Cellular compatibility of nanocomposite scaffolds based on hydroxyapatite entrapped in cellulose network for bone repair

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ABSTRACT

In the past few decades, artificial graft materials for bone tissue engineering have gained much importance. The purpose of this research is to develop and characterize a novel 3D nanocomposite scaffold that closely mimics the properties of real bone, such as morphology, composition, and mechanical characteristics. This novel porous 3D nanocomposite scaffold is composed of polyacrylamide grafted cellulose, and hydroxyapatite. The morphology, structure, and mechanical properties of cellulose-graft-polyacrylamide containing different amounts of nano-hydroxyapatite were characterized. The swelling behavior of the scaffolds was examined in both water and phosphate buffer saline (PBS) solution. The cytotoxicity of the scaffolds was determined by MTT assays on human fibroblast gum (HuGu) cells. Results showed that the nanocomposite scaffolds were highly porous with maximum porosity of 85.7 % interconnected with a pore size of around 72–125 μm . The results of cell culture experiments showed that the scaffold extracts do not have cytotoxicity in any concentration. Our results suggested that the introduced scaffolds are comparable with the trabecular bone from the compositional, structural, and mechanical perspectives and have a great potential to be used as bone substitute.

Keywords: Bone tissue engineering; Cellulose; Hydroxyapatite; Nanocomposite

1. Introduction

Bone repair is a major issue in maxillofacial orthopedic surgery because of various bone diseases, such as bone infections, bone tumors, skeletal abnormalities, and bone loss by trauma¹. The traditional treatments for bone defects include autografts, allografts, and xenografts. Although autografts are well suited for bone regeneration, they have to be dissected from bone tissue from another site. This process not only causes donor site morbidity but also difficulty in constructing a graft of the desired shape using the dissected tissue^{2,3}. Moreover, pathogen transfer or immune reaction to allografting and xenografting is a possibility^{4,5}. To overcome the above-mentioned drawbacks, the fabrication of an ideal synthetic bone graft has been devoted to bone tissue engineering, in which a 3D porous scaffold of ceramic (i.e., calcium phosphate)⁶, synthetic polymers (i.e., PVA, PCL, and PLGA)⁷⁻⁹, natural polymers (i.e., chitosan, gelatin, and cellulose)¹⁰⁻¹², or their composites¹³⁻¹⁵ is used to mimic the natural bone behavior to some extent.

As an interesting biomaterial with potential orthopedic and dental applications hydroxyapatite (HA) has good mechanical properties¹⁶, osteoconductivity¹⁷, bioactivity¹⁸, and bone bonding ability¹⁹. HA is not biodegradable and can be removed and remodeled in the host. If implanted directly, it may dislocate within the tissue²⁰. To eliminate such an undesired mobility, HA is usually mixed with natural or synthetic polymers, such as cellulose (Cel) and polyacrylamide (PAAm)²¹⁻²⁴. Cel is the most abundant material in most plants and bacteria. It degrades slowly because of the absence of enzymes that attack the linkage. Cel is also a promising material for biomedical applications, including drug delivery and tissue engineering, because of its biocompatibility, non-toxicity, reactive surfaces for protein binding, biodegradability, readily

modified properties, and mechanical strength and resistance to breakdown in vivo ²⁵. However, only a few studies have been conducted on the application of Cel as a scaffold for cell culturing because of the absence of an intrinsic 3D architecture. Further, Cel has some other drawbacks such as low mechanical properties. As a result, the use of another synthetic or natural polymer may provide additional 3D architecture for Cel based scaffolds. PAAm is a synthesized polymer used in biomedical engineering and tissue engineering ²⁶⁻²⁹ because of its non-toxic and biologically inertness, capacity for preserving its shape and mechanical strength, and convenient adjustability of mechanical, chemical, and biophysical properties ^{30,31}. However, brittleness as its poor mechanical property caused by the lack of an efficient energy dissipation mechanism and the irregular distribution of cross-linking points have limited further research on using PAAm solely ^{32,33}.

The work aimed to develop and characterize a new 3D nanocomposite scaffold made of Cel, PAAm, and nanopowders of HA (n-HA) for use in bone repair applications to solve the above-mentioned shortcomings. To this end, hydroxyapatite/cellulose-graft-polyacrylamide (HA/Cel-g-PAAm) nanocomposite scaffolds were synthesized by freeze-drying method. The morphology and structural characteristics were observed by scanning electron microscopy (SEM), Fourier transform infrared spectroscopy (FTIR), energy-dispersive X-ray spectroscopy (EDX), and X-ray diffraction analysis (XRD). The biocompatibility of the scaffolds was tested by culturing HuGu cells in vitro. The mechanical properties of the scaffolds were measured by tensile test instrument. The swelling behavior of the scaffolds was examined in both water and phosphate buffer saline (PBS) solution. To our knowledge, this study is the first on the HA/Cel-g-PAAm composite for 3D scaffolds.

2. Materials and methods

2.1 Materials

Microcrystalline cellulose extracted from cotton linters with 20 μm particle size and 0.5 g.L^{-1} bulk density (reported by the manufacturer, Aldrich) was used as a basic of composite construction. The cellulose was dissolved in a 5 % w/v mixture of dimethylformamide and lithium chloride (TAT, Iran). n-HA (CAM implants, Netherlands), potassium persulfate (BDH Chemicals, UK) as an initiator, acrylamide (Merck) as a monomer, and N,N'-methylene-bis-acrylamide (Aldrich) as a crosslinker were applied. Sodium chloride, potassium chloride, sodium hydrogen phosphate, and di-potassium hydrogen phosphate were used as received (TAT, Iran) for the preparation of PBS solution.

2.2 Synthesis of nanocomposite scaffolds

In this study, the nanocomposite scaffolds were prepared using the prepared HA/Cel-g-PAAm nanocomposite powders, which were synthesized through free radical polymerization. First, Cel (0.8 g) was dissolved in a mixture of lithium chloride (3 g) and dimethylformamide solution (60 mL) and stirred at 70 °C in a flask fitted to an argon gas inlet. After complete dissolving of Cel, potassium persulfate (0.06 g) was added to the Cel solution. Third, acrylamide (1.2 g), N,N'-methylene-bis-acrylamide (0.03 g), and different amounts of n-HA were added gently into the initiated Cel solution, and the solution was stirred under argon gas at 68 °C. After 3 h and complete graft polymerization of acrylamide onto Cel, the prepared nanocomposite powder was dispersed in distilled water many times to remove unreacted monomer, initiator, and solvent. Finally, the prepared nanocomposite powders were vacuum-filtered and dried at 50 °C in an oven overnight. Using this method, five different nanocomposite powders containing 0.18, 0.36,

0.54, 0.72, and 0.90 g of n-HA, namely, H1, H2, H3, H4, and H5, respectively were synthesized. To prepare the scaffolds, 0.1 g of HA/Cel-g-PAAm nanocomposite powders was dispersed in a diluted acetic acid solution (4 %) and stirred at room temperature for 72 h. After sonication for 90 s, the solutions were placed into plastic molds and kept in a freezer at -20 °C for 24 h. Finally, the solidified mixtures were transferred into a lyophilizer (FD-10, Pishtaz Engineering Co., IRAN) and then freeze-dried up to -56 °C (vacuum 6.2 mbar) for 8 h and then warmed to -5 °C (1.4 mbar) for 6 h and to 24 °C (0.98 mbar) for 2 days in the main drying phase (see Scheme 1).

Scheme 1

2.3 Characterization of the prepared scaffolds

The synthesis of HA/Cel-g-PAAm nanocomposite powders was verified by a FTIR spectrophotometer (Perkin-Elmer 8700, Japan) in the range of 400–4000 cm⁻¹. The major phases

of the scaffolds were analyzed using X-rays diffractometer (EQuniox 300, France) using Cu-K α radiation (1.54 Å) at a 2 θ range of 10-70° with voltage and current settings of 40 kV and 30 mA, respectively. The morphology and chemical composition of the prepared scaffolds were characterized using SEM-EDS (Tescan Vega 3 LMU with Bruker Quantax EDS, Czech Republic). The mechanical properties of the prepared scaffolds were tested by SANTAM compression test apparatus (ASTM D5024-95, Iran) at a loading rate of 0.5 mm/min using the circular shape scaffolds with dimensions 5 mm diameter and 10 mm height. The elastic modulus and compressive strength of the scaffolds among the other mechanical properties were selected to be calculated using the stress–strain curve. The elastic modulus is the slope of the initial linear portion, and the compressive strength is the maximum point of the curve.

2.4 Porosity determination of the scaffolds

The apparent porosity percentage of the prepared nanocomposite scaffolds was determined through liquid displacement method³⁴. At first, the scaffolds were placed in a graduated cylinder of water and kept under water to fill the pores of scaffolds. Then, the filled pore scaffolds were removed from water. The porosity (%) of the prepared scaffolds were calculated using the following equation:

$$\text{porosity (\%)} = \frac{V_1 - V_3}{V_2 - V_3} \times 100 \quad (1)$$

Where V_1 is initial volume of water before immersion of the scaffold, V_2 is volume of water after immersion of the scaffold and V_3 is final volume of water after removing of filled pores scaffold. In addition, the pore size of the prepared scaffolds in 10 different cross-sectional SEM micrographs representing different regions of the scaffold was calculated using ImageJ software. The dimensions of more than 50 pores were measured and averaged to obtain a mean pore size.

2.5 Swelling behavior of the prepared scaffolds

The swelling behavior and the structural stability of scaffolds are critical to their practical use in tissue engineering³⁵. The swelling properties of the synthesized nanocomposite scaffolds were evaluated in water and PBS solution. The dried pieces of the prepared nanocomposite scaffolds were weighed and immersed in water and PBS solution. The scaffolds began to soak and imprison liquids in their macropores for a time period until equilibrium was reached. At the equilibrium stage with further increases in time, large differences in the swelling percentage of the scaffolds were not observed. The swollen nanocomposite scaffolds were blotted and weighed after removing excess water on their surface. The swelling percentage was determined using the following equation³⁶:

$$\text{Swelling (\%)} = \frac{W_s - W_d}{W_d} \times 100 \quad (2)$$

Where W_s and W_d are the weights of the swollen and the dried scaffolds, respectively.

Note that the PBS solution was prepared by dissolving four salts, namely, NaCl (8 g), KCl (0.2 g), Na_2HPO_4 (1.44 g), and KH_2PO_4 (0.24 g), in distilled water (800 mL). After adjusting the pH of the solution to 7.4 with HCl (1 mol.L^{-1}), the total volume of the solution reached 1 L .

2.6 Cytotoxicity evaluation of the prepared scaffolds

Human gum fibroblast cells (HuGu) from the Iranian Biological Resources Center (IBRC) were cultured in Dulbecco's Minimum Eagle's Medium (DMEM) supplemented with 2 mmol.L^{-1} glutamine and 10% fetal bovine serum (FBS) and maintained in an 5 % CO_2 incubator at 37°C . When the cells reached the stage of confluence, they were harvested by trypsinization and were added a fresh culture medium to create a cell suspension. The 3-(4,5-dimethylthiazol-2-yl)-2,5-

diphenyltetrazolium-bromide (MTT, Aldrich) assay, which was based on the extraction method, was applied to evaluate the cytotoxic effect of the prepared scaffolds. A cell suspension with a concentration of 5000 cells in 100 μL of medium was loaded into the well of a 96-well flat-bottom culture plate and incubated at 37 °C in an incubator. After seeding of the fibroblast cells, they were treated with seven different concentrations of H1 and H5 scaffold extracts (0.03-2.00 mg.mL^{-1}). After different incubation times (24, 48, and 72 h) and the aspiration of the medium, 20 μL of MTT solution (5 mg.mL^{-1} of PBS) was added to each well, and then incubation was conducted at 37°C for 4 h. The upper solvent was removed, and 200 μL of dimethyl sulfoxide (DMSO) was added to dissolve the precipitated formazan crystals for 6 h at 37°C. Three parallel replicates were analyzed for each sample. The controls (empty wells without scaffolds extracts) were treated in the same manner. Finally, the plate cover was removed, and the optical density (OD) values were recorded at 570 nm by the absorbance microplate reader (Bio-Tek, ELx-800, USA). The cell viability was calculated according the following equation ³⁷:

$$\text{Cell viability (\%)} = \frac{\text{OD Sample}}{\text{OD Control}} * 100 \quad (3)$$

3. Results and Discussion

3.1 Characterizations of the prepared scaffolds

3.1.1 Fourier transforms infrared (FTIR) analysis

To confirm the graft polymerization of acrylamide onto Cel and to show the presence of various n-HA amounts, the FTIR spectra of the prepared nanocomposite scaffolds were investigated (Figure 1). In the spectra of all samples, the broad peak in the 896–1235 cm^{-1} wavenumber range was related to the pyranoid ring of the Cel. The strongest and broadest peak associated with the asymmetric C-O-C bridge stretching of the Cel ring was observed at approximately 1082 cm^{-1} in

the spectra of all samples. In addition, the broad absorption peak between $3,000\text{ cm}^{-1}$ and $3,700\text{ cm}^{-1}$ belongs to the stretching vibration of the hydroxyl groups of Cel and n-HA observed. The absorption band at $1,650\text{ cm}^{-1}$ attributed to the stretching vibration of C=O bonds in PAAm confirmed the graft polymerization of PAAm onto Cel. The modifications that occurred during the interaction of n-HA with Cel-g-PAAm can be confirmed by the presence of three absorption bands at 1092 cm^{-1} , 1029 cm^{-1} and 961 cm^{-1} , which correspond to the symmetric and asymmetric stretching modes of phosphate groups (PO_4^{3-}) in n-HA ³⁸. The absorption peaks were from 566 cm^{-1} to 633 cm^{-1} and were assigned to the asymmetric bending modes of the O-P-O bonds of the phosphate groups in n-HA ³⁹. The intensity of the peaks assigned to n-HA was improved by increasing the amounts of n-HA in the prepared scaffolds (H5> H4> H3> H2> H1). These supportive results confirmed the graft polymerization of AAm onto Cel and the synthesis of Cel-g-PAAm-based nanocomposites with different n-HA contents.

Figure 1

3.1.2 X-ray diffraction (XRD) analysis

Figure 2 shows the XRD patterns of the synthesized nanocomposite scaffolds. For all of the samples, the crystalline peaks observed at approximately 23.2°, 26°, 29.3°, 32.2°, 46.6°, and 49.4° confirmed the presence of n-HA in the scaffold structure⁴⁰. As shown in Figure 2, the intensity of the mentioned peaks clearly increased by the increase in the amounts of n-HA in

samples H1 to H5. Furthermore, a diffraction peak was detected at $2\theta=16^\circ$, which was assigned to the reflection of Cel-g-PAAm.

Figure 2

3.1.3 Scanning electron microscopy (SEM) and energy-dispersive X-ray (EDS) analysis

SEM images of the prepared nanocomposite scaffolds are shown in Figure 3. All the scaffolds had irregular interconnected pores. These sub-micro pores created a rough texture on the sample surface, which is essential for cell attachment and proliferation. The average sizes of these pores are listed in Table 1. The size was in the range of 72-125 μm , which is appropriate for the

vascularization process in tissue engineering applications. In addition, the maximum porosity percentage was calculated 85.7 % for the H5 scaffold. As shown in Table 1, increasing the amount of n-HA in the composition of the scaffolds from H1 to H5 led to the increase in the porosity percentage, but it decreased the pore size of the scaffolds because of the increase in crystallinity of the prepared nanocomposite scaffolds. Manicini et al. found that the porosity of the scaffolds increased with the increase in crystallinity ⁴¹. The results show that the prepared scaffolds are similar in appearance to the cancellous bone with 75 %–85 % porosity and 300–600 μm diameter size.

Figure 3

Table 1. Average pore size, porosity and mechanical properties of the prepared nanocomposite scaffolds

Sample	Pore size (μm)	Porosity (%)	Compressive strength (MPa)	Elastic modulus (GPa)
H1	125 ± 4	67.3 ± 2	1.21 ± 0.1	0.11 ± 0.01
H2	110 ± 5	73.8 ± 3	3.32 ± 0.3	0.49 ± 0.02
H3	94 ± 2	76.1 ± 1	5.97 ± 0.2	0.69 ± 0.02
H4	83 ± 2	82.4 ± 2	1.67 ± 0.1	0.78 ± 0.01
H5	72 ± 3	85.7 ± 3	1.59 ± 0.3	0.89 ± 0.01
Trabecular bone ^{46,47}	300-600	75-85	2-6	0.1-0.3

The X-ray emitted from a random place in the wall's pore of the H5 nanocomposite scaffold during bombardment by an electron beam was analyzed, and the EDS spectrum was obtained. Figure 3 displays the main signals for Cel (i.e., carbon and oxygen), AAm (i.e., carbon, oxygen, and nitrogen), and n-HA (i.e., calcium, phosphorus, and oxygen). According to the EDS spectrum, the walls of the pores are composed of HA/Cel-g-PAAm nanocomposites within the scaffolds.

3.2 Mechanical properties

When using porous structures for tissue engineering scaffolds, the structure must retain sufficient mechanical properties to fulfill the requirements of structural integrity once implanted in the host tissue ⁴². The elastic modulus and compressive strength of the scaffolds were calculated and they are listed in the Table 1. The elastic modulus was the slope of the initial linear portion and the compressive strength was the maximum point of the curve. As shown in Table 1, the elastic modulus of nanocomposite scaffolds was enhanced by increasing the amount of n-HA in samples

H1 to H5 because of the high elastic modulus (an intrinsic property) of ceramics compared with polymers. Although the compressive strength of the scaffolds was expected to increase by increasing the n-HA contents and subsequently increasing the crystallinity of nanocomposite, the results indicated that sample H3 instead of H5 had the highest compressive strength. This behavior can be explained by the fact that the compressive strength is affected by the other factors, such as porosity, aside from crystallinity. As, a high porosity causes a decrease in the mechanical strength of porous materials, the H4 and H5 nanocomposite scaffolds with the higher porosity than H3 had a lower compressive strength.

3.3 Swelling behavior of composite

As shown in Figure 4, the structural stability of the scaffolds in water and PBS solution, which matches with the human body solution from the perspective of osmolarity and ion concentrations, was evaluated in terms of swelling percentage. When the scaffold immersed in the liquid, the water molecules were in contact with the pores of the scaffold. The osmotic pressure difference between the solution and the scaffold increased. Thus, the scaffold started to uptake water molecules to overcome this difference ⁴³. The molecules penetrated inside the nanocomposite scaffold network through its pores. Therefore, the high swelling rate was observed at the beginning. Clearly, this swelling system could not continue forever. The osmotic pressure difference between the solution and the scaffold might be reduced by the increasing water scaffold interactions until the equilibrium state was balanced ⁴⁴. The results exhibited that the swelling percentage of the scaffolds was inversely proportional to the n-HA concentration in the nanocomposite and that the equilibrium swelling ratio decreased from H1 to H5. The result could be due to the low hydrophilicity of n-HA particles compared with that of Cel-g-PAAm.

Furthermore, the swelling ratio reached equilibrium in the PBS solution more quickly than that in distilled water. The maximum swelling of H1 in distilled water was 115.8 %, which was greater than 69.3 % in the PBS solution. These results can be explained using the role of n-HA as a crosslinker. The existence of n-HA resulted in the generation of ionic interactions with the mono- and multivalent ions in the PBS solution. Thus, it led to the reduced initial osmotic pressure difference between the external solution and the scaffold. Therefore, the swelling capacity of the nanocomposite scaffolds in the PBS solution decreased.

Figure 4

3.4 Cytotoxicity of the prepared scaffolds

Biomaterial cytotoxicity can emanate from the original material itself and from the by-products that may leach out from the material ⁴⁵. The cytotoxicity of the seven scaffolds' (H1 and H5) extracts was estimated by evaluating the viability of HuGu cells using an MTT assay. As shown in Figure 5, extracts of both scaffolds with different concentrations have good cell viability, and they were higher than that of the control samples after 72 h of culturing, thus indicating a higher proliferation rate. From the results of OD and cell viability curves for both scaffolds, the prepared nanocomposite scaffolds with different n-HA contents in the concentration of each extract is not cytotoxic. In addition, the optical imaging of the HuGu fibroblastic cells in Figure 6 shows that the number of cells increased during the culturing time. After 72 h, the cells were densely arranged and seemed to be pile up on each other. The prepared cytocompatible and non-toxic scaffolds can be concluded to potentially be used in biomedical applications.

Figure 5

Figure 6

4. Conclusions

In summary, by employing the freeze-drying method, HA/Cel-g-PAAm nanocomposite scaffolds were successfully manufactured. Microscopy images demonstrated that these novel scaffolds

have a porous, interconnected, and rough structure. The results confirmed that by increasing the n-HA amounts in the structure of scaffolds, pore size decreased from 125 μm to 72 μm , while porosity increased from 67.3 % to 85.7 %. Structural analysis results showed that n-HA powders were uniformly dispersed and successfully incorporated into the Cel-g-PAAm matrix. Compared with the trabecular bone, the n-HA/Cel-g-PAAm scaffolds showed close mechanical strength, which could be controlled by varying the amount of n-HA. HuGu cells cultured in the n-HA/Cel-g-PAAm scaffolds showed that the scaffolds are non-toxic and have good biocompatibility. Based on our observations, the introduced novel scaffolds are considered a promising candidate for bone tissue engineering application.

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Figure captions

Scheme 1. The schematic illustration for the preparation of scaffold

Fig. 1 The peaks in FTIR spectra of the prepared nanocomposite scaffolds (H1-H5) in the range of 400-4000 cm^{-1}

Fig. 2 The XRD patterns of the prepared nanocomposite scaffolds (H1-H5)

Fig. 3 The SEM images of the prepared nanocomposite scaffolds. EDS point analysis for elemental composition of the prepared nanocomposite scaffold (H5)

Fig. 4 The swelling behavior of the prepared nanocomposite scaffolds (H1-H5) in distilled water and PBS solution

Fig. 5 MTT assay of HUGU cells for the scaffolds (H1 and H5) extracts in terms of optical density values and the normalized cell viability (%) after 24, 48, and 72 h.

Fig. 6 Optical images of the cell morphology in the presence of the scaffolds (H1 and H5) extracts after 24, 48, and 72 h.

HA/Cel-g-PAAm
nanocomposite powder

Freeze dryer

HA/Cel-g-PAAm
nanocomposite scaffold

SEM image