could be explained by considering the SFN as a reinforcing filler. However, when the alginate concentration increased up to 1.5%, SNF addition illustrated a critical point at 1.5% concentration. In other words, the addition of SNF first improved the modulus, while further addition reduced the modulus. For instance, in samples 4, 5, and 6, with the same alginate concentration (1.5% w/v), the hydrogel's modulus increased from 63.65 to 92.97 kPa when the

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3 SFN concentration increased to 1.5% w/v (samples 4 and 5), it then dropped to 86.08 kPa at a
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5 higher loading of SNF (3% w/v) in sample 6. The same trend was detected for samples 7, 8, and
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10 This behavior could be explained by considering that an increase in the SFN content in the
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12 samples exacerbated the diffusion of cross-linking agents into the hydrogel and caused an
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14 incomplete crosslinking, consequently reducing the compression modulus. Furthermore, SFN
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16 played a filler role in the hydrogels; when its concentration reached a critical value, fracture
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18 initiating points such as agglomerated nanofibers and un-crosslinked random points increased
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20 resulted in a decrease in the compression modulus of hydrogel [45].
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24 Cartilage possesses viscoelastic materials, so the alternatives must have considerable mechanical
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26 attributes. A cartilage scaffold should resemble the natural cartilage tissue; therefore, ECM may
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28 be considered a suitable material for scaffold fabrication [46]. However, the ECM-based
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30 scaffolds display lower biomechanical strength in comparison with the native tissue
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32 [15,18,47,48]. Consequently, materials with intrinsic superior mechanical properties can be used
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34 along with cartilage ECM to obtain high mechanical strength for ECM-based scaffolds. In this
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36 work, SFN was used as a reinforcing component to increase the mechanical strength of the
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38 prepared hydrogels. The compressive moduli of the synthesized hydrogels were inferior to those
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40 of the natural articular cartilage (0.5-1 MPa) [49]. This difference was expected as ECM and
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42 alginate are both natural polymers, but SFN had a positive impact on the mechanical properties
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44 of the hydrogels. The compression modulus of the optimum hydrogel in the present work was
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46 significantly enhanced compared to previous studies either on pure alginate crosslinked using
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48 CaCO_3 [50] or on cartilage ECM-derived hydrogels for tissue engineering applications [51], due
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50 to the addition of the SFN and the IPN system formation.
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Table 2. Experimental central design runs and corresponding results

Sample number	Alginate concentration (%w/v)	SFN concentration (%w/v)	Compression Modulus (kPa)
1	1	0	27.36 ± 1.15
2	1	1.5	39.49 ± 1.86
3	1	3	46.67 ± 1.96
4	1.5	0	63.65 ± 2.61
5	1.5	1.5	92.97 ± 4.56
6	1.5	3	86.08 ± 3.70
7	2	0	74.86 ± 3.44
8	2	1.5	81.43 ± 3.83
9	2	3	61.98 ± 2.54

Designing a hydrogel with appropriate gelation time besides suitable mechanical performance is crucial in applications of *in situ* forming hydrogels for cartilage tissue engineering. Extended or short gelation times can cause unwanted cell migration and diffusion of the scaffold to uninjured areas or gelation before injection. Although in most studies, alginate gelation has been induced by the addition of calcium chloride (CaCl₂), it typically leads to rapid and poorly controlled gelation due to its high solubility in aqueous solutions [11]. In this work, CaCO₃ along with GDL was used to increase the alginate's gelation time. Since Ca²⁺ cations in CaCO₃ release as soon as the pH of the solution is acidic [52], GDL was used to lower the solution's pH slowly to yield a proper gelation time for the *in situ* forming a hydrogel. The gelation time was determined using the vial tilting method, and its value was measured to be approximately 10 s for all the samples.

Although the enzymic reaction mediated by H₂O₂ and HRP results in faster gelation than the ionic reaction of alginate, this gelation time was applicable using a dual syringe. Moreover, this hydrogel may also have a robust adhesion to the tissue, which is one of the key parameters for designing and synthesizing *in situ* forming hydrogels. Thi et al. [53] showed that using HRP as a crosslinking agent resulted in strong bonds production between the Ph groups attached to the polymer and the tyrosine available in body tissue. This bond could increase the adhesion strength to the tissue more than three times compared to clinical fibrin glues [54]. Therefore, it could be expected that the fabricated hydrogel might be considered as an *in situ* forming tissue adhesive hydrogel, but further investigations are required to confirm.

3.4. Optimization of the IPN composite by Response Surface Methodology (RSM)

Table 2 results were used to determine the sample with an optimum compression modulus. The following quadratic equation was extracted from Design-Expert software to investigate the effect of process parameters, including concentrations of SNF and alginate on the compression modulus ($R^2 > 0.99$):

$$\frac{1}{\sqrt{\text{Compression modulus}}} = 0.50282 - 0.048431 \times C_S - 0.42864 \times C_A + 0.018746 \times C_A \times C_S + 0.11759 \times C_A^2 + 4.88512 \times 10^{-3} \times C_S^2 \quad (5)$$

where C_S and C_A are concentrations (w/v %) of SFN and alginate, respectively.

The ANOVA analysis was done to investigate the accuracy of the suggested model. R-squared values, adjusted R-squared, predicted R-squared, and adequate precision were 0.9994, 0.9984, 0.9952, and 92.714, respectively. The difference between the predicted R-squared and adjusted R-squared of the model was less than 0.2, indicating the agreement between these parameters [55-57]. Furthermore, the signal to noise ratio of the model describing by adequate precision was 92.714 revealing an adequate signal for the model (it should be greater than 4). The R-squared

and adjusted R-squared of the model were both higher than 0.9. The negligible difference between R-squared and predicted R-squared values could be used as proof that this model could predict new results [58-60]. Moreover, the P-values of the model and all involved parameters were less than 0.05, indicating that the selected parameters had a significant impact on the model.

The numerical optimization was done to have the highest possible compression modulus. Accordingly, the model provided optimum values for the concentrations of alginate and SFN, as 1.685 and 1.724 % w/v, respectively. Due to these optimum values, the highest possible compression modulus was 100.13 kPa. The optimum hydrogel was prepared based on the optimum values, and its compression modulus was measured to be 102.97 ± 4.75 kPa, which is in good agreement with the software suggested value.

3.5. Morphology

Cross-sectional SEM images of the optimum samples (with and without SFN) are illustrated in **Figure 4**, indicating that both prepared hydrogels had an interconnected porous morphology. Furthermore, the addition of SFN had an impact on the pore size of the hydrogel. When the fibers introduced to the system, they formed a tight network structure resulting in a reduction in the average pore size from 600 ± 50 μ m to 225 ± 25 μ m in the hydrogel due to the role of SFN as a filler. Nonetheless, the resulting mean pore size was adequate for the viability and functionality of the chondrocytes after adding the SFN [21]. In addition, changes in pore size will affect tortuosity and compactness, which can cause a slight decrease in the water uptake of the scaffold. In general, enhanced cell adhesion and intracellular signaling can be attributed to smaller pores, and the effectiveness of nutrient supply and oxygen diffusion can be provided by larger pores [61]. However, it must be noted that the proper pore size is variable for different tissues. It has also

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3 been reported that tissue regeneration can be affected by pore size as the promotion of inherent
4 cell function would be facilitated in a certain size range [62]. Lien et al. [63] reported an abnormal
5 and de-differentiated form of chondrocytes in the pores with a smaller size and normal phenotypes
6 in the larger ones. They concluded a pore size preference between 250 μm to 500 μm that could
7 be considered optimum in improving cell proliferation and ECM production. It is also worth
8 mentioning that introducing the SFN to the hydrogel increased the pore size distribution's
9 uniformity. Moreover, the morphology and the dispersion of the SFN within the hydrogel matrix
10 are demonstrated in **Figure 4c** and **Figure 4d**, which might resemble the role of collagen fibers
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Figure 4. Morphological and physical characterizations of the optimum hydrogel. a) SEM images of hydrogel with SFN and b) without SFN. c, d) Magnified images of two-pore sections within the hydrogel (The corresponding enlarged area indicated by ** and * are shown in images c and d, respectively); Pore size of the sample containing SFN nanofibers. e) Swelling ratio of the optimum hydrogels ($\square$) with and ($\circ$) without nanofibers in 48 hours. Error bars correspond to mean $\pm$ SD for $n = 3$. f) Weight loss of the optimum hydrogels ($\square$) with and ($\circ$) without SFN in 28 days. Error bars correspond to mean $\pm$ SD for $n = 3$.

3.6. Swelling ratio

The swelling behavior of hydrogels is one of the interesting features of the hydrogels, favoring cell infiltration and migration [64-66]. Therefore, the swelling rate of the hydrogels was studied over 48 h. The results are illustrated in **Figure 4e**. The results indicated a 20% reduction in swelling ratio by adding the SFN to the hydrogel, which could be explained by considering that the elastic forces of the SFN caused a counterbalance against the swelling pressure resulted in a water content decrease [67]. By this effect, the degree of swelling could be related to the compressive elastic modulus inversely. Therefore, if the swelling capacity increases, the compressive elastic modulus will decrease. The correlation between the addition of the SFN and the water content of the hydrogel can also be related to the effect of methanol treatment of the electrospun SFN. Due to the low stability of the electrospun SFN, modification by methanol was used to increase the structure and shape stability of the obtained nanofibers. Moreover, this step improved the mechanical and elastic properties of the nanofibers significantly. This advantage has been ascribed to the formation of β -sheet crystals in the SFN [68]. However, this treatment resulted in a limitation in the swelling capacity of the SFN, and consequently, the water uptake of the scaffold was affected [69]. The SFN-free sample reached the equilibrium state sooner than the other sample, which implied the constraining effect of adding the SFN to the hydrogel to reach an equilibrium state. The reason could be related to a decrease in water permeability of the hydrogel due to the presence of the SFN. The fibers may trap the water within the pores of the hydrogel and extend the equilibrium time.

3.7. *In-vitro* biodegradation

It can be indicated that optimal tissue regeneration requires a balance between the degradation rate of the scaffold and the new tissue formation rate [70]. The corresponding results of *in vitro* biodegradation of the prepared hydrogels are illustrated in **Figure 4f**. The biodegradation results

were evaluated for four weeks because this period is critical for cartilage regeneration after grafting the scaffold [71]. A slight difference between the results for the samples with and without the SFN was detected. The hydrogel incorporated with the SFN showed 50% mass loss after 28 d, which is 10% less than the SFN-free sample due to the lower degradation rate of the SFN compared to the other components of the hydrogel.

3.8. Viability and proliferation of the chondrocyte cells

In-vitro viability of the cells seeded into the hydrogel was investigated using the MTT assay. The viability of human chondrocyte cells encapsulated in the optimum sample is shown in **Figure 5a**. One day after encapsulating the cells, cell density faced a significant drop compared to the 2D control group, probably due to the slower diffusion of the nutrients into the 3D hydrogel. Over time, cell density increased in both samples as on day 7. The difference between the control samples and IPN ones can be explained by the difference in 2D and 3D culture of the cells, respectively. The results indicate that the hydrogel provided a suitable microenvironment for cell growth and viability. A previous study by Zuluaga-Vélez et al. [72] proved that using silk-hydrogels positively affects cell attachment and proliferation. Their study found that the number of cells used in the control group was considerably more than the cells encapsulated in hydrogels. However, the difference between the cells in the control group and the cells encapsulated in the hydrogels was much lower in this work.

Figure 5. Characterization of the biological performance of the optimum hydrogel. a) Viability of the encapsulated human chondrocyte cells in the hydrogel. b) The amount of GAG and Col II secretions on 7, 14, and 21 days after cell culture. Data are represented as mean $\pm$ SD (n=3). ** and * represent significant differences between groups at $p \leq 0.01$ and $p \leq 0.05$, respectively.

3.9. Glycosaminoglycan and collagen II content determination

The measured GAG and collagen II contents are shown in **Figure 5b** for the cell encapsulated hydrogels. The GAG and collagen II content values increased slightly on days 14 and 21.

Statistical analysis demonstrated a significant difference in collagen II secretion between day 7 and day 14, in good agreement with the literature [30]. GAG and collagen II are present abundantly in the ECM of hyaline cartilage, while connective tissues like tendons and ligaments mainly contain collagen I [73]. Furthermore, there is collagen type X, which can be related to the hypertrophy of chondrocytes. This type is mostly limited to the deep zone of cartilage and also is available in the adjoining calcified articular cartilage of an adult [74]. The obtained results confirm that the chondrocytes retained the chondrogenic phenotype supported by the data, illustrating the GAG and collagen II secretions inside the media. Moreover, the secretion of ECM by chondrocytes and cellular microenvironmental changes had a considerable impact on the mechanical properties of the implanted scaffold. Igarashi et al. [75] reported the application of alginate and bone marrow stromal cells (BMSCs) to fabricate an *in situ* forming cellular implant. They confirmed that the compression modulus of the implants was enhanced significantly after 12 weeks and reached 80% of the natural cartilage as a result of ECM secretion.

It is worth mentioning that since the cartilage degeneration is usually caused by macrophage activation and causes polarization towards the pro-inflammatory M1 phenotype [76] and consequently an abnormal immune response in the joint, it is vital to apply appropriate immunomodulatory approaches such as using biomaterials with immunomodulatory capacity, the delivery of cells or anti-inflammatory proteins [77-79]. Engineering appropriate immunomodulatory approaches to tune the inflammatory response in order to improve cartilage tissue engineering should be addressed in future studies.

4. Conclusion

An injectable hydrogel based on modified decellularized cartilage ECM, alginate, and SFN was introduced in this study. The synthesized hydrogel was optimized regarding its mechanical strength (compression modulus) using the response surface methodology. The corresponding concentrations of alginate and SFN for the optimum hydrogel were 1.685 and 1.724 % w/v, respectively. These values resulted in a compression modulus of 102.97 ± 4.75 kPa for the optimum hydrogel. It was further characterized in morphology, swelling ratio, cell viability, biodegradation, GAG, and collagen II secretions. In summary, the cell viability and GAG and collagen II content measurements showed this hydrogel's considerable capability in mimicking the natural cartilage. Overall, the obtained results highlight the high potential of the fabricated *in situ* forming hydrogel for further cartilage tissue engineering studies. Ongoing studies are focused on further improving this hydrogel's mechanical strength to match the native cartilage tissue.

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Figure 1

90x106mm (300 x 300 DPI)

Figure 2

336x577mm (120 x 120 DPI)

Figure 3

131x65mm (120 x 120 DPI)

Figure 4

131x126mm (120 x 120 DPI)

84x118mm (120 x 120 DPI)