Fabrication and Characterization of Nano-Hydroxyapatite\ Chitosan Biocomposites with and without Citric Acid Addition

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BIOCOMPOSITES composed of carbonated apatite and chitosan in the presence or absence of citric acid were fabricated through a co-precipitation method. The prepared biocomposites were characterized by Fourier transformed infrared spectroscopy (FT-IR), X- ray diffraction (XRD), Thermo gravimetric analysis (TGA), Transmission electron microscopy (TEM), and Scanning electron microscopy (SEM). The results proved that the presence of citric acid in the composite accelerated HA formation and reduced its particle size. Also, the compressive strength increased for HA/chitosan composite compared to HA alone and more increased with addition of citric acid which enhanced the binding strength between HA particles. The HA/chitosan composite with ratio 70% HA and 30% chitosan, especially with citric acid, may be promising for bone grafting and bone tissue engineering applications.

Keywords: Apatite, Chitosan, Biocomposites, Citric acid and TEM.

Hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) has been used for orthopedic and dental implants because it has a similar chemical composition and structure to the mineral phase of human bone[1]. Because of its low fracture toughness, hardness and brittleness, HA can`t serve as a bulk implant material under the high physiological loading conditions[2]. Carbonate apatite has been used as a biocompatible and osteoconductive substitute in the field of hard tissue repair and regeneration, especially nano-carbonate apatite elicits cell-biomaterial interactions because of its better resorption and close surface contact with the surrounding tissue[3].

Therefore, novel composites of hydroxyapatite and organic polymers have become of great interest to compensate the weak mechanical points of hydroxyapatite[4]. Chitosan possesses biocompatible, biodegradable[5], and anti-bacterial properties[6]. In addition, chitosan is flexible and has a high resistance up heating due to the intra- molecular hydrogen bonds formed between hydroxyl and amino groups [4]. Chitosan has been used as substitute for bone[7], cartilage [8] and nerve [6].

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Citric acid ($C_6H_8O_7$), which has a hydroxyl and three carboxyl groups in its molecule, is found in bone in the form of citrate in 0.9 wt% [9]. Citric acid plays an important role in citric acid cycle of humans. In general, chitosan and organic acid forms a chitosan-organic acid complex[4]. Hydroxyapatite/ polymer composites have osteoconductivity due to the presence of hydroxyapatite [10]. A composite biomaterial of HA and chitosan increased osteoconductivity and biodegradation together with sufficient mechanical strength for orthopedic use [11].

The objective of the present work is to prepare and characterize the nano HA/ chitosan polymeric composites in the presence and absence of citric acid. The compressive strength of the composites was also measured to evaluate the role of citric acid in the composite strength.

Materials and Methods

Materials

The starting materials for preparing hydroxyapatite (HA) are calcium hydroxide ($Ca(OH)_2$) and orthophosphoric acid (85% H_3PO_4) (Laboratory Rasayan). Other materials were chitosan polymer (viscosity 800 cps) with high molecular weight (M_r= 600,000) and deacetylation of 85% (Sigma-Aldrich), anhydrous citric acid (Morgan Chemical Ind. Co., Egypt), ethanol and 96% acetic acid (EL-Nasr Pharmaceutical Co., Egypt).

Methods

Preparation

Preparation of hydroxyapatite/chitosan composites: The compositions of hydroxyapatite/chitosan composites were given in Table 1. The Notations of the prepared composites were mentioned in Table 2. Preparation of HAC1 composite with weight ratio composition [(HA: chitosan (80:20)] was mentioned as follows: The chitosan solution with a concentration of 4% was prepared by dissolving chitosan (4 g) into 2% acetic acid with stirring for 5 hr to get a perfectly transparent solution. Afterwards, this solution was mixed with 9.37 % solution of H_3PO_4 and dropped slowly onto the 11.81 % ethanol solution of Ca $(OH)_2$ with vigorous stirring, kept for 24 hr after dropping and then the obtained paste was aged for another 24 hr. Finally, the precipitate was filtered, washed with distilled water to remove the excess of NaOH solution and then dried in a vacuum oven at 70°C.

Preparation of hydroxyapatite/chitosan/citric acid composites: Chitosan was dissolved in 0.2M citric acid instead of 2% acetic acid to prepare chitosan solution. Then, hydroxyapatite/chitosan/citric acid composites with different weight ratios of hydroxyapatite and chitosan polymer were fabricated by the same method of hydroxyapatite/chitosan composites preparation as in Table 1.

TABLE 1. The Compositions of hydroxyapatite/chitosan composites.

Samples	HA/chitosan (weight ratio)	Ca (OH)$_2$ (g)	H$_3$PO$_4$ (g)	Chitosan (g)
HA powder	100/0	14.76	11.71	--
HAC1 comp.	80/20	11.81	9.37	4.00
HAC2 comp.	70/30	10.33	8.20	6.00
HAC3 comp.	60/40	8.86	7.03	8.00
HAC4 comp.	50/50	7.38	5.86	10.00
HAC5 comp.	20/80	2.95	2.34	16.00

TABLE 2. The notations of hydroxyapatite/chitosan composites.

| HAC1 comp.	HAC2 comp.	HAC3 comp.	HAC4 comp.	HAC5 comp.
80%HA/20% chitosan composite	70%HA/3% chitosan composite	60%HA/40% chitosan composite	50%HA/50% chitosan composite	40%HA/60% chitosan composite

Sample characterization

The FT-IR spectrum was assessed in the range of 400 to 4000 cm^{-1} using Jasco, FT/IR 300 E, Fourier Transform Infrared Spectrometer, Serial No. 4140109, and Japan. The phase purity of samples was examined by X-ray diffractometer (Philips P.W: 1730) with Cu K$_\alpha$ target, C/S 1000. The thermo-gravimetric analysis of the samples was carried out using Shimadu TGA-50H under N$_2$ flow over rate 30 ml/min, Rate: 10°/min. The particle size of the HA powder for the composites was examined using the transmission electron microscope unit (JEM-1230 Japan), 0.2nm Resolution, 600 KX Magnification. The morphology of the prepared samples was examined by means of SEM, JXA 840A Electron Probe Microanalyzer (JEOL, Japan). The compressive strength was measured using Instron 5500 R the universal testing machine, USA; at a cross head speed of 1 mm/min and load cell was 10 KN. The average value was taken of three samples to ensure the results and size of the measured samples was 1 cm diameter × 1 cm thickness and shape of the sample was having a cylindrical shape.

Results and Discussions

FT-IR analysis

Figure 1 represents the FT-IR spectra of HA, chitosan and their composites. The bands of HA appeared at 3641 and 3440 cm^{-1} in the HA powder and were assigned to the stretching modes of vibration of OH group. In addition, the bands at 1457, 1460, 1422 and 877 cm^{-1} also appeared and were assigned to the CO$_3^{2-}$ groups. Also, the characteristic bands for PO$_4^{3-}$ groups were recorded at 1028, 603 and 569 cm^{-1} in HA powder. Shen *et al*. [12] proved that the band appeared at 1027 cm^{-1} which was assigned for the PO$_4^{3-}$ stretching mode. The bands of chitosan in chitosan powder appeared at 3446 cm^{-1} for OH, at 1652 cm^{-1} for amide I, at 1550-1540 cm^{-1} for amide II, at 1118 cm^{-1} for the C-O and at 2921 and 1458 cm^{-1} for the CH band[13]. Figure 1 also shows the FT-IR of HAC composites. The intensity of HA bands in all HAC composites such as OH at 3641, 3562 and at 3440 cm^{-1}, CO$_3^{2-}$ at 1457, 1460, 1422 and 877 cm^{-1} and PO$_4^{3-}$ at 1028, 603 and at 569 cm^{-1} were reduced compared to the original bands in the

HA powder proving the coating effect, especially in the case of the HAC2 composite. Also, the bands of chitosan in the spectra of HAC composites such as OH at 3446 cm^{-1}, CH at 2921 cm^{-1} and amide I at 1652 cm^{-1} were reduced compared to original polymer denoting interactions through hydrogen bonds as follows: (1) The OH of HA and chitosan, (2) the calcium of the HA with OH or amino group of chitosan, (3) the phosphate of the HA with the amino group of chitosan [14].

The FT-IR spectra of HA, chitosan, citric acid and their composites are shown in Fig. 2. The bands appeared for the citric acid powder were OH at 3500 and 3447 cm^{-1}, CH at 2988,1428, 1391,1356 and 1300 cm^{-1}, C-O at 1176, 1140, 1086 and 1086 cm^{-1} and C=O at 1750 and 1702 cm^{-1}[15]. It is clear that there is high reduction in the intensity of the bands in the HAC/citric acid composites compared to the same composites without citric acid proving high coating. Some bands such as OH at 3446 cm^{-1}, CH at 1356 & 1300 cm^{-1} and C-O at 1052 cm^{-1} were shifted to 3418, 1337, 1317, and 1031, respectively, proving interaction between hydroxyl groups of citric acid, chitosan polymer and HA through hydrogen bonding. Also, citric acid strongly interacted with calcium ions via its COO^{-} groups forming calcium citrate complex.
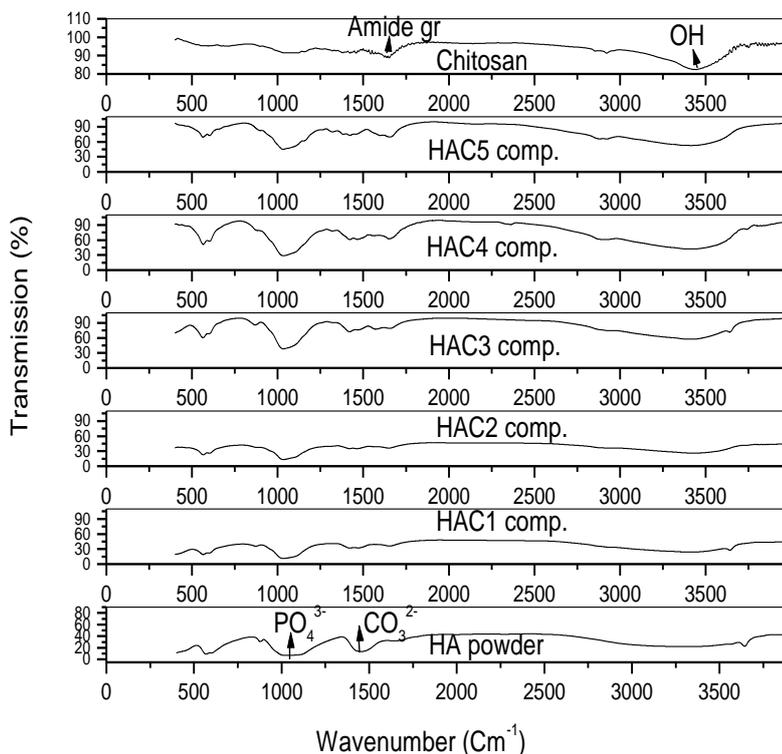
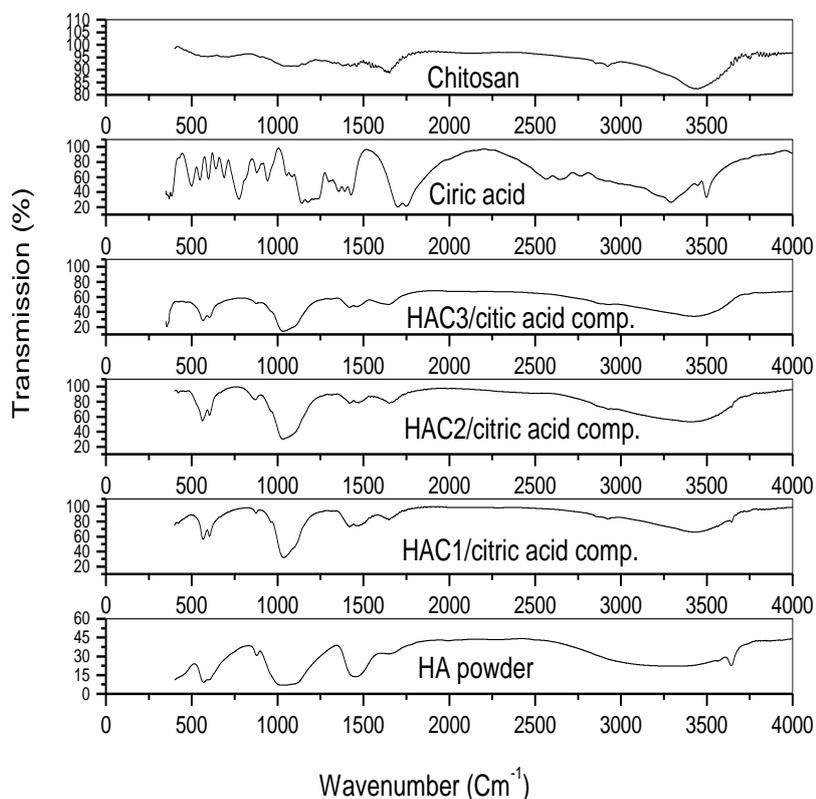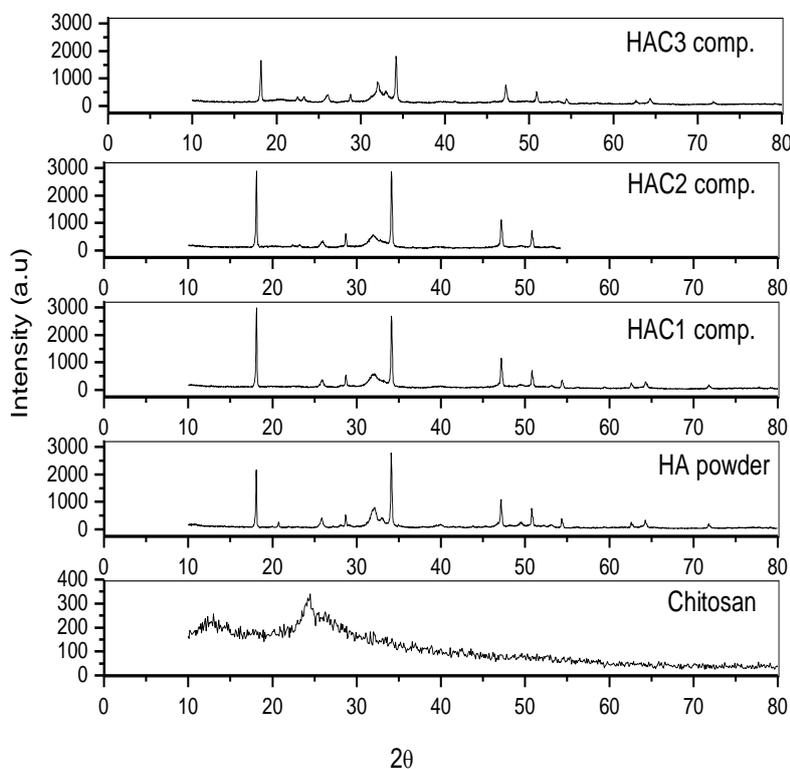

Fig. 1. The FT-IR of HA, chitosan and HAC composites.

Fig. 2. The FT-IR of HA, chitosan, citric acid and HAC/citric acid composites.

Phase analysis

Figure 3 shows the patterns of HA powder, chitosan and their composites. The spectrum of HA powder has the diffraction peaks of d-spacing (Å) equal to 4.06, 3.4, 3.1, 2.8, 2.7, 2.6, 1.79, 1.7, 1.48, 1.4 and 1.3 (Card No.9.432). The broadening diffraction peaks from $2\theta=30-35$ for the prepared HA denote that HA is composed of small crystals and are poorly crystalline[16]. The peaks of calcium hydroxide appeared in the prepared HA powder at the d-spacing equal to 4.9, 3.1, 2.6, 1.9, 1.79, 1.68 and 1.4 (Card No.87-0673) proving that part of calcium hydroxide is un-reacted to form further HA particles (Fig. 3). In this domain, Liang *et al*. [17] reported that the calcium hydroxide promotes the repair of bone tissues by providing rich Ca^{2+} ions and alkaline environment to deposit the minerals and stimulate calcification enzyme activity of osteoblasts. Several experimental studies have shown that calcium hydroxide may possess antimicrobial [18] and anti-inflammatory properties [19]. Therefore, the presence of calcium hydroxide in HA powder within the HAC composites is important for bone grafting and bio-applications.

Fig. 3. The XRD of HA powder, chitosan and HAC composites .

The XRD patterns of HA, chitosan and HAC composites are shown in Fig. 3. The peaks of chitosan appeared at 2θ within the range of (20-30) and it has a hump peak because of its amorphous structure [13]. The intensity of all peaks of HA and calcium hydroxide in the HAC composites was reduced compared to those in HA powder, especially as in the HAC3 composite due to coating of HA particles by chitosan polymer. The broad peak of chitosan completely disappeared in three composites due to the effect of HA formation. Figure 4 shows the XRD patterns of HA, chitosan and their composites in the presence of citric acid. The main peak intensity of HA at d-spacing =2.8 in the HAC1/citric acid composite was gradually increased compared to the HA powder till reaching a maximum intensity in HAC3/citric acid composite denoting enhancement of HA formation as a result of the presence of citric acid.
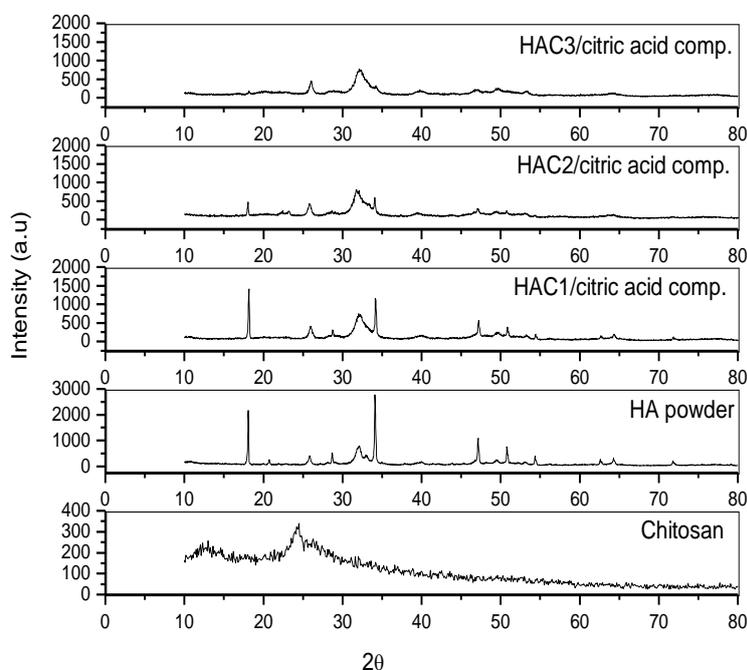
Fig. 4. The XRD of HA powder, chitosan and HAC/citric acid composites .

In this text, Shen *et al.*[12] reported that citric acid has a strongly chelating ability of calcium ions to its carboxylate groups, furthermore, the presence of citric acid with high chitosan concentration, enhancing nucleation center for HA formation via OH- & COO- of citric acid and OH- & NH_3^+ of chitosan. While the peaks of citric acid appeared in HAC1/citric acid composite and their intensities were gradually reduced with increase of chitosan and completely disappeared as in HAC3/citric acid composite denoting high coating. By comparing Fig. 3 and Fig. 4, it is noted that the intensity of HA peaks in the three HAC/citric acid composites is highly reduced compared to HAC composites denoting increase of coating by chitosan polymer on HA particles in the presence of citric acid.

TGA analysis

The TGA of HA powder and their C composites with chitosan polymer are shown in Fig. 5. The TGA analysis shows that the weight loss of C composites increased by increasing the chitosan content accompanying release of absorbed and structural water molecules, NH_3^+ and CO_2 gases.   The weight losses of the HAC composites were 22.598, 26.598, and 29.801% in HAC1, HAC2 and HAC3 composites, respectively, due to gradual increase of chitosan content. These values were high compared to the HA (12.589%) proving coating of chitosan polymer onto the HA particles. The TGA also revealed that the HAC composites are thermally stable above 600 °C due to the presence of HA phase so it is believed that chitosan was perfectly incorporated into the composites [2].
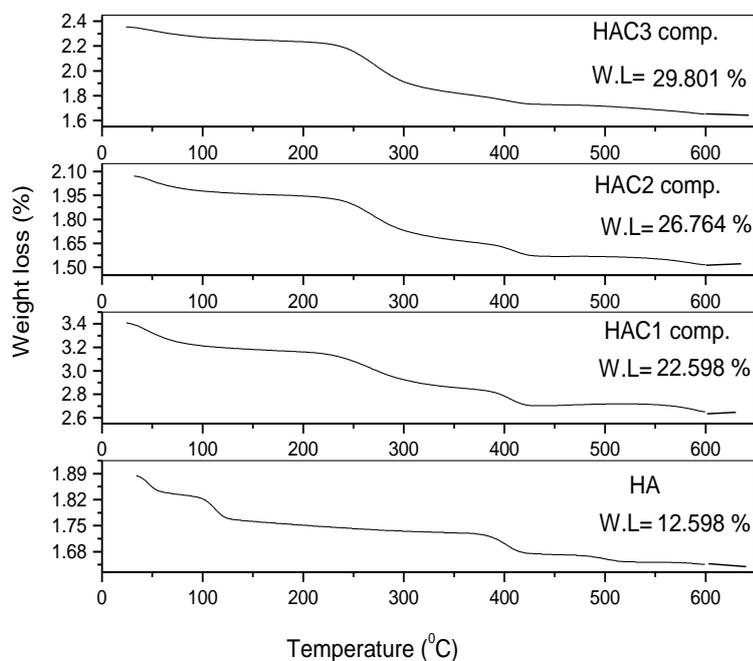
Fig. 5. The TGA of HA and its composites with chitosan polymer.

Compressive strength

Table 3 shows the changes in the compressive strength of the samples. It is obvious that the compressive strength was firstly increased with the increase of the chitosan content and reached a maximum value (19.6 MPa) for HAC2 composite. Afterwards, the compressive strength was decreased to (12.84 MPa) as in the HAC3 composite as a result of the reduction of interaction between two matrices. The values of compressive strength of all HAC composites were higher than that of the prepared HA (9.66 MPa) proving the role of chitosan polymer matrix in enhancement of the bonding strength between the HA particles within the composite[14]. The compressive strength of the HAC/citric acid composites increased especially in the case of HAC2/citric acid composite recording 30.5 MPa in comparison with the HAC composites. The citric acid with (0.2 M) concentration is good agreement to enhance the compressive strength of HAC composites because of the bonding strength which increased between HA particles with addition of citric acid [4].

TABLE 3. The compressive strength (MPa) of the prepared HA and its composites.

Sample	HA/chitosan (Weight ratio)	HA	HAC composite	HAC/citric acid composite
HA	100/0	9.66	--	--
HAC1 Comp.	80/20	--	10.58	13.76
HAC2 Comp.	70/30	--	19.6	30.5
HAC3 Comp.	60/40	--	12.48	14.6

TEM photographs

The TEM photographs of the prepared HA sample, HAC2 and HAC2/citric acid composite are shown in Fig.6 and Table 4. For HA powder, the crystallites are envisioned as needle and sphere-like in shape. The mean size of the needle is 7 nm in length and 5nm in width. For HAC2 composite, the size of needle crystals was increased up to 38.66 nm in length and to 2.5 nm in width compared to that of the HA sample proving growth of HA crystals due to the presence of the chitosan polymer (Fig.6a and b). In this domain, the carbonate apatite in the nano level has an important impact on the cell biomaterials interaction because of its better resorption and close surface contact with the surrounding tissue [3].

a
b
c
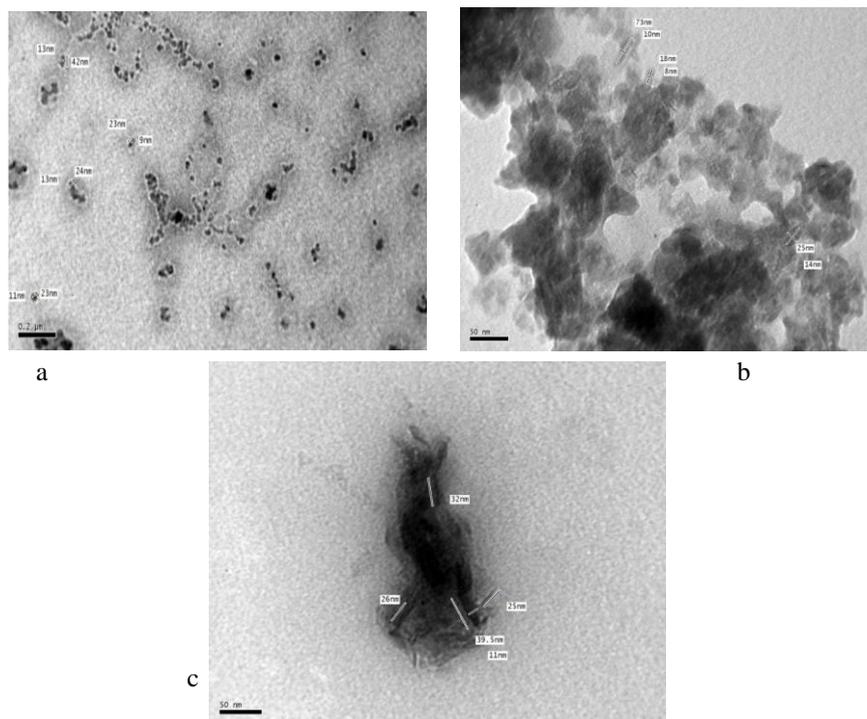
Fig. 6. The TEM images for (a) HA powder, (b) HAC2 composite and (c) HAC2/citric acid composite.
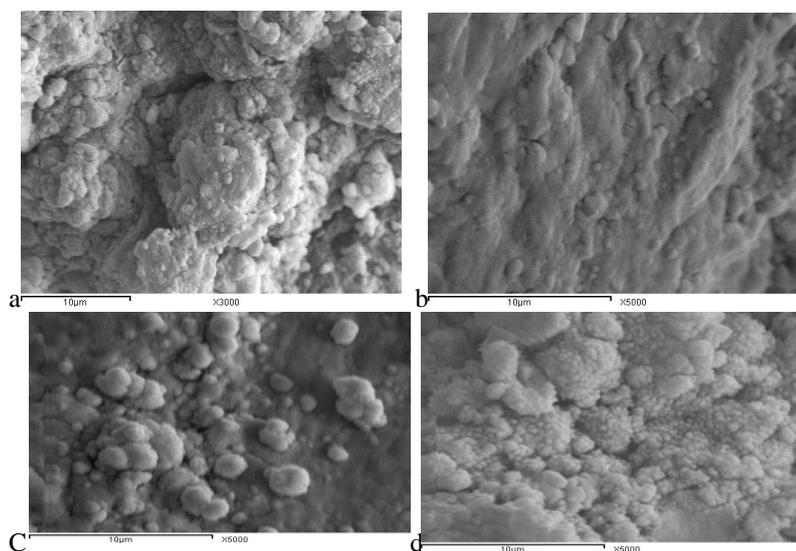
For HAC2/citric acid composite, the TEM image shows nano-sized HA particles increase to 30.6 nm in length compared to HA sample (7nm) and they decreased compared to HAC2 composite (38.66 nm) (Fig.6c). This result may be caused by different intensities of interactions between ions with the addition of citric acid. The abundant supply of COO$^-$ coordination sites available for complexation with the Ca^{2+} ions produced a very large number of nuclei for the growth of nano-HA crystallites that were not able to grow largely [19].

TABLE 4. The crystal size of (nm) of HA and its composites.

Sample	HA powder	HAC2 composite	HAC2/citric acid composite
Length (nm)	7	38.66	30.6
Width (nm)	2.8	10.66	8.34

SEM morphology

Figure 7 shows the surface morphology of HAC2 and HAC2/citric acid composites. For the HAC2 composite, Fig.7a shows that the surface is rough due to the formation of HA particles in and onto the structure of chitosan polymeric matrix. Figure 7 b reveals that the surface is smooth, in which HA particles are uniformly dispersed into the chitosan matrix structure and were completely coated by the chitosan polymer proving integration. Regarding the SEM image of the HAC2/citric acid composite, the surface of the composite was smooth and there are many small spherical HA particles distributed, imbedded and coated within the composite structure denoting homogeneity and integration between the components of the composite (Fig.7c & d). According to the SEM images, the HAC2/citric acid composite has high formation and nucleation of apatite compared with the HAC2 composite proving a vital role of citric acid in the acceleration of HA formation and its nucleation [20].

Fig. 7. The SEM images for (a, b) HAC2 composite and (c, d) HAC2/citric acid composite.

Conclusions

The characterization of the samples proved that the formed HA was carbonated hydroxyapatite (CHA). The formation and coating of CHA particles increased by increasing the chitosan content and also were highly increased after the addition of citric acid. Also, the TEM data proved that the size of CHA particles had the nano-range scale and was increased in HAC2 composites proving the vital role of chitosan in the growth of HA particles but was decreased in HAC2/citric acid composite due to citric acid addition. The compressive strength increased with increasing the content of chitosan as in HAC2 composite and was highly increased as in HAC2/citric acid composite, because citric acid enhanced the binding strength between HA particles and chitosan polymer. The HAC composites with ratio 70% HA and 30% C, especially with citric acid, are expected to be attractive for bone grafting and bone tissue engineering applications.

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تحضير و توصيف متوالفات حيوية نانومترية من الهيدروكسى اباتيت وبوليمر الكيتوزان فى وجود وغياب حمض الستريك

خالد رزق محمد ، عايدة عبدالكريم سلامه * **و زينب محمد الرشيدى**

قسم المواد الحيوية ـ المركز القومى للبحوث و*قسم الفيزياء الحيوية ـ جامعة الازهر ـ القاهرة ـ مصر.

تم تحضير متوالفات الحيوية من كربونات الاباتيت و بوليمر الكيتوزان فى وجود وغياب حمض الستريك من خلال طريقة الترسيب. تم توصيف المتوالفات المحضرة بواسطة الاشعة تحت الحمراء و حيود الاشعة السينية وتحليل الفقد فى الوزن واختبار حجم الحبيبات و كذلك التركيب السطحى للمتوالفات. وقد برهنت النتائج على اهمية اضافة حمض الستريك اثناء عملية تحضير المتوالفات والذى سرع ذلك من تكوين الهيدروكسى اباتيت وقلل من حجم حبيباتها. ايضا اتضح ان قوة الضغط للمتوالفات المحضرة كانت اعلى مقارنة بالهيدروكسى اباتيت المحضرة وزادت اكثر فى وجود حمض الستريك الذى حسن من قوة الترابط والاتحاد بين حبيبات الهيدروكسى اباتيت و الكيتوزان عن طريق مجموعات حمض الستريك الفعالة. بناءا على النتائج السابقة امكن تحضير متوالفات محتوية على 70 % هيدروكسى اباتيت و 30% كيتوزان فى وجود حمض الستريك تتميز بان لها تطبيقات واعدة فى المستقبل فى مجال استبدال العظام و هندسة الانسجة العظمية.