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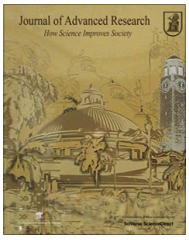

ORIGINAL ARTICLE

In vitro study of nano-hydroxyapatite/chitosan–gelatin composites for bio-applications

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ABSTRACT

The present work aims to study the *in vitro* properties of nano-hydroxyapatite/chitosan–gelatin composite materials. *In vitro* behavior was performed in simulated body fluid (SBF) to verify the formation of apatite layer onto the composite surfaces. The *in vitro* data proved the deposition of calcium and phosphorus ions onto hydroxyapatite /polymeric composite surfaces especially those containing high concentrations of polymer content. The degradation of the composites decreased with increase in the polymeric matrix content and highly decreased in the presence of citric acid (CA), especially these composites which contain 30% polymeric content. The water absorption of the composites increased with increase in the polymeric content and highly increased with CA addition. The Fourier transformed infrared reflectance (FT-IR) and scanning electron microscope (SEM) for the composites confirmed the formation of bone-like apatite layer on the composite surfaces, especially those containing high content of polymers (30%) with 0.2 M of CA. These promising composites have suitable properties for bio-applications such as bone grafting and bone tissue engineering applications in the future.

Introduction

Hydroxyapatite (HA) is derived either from natural sources or from synthetic sources and regarded as bioactive substance, since it forms a strong chemical bond with host bone tissue, and hence, it is recognized as a good bone graft material. HA is not only bioactive but also osteoconductive, non-toxic and non-immunogenic, and its structure is crystallographically similar to that of bone mineral [1]. Li et al. reported that nano-hydroxyapatite (nHA) precipitates may have higher solubility and therefore affect the biological responses [2]. It has been shown that HA and its composites are suitable for attachment, proliferation, and differentiation of mesenchymal stem cells (MSCs), owing to their structure and chemical compositions [3]. Kong et al. [4] reported that the apatite-coated chitosan/nano-hydroxyapatite composite scaffolds cells presented better proliferation than on apatite-coated chitosan scaffolds. Therefore, the addition of nano-hydroxyapatite improved the bioactivity of chitosan/nano-hydroxyapatite composite scaffolds. Also, Xianmiao et al. [5] reported that the morphology and behavior of bone marrow stem cell (BMSC) cultured *in vitro* with the n-HA/chitosan (CS) composite membranes are

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observed under phase-contrast microscope. In chitosan–nHA scaffolds, pre-osteoblasts have high affinity to the surface of chitosan–nHA composite, which is attributed to its increase in surface area and composition [6].

For the increase in bioactivity and mechanical property, some composites of polymer and bioactive ceramics have been developed for bone tissue engineering. Among these composites, HAp/polymer composites have attracted much attention since such composites may have osteoconductivity due to the presence of HAp. Thus, HA/polymer composite scaffolds are of interest for biomedical applications [7]. HA–collagen composite is similar microstructure to native bone and showed osteoclastic resorption and good osteoconductivity [8] but the high cost of collagen, which limits its clinical application in healing bone defect to its insouciant formability and flexibility.

Polymers such as chitosan have a higher degradation rate than bioceramics; chitosan is a unique polysaccharide based biopolymer that shares a number of chemical and structural similarities with collagen [9]. Recently, chitosan (CS) being a natural biodegradable cationic polymer with good biocompatibility, much attention has been paid to chitosan-based biomedical materials [10]. Therefore, incorporation of HA into a chitosan polymer matrix has been shown to increase osteoconductivity and biodegradability with significant enhancement of mechanical strength [11]. Another example for natural polymer is gelatin, which is a biodegradable polymer with many attractive properties, such as excellent biocompatibility, non-antigenicity, plasticity and adhesiveness, and it is widely used in biomedical and pharmaceutical fields. Thus, gelatin was selected as a suitable candidate blended with chitosan [12]. Also, gelatin is blended with chitosan to improve the biological activity since (i) gelatin promotes cell adhesion and migration and (ii) forms a polyelectrolyte complex [13]. Citric acid found in bone in the form of citrate in 0.9 wt% [14]. The three carboxyl of citric acid provide more nucleation sites to formation of ultra fine nano-sized carbonate apatite and the increase in citric acid benefit the bone resorption and ossification through the formation of dissociated calcium citrate complexes in the surrounding body fluid [15].

The current work is aimed of the evaluation of bioactivity for the prepared nano-HA/chitosan–gelatin composites in the presence and absence of citric acid via *in vitro* study in the simulated body fluid (SBF). The *in vitro* study includes two assessments: the first is the measurement of calcium (Ca^{2+}) and phosphate PO_4^{3-} ions in SBF after withdrawal of the composite. The second is the degradation behavior, water absorption ability, FT-IR, and SEM-EDX analyses for these composites to confirm the bone-like apatite layer formation on their surfaces.

Experimental

Preparation of the biocomposites

The biocomposites were prepared according to Mohamed et al. [16]. Table 1 shows compositions for preparation of HA/chitosan–gelatin composites (HACG composites). Preparation of HA80CG20 composites with weight ratio of composition [(HA/chitosan–gelatin) (80:20)]. The chitosan–gelatin solution with a concentration of 4% was prepared by dissolving chitosan (2 g) into 2% acetic acid (50 ml) with stirring for 5 h to get a perfectly transparent solution and dissolving gelatin (2 g) into distilled water (50 ml). The chitosan and gelatin solutions were mixed together and then mixed with 9.37% solution of H_3PO_4. This solution was dropped slowly into 11.81% ethanol solution of Ca $(OH)_2$ with vigorous stirring. The pH of the mixture was adjusted with NaOH solution up to 10. The stirring was kept for 24 h after dropping and then the paste obtained was aged for another 24 h. Finally, the precipitate was filtered and washed with distilled water to remove the excess NaOH and dried in a vacuum oven at 70 °C. Preparation of HA/chitosan–gelatin composites in the presence of citric acid (CA) (HACGCA composites) was also performed according to Table 1. Dissolve each of chitosan and gelatin polymers in 0.2 M of CA solution. The above steps for preparation of HACGCA composites were repeated to obtain different HACGCA composites with various weight ratios of HA and chitosan–gelatin mixture, respectively.

In vitro behavior

Solution analysis

The SBF solution and solid composites were assessed pre- and post-immersion for different periods. The notations and compositions of HA/chitosan–gelatin composites (HACG composites) were shown in Table 1. To study the bioactivity, the composites were soaked in SBF which is proposed by Kokubo and Takadama [17] at body temperature (37 °C) and pH: 7.4 for different periods up to 28 days. The SBF has a composition similar to human blood plasma and has been extensively used for *in vitro* test. After the immersion periods, the solutions were analyzed by spectrophotometer to detect the calcium ions at $\lambda = 570$ nm and phosphorus ions concentration at $\lambda = 675$ nm [18]. Each test was repeated three times, and the average value was taken to confirm the results.

Table 1 Notations and compositions of HA/chitosan–gelatin composite materials (HACG composites).

Notations	HA/chitosan–gelatin (%)	$Ca(OH)_2$ (g)	H_3PO_4 (g)	Chitosan (g)	Gelatin (g)	Citric acid (CA) (%)
HA	100% HA	14.76	11.71	–	–	–
HA80CG20 comp.	80% HA:20% CG	11.81	9.37	2.00	2.00	–
HA80CG20CA comp.	80% HA:20% CG/CA	11.81	9.37	2.00	2.00	3.84
HA70CG30 comp.	70% HA:30% CG	10.33	8.20	3.00	3.00	–
HA70CG30CA comp.	70% HA:30% CG/CA	10.33	8.20	3.00	3.00	3.84
HA60CG40	60% HA:40% CG	8.86	7.30	4.00	4.00	–
HA60CG40CA comp.	60% HA:40% CG/CA	8.86	7.30	4.00	4.00	3.84

NB: The ratio between chitosan (C) and gelatin (G) is 1:1 in all composites.