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Biocompatible nanocomposite scaffolds based on copolymer-grafted chitosan for bone tissue engineering with drug delivery capability

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ABSTRACT

Significant efforts have been made to develop a suitable biocompatible scaffold for bone tissue engineering. In this work, a chitosan-*graft*-poly(acrylic acid-*co*-acrylamide)/hydroxyapatite nanocomposite scaffold was synthesized through a novel multi-step route. The prepared scaffolds were characterized for crystallinity, morphology, elemental analysis, chemical bonds, and pores size in their structure. The mechanical properties (i.e. compressive strength and elastic modulus) of the scaffolds were examined. Further, the biocompatibility of scaffolds was determined by MTT assays on HUGU cells. The result of cell culture experiments demonstrated that the prepared scaffolds have good cytocompatibility without any cytotoxicity, and with the incorporation of hydroxyapatite in their structure improves cell viability and proliferation. Finally, celecoxib as a model drug was efficiently loaded into the prepared scaffolds because of the large specific surface area. The in vitro release of the drug displayed a biphasic pattern with a low initial burst and a sustained release of up to 14 days. Furthermore, different release kinetic models were employed for the description of the release process. The results suggested that the prepared cytocompatible and non-toxic nanocomposite scaffolds might be efficient implants and drug carriers in bone-tissue engineering.

1. Introduction

The typical goal of a bone tissue engineering approach is to develop bone graft replacements that can repair bone defects without the need for allografts or autografts [1]. With this approach, the porous scaffold serves an important role in the manipulation of the functions of osteoblasts and a central role in the guidance of new bone formation into desired shapes. On the other hand, a sharp rise in musculoskeletal diseases and disorders often demands a drug treatment at the specific defect site [2,3]. Local drug delivery systems are alternative tools in modern medicine because they can assure the optimal level of the drug for longer periods of time and the reduction in possible undesirable side effects. In bone tissue engineering, the term "drug" refers to therapeutic agents such as antibiotics, anti-cancers or anti-inflammatories [4,5]. The potential scaffold as a drug carrier must have the ability to incorporate a drug either physically or chemically, retain the drug at the defect site, and deliver the drug in a controlled manner over time [6–8]. This procedure can simplify treatment for the patient as surgery and drug delivery that combined into one procedure rather than requiring post-surgical systemic and oral administration.

In recent years, chitosan and its applications in the field of tissue engineering have attracted considerable attention [9,10]. It has been studied as a useful biomaterial in diverse tissue engineering applications because of its hydrophilic surface that promotes cell adhesion, proliferation and differentiation, good biocompatibility and host response, biodegradability by lysozyme and other enzymes, bactericidal/bacteriostatic activity, and the capacity to maintain a predefined shape after cross-linking [11–14]. However, chitosan is fragile and does not have enough mechanical properties suitable for bone tissue engineering. The principal advantage of synthetic materials is that their porosity, mechanical strength, and degradation rate can be easily tuned by varying the structures of the polymer and/or the cross-linker. An example of that is poly(acrylic acid), which is a known biocompatible anionic polymer that has been used as a Food and Drug Administration (FDA) approved basic component of a bone cement implant [15]. Another example is polyacrylamide, which is also a synthesized polymer made of a reactive acrylamide monomer [16]. Although the toxicity of the acrylamide monomer has been documented, polyacrylamide is widely used in biomedical applications and drug delivery [17,18]. The mechanical properties of the mentioned polymers still need to be improved which can be achieved by forming a composite of polymer-ceramic materials [19]. Hydroxyapatite (HAp) is one such ceramic, which is a natural mineral constituent of human bone and teeth [20,21]. Biocompatibility, excellent bioactivity and osteoconductivity, being

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non-toxic and non-inflammatory, and excellent chemical and biological affinity with bony tissue of HAp are some of the reasons that HAp is increasingly being explored as bone substitute and drug delivery systems for numerous applications in nano-medicine, orthopedics, and dentistry [4,22,23]. To our knowledge, experimental investigations concerning the composition and synthesis of chitosan-*graft*-poly(acrylic acid-*co*-acrylamide)/n-HAp scaffold as a bone substitute with drug carrier potential have not yet been undertaken.

The aim of the present study is to not only introduce a novel nanocomposite scaffold as a bone substitute, but also reduce the risk of implant failures with anti-inflammatory drug delivery potential. To this end, novel composite scaffolds containing chitosan-*graft*-poly(acrylic acid-*co*-acrylamide)/n-HAp using a freeze-drying method were introduced. These scaffolds were characterized by Fourier transform infrared spectroscopy (FTIR), X-ray diffraction analysis (XRD), scanning electron microscopy (SEM), and energy-dispersive X-ray spectroscopy (EDS). Prior to the estimation of drug delivery, the evaluation and verification of their biocompatibility and non-toxicity in the presence of HUGU fibroblastic cells were examined. Drug loading and releasing characteristics of the prepared nanocomposite scaffolds and their kinetics of release were investigated using celecoxib as a model drug. Celecoxib is a selective COX-2 inhibitor drug and is the only non-steroidal anti-inflammatory drug (NSAID) that has been approved by the FDA for adjuvant treatment of patients with familial adenomatous polyposis to date [24–27]. Celecoxib has also been shown to inhibit the surviving protein expression in human cancer cells [28–30]. We hypothesized that the celecoxib-loaded scaffold could provide a novel and effective drug carrier to bone healing.

2. Materials and methods

2.1. Materials preparation

Chitosan (average molecular weight, 300,000 $g \cdot mol^{-1}$ and degree of deacetylation (DD), 95%, Aldrich) was dissolved in a dilute solution of acetic acid (Merck). Acrylamide (Merck) and acrylic acid (Merck) were used as monomers, and *N*,*N*′-methylene-bis-acrylamide (Aldrich) and glutaraldehyde (25% in H_2O, Aldrich) were crosslinkers. Celecoxib as a model drug was extracted from a commercial pill (Celexib® 200, DarouPakhsh Pharmaceutical Mfg. Co. Iran). Nano-HAp was prepared using disodium hydrogen orthophosphate (Analar) and calcium chloride (Aldrich). Sodium hydroxide (Aldrich), sodium chloride (Merck), potassium chloride (Merck), disodium hydrogen phosphate (Merck), potassium dihydrogen phosphate (Merck) and ethanol (Merck) were used as received.

2.2. Synthesis of nano-hydroxyapatite (n-HAp)

In this study, the n-HAP was synthesized through a micro-emulsion technique [31]. A calcium chloride solution (2 $mol \cdot L^{-1}$) was prepared in a three-necked flask, connected to nitrogen gas, and the temperature was increased to 65 °C. Then, 100 mL of disodium hydrogen phosphate (1.2 $mol \cdot L^{-1}$) solution was added drop by drop to the calcium chloride solution at a rate of 2 $mL \cdot min^{-1}$ while stirring vigorously. The pH of the solution during the reaction of calcium chloride with disodium hydrogen phosphate decreased due to the preparation of hydrochloric acid (see reaction (1)). Therefore, the pH was controlled at 10 ± 0.5 by the addition of a sodium hydroxide solution (1 $mol \cdot L^{-1}$). The product was filtered and washed several times with distilled water and dried overnight in an oven at 60 °C. Thus, n-HAp was formed through the following reaction:

$$10CaCl_2 + 6Na_2HPO_4 + 2H_2O \rightarrow Ca_{10}(PO_4)_6(OH)_2 + 12NaCl + 8HCl \quad (1)$$

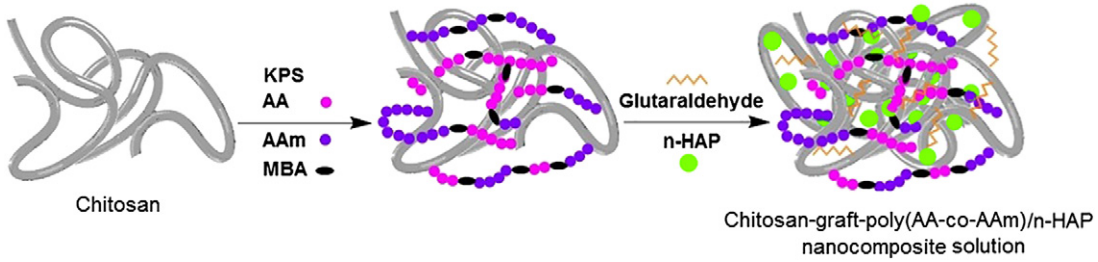
2.3. Fabrication of copolymer grafted chitosan nanocomposite scaffolds

The chitosan-*graft*-poly(acrylic acid-*co*-acrylamide)/n-HAP nanocomposite scaffolds were synthesized using the following three steps: (I) the preparation of acrylic acid-co-acrylamide (AA-co-AAm)/HAP copolymer grafted chitosan nanocomposite solution; (II) the freeze-drying of the solution and the washing, filtering, and dissolving of nanocomposite powder in a suitable solvent; and (III) the freeze-drying of a molded nanocomposite solution and the preparation of scaffolds.

First, a chitosan solution (1.6% W/V) was prepared by dissolving chitosan powder in diluted acetic acid solution (2% V/V) in a two-necked round-bottom flask fitted with a nitrogen gas inlet in a water bath at 60 °C with continuous stirring. Second, the initiator, potassium persulfate (0.04 g), was added to the chitosan solution. After 15 min, acrylic acid (0.25 mL), acrylamide (0.25 mg) and *N*,*N*′-methylene-bis-acrylamide (0.04%) were introduced into the above solution in the flask as monomers and a crosslinker, respectively. After stirring the mixture for 30 min, the different amounts of n-HAp powder and glutaraldehyde (25 wt%) were added to the solution, which was then stirred vigorously for 3 h. Finally, the graft polymerization and crosslinking was stopped by cooling the solution and removing the nitrogen gas flow before reaching the gelation point. As shown in Scheme 1, the product of Step 1 was named as the nanocomposite solution, and it was used in the synthesis of the nanocomposite scaffolds. By employing this method, different nanocomposites with different weight ratios of chitosan/n-HAp 100:0, 100:25, 100:50, and 100:75 were synthesized and named S-0, S-1, S-2, and S-3, respectively.

In Step II, the prepared nanocomposite solution was transferred into a freeze-dryer (FD-10, Pishtaz Engineering Co., Iran) for 48 days. The obtained nanocomposite powder was dispersed in a large volume of distilled water several times to dissolve and remove the remaining unreacted monomers, potassium persulfate, and *N*,*N*′-methylene-bis-acrylamide. After that, the vacuum-filtered powder was dried in an oven at 50 °C overnight. Finally, the nanocomposite powder was dissolved in a solution of diluted acetic acid (10%) at room temperature with vigorous stirring overnight.

For the synthesis of the grafted nanocomposite scaffold in Step III, the crosslinked chitosan-*graft*-poly(AA-*co*-AAm) solution containing HAp, which was prepared in Step II was cast into an aluminum mold and transferred to a freezer at −20 °C for 24 h. The solidified mixture was then placed in a lyophilizer and faced three phases: (I) freezing up to −70 °C (vacuum 6.4 mbar) for 24 h; (II) warming to −15 °C



Scheme 1. The schematic illustration of the synthesis of chitosan-*graft*-poly(AA-*co*-AAm)/n-HAp nanocomposite.