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# From crabshell to chitosan-hydroxyapatite composite material via a biomorphic mineralization synthesis method

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**Abstract** Hydroxyapatite–polymer composite materials, as biological bone tissue materials, have become an important research direction. In this paper, the calcium carbonate from the crabshells was transformed into hydroxyapatite by a hydrothermal process. According to the method that we called Biomorphic Mineralization synthesis, we obtained a novel kind of hydroxyapatite-chitosan composite materials which reserved the natural perfect structure of the original crabshells. Benefited from its fine micro-structure as the crabshells, this kind of materials held a high value of tensile modulus, which is expected to be promising bone tissue engineering applications.

## 1 Introduction

Hydroxyapatite (HAP) is the main component of teeth and bones in vertebrates. With very good biocompatibility and bioactivity, it is considered the most potential materials for bone tissue engineering [1, 2]. Sintered hydroxyapatite can be utilized as bone and tooth implant materials. However, its toughness and strength are far less than the requirements of underlying bone tissue [3, 4]. So it is natural to make inorganic or organic matrix composite materials to improve the mechanical properties. In order to make the composite be satisfied with the requirements to biocompatibility, bone induction activity, and strength, a large number of inorganic and organic materials have been studied [5–7]. In recent years [8–10], chitosan has been taken more and more attention.

Chitosan is a promising polymer because of its excellent biocompatibility, biodegradability and structural similarity to the glycosaminoglycans [2]. It has been extensively used in bone tissue engineering since it was shown to promote growth and mineral rich matrix deposition by osteoblasts in culture [11, 12]. In spite of that, pure chitosan matrices have previously been shown to have low mechanical strength under physiological conditions, which limited their use [2, 13]. Therefore, a composite biomaterial of HAP and chitosan is expected to show increased osteoconductivity and biodegradation together with sufficient mechanical strength for bone tissue engineering [11].

So far, the mechanical properties of the HAP-CTS based materials obtained by different methods still can not be satisfactory. Feng Zhao et al. [14] prepared a hydroxyapatite/chitosan–gelatin materials by phase separation method, and its bending strength could be 32 MPa; Yamaguchi et al. [15] produced a chitosan/hydroxyapatite (HAP) composites with a homogeneous nanostructure by a co-precipitation method, whose tensile modulus could reach 180 MPa, and bending strength can reach 10 MPa; Qiaoling Hu et al. [16] addressed that a kind of biodegradable chitosan/hydroxyapatite nano-composite rods was prepared via in situ hybridization, and the initial mechanical properties of bending strength and modulus of this composite reach 86 MPa and 3.4 GPa, which is similar to that of pure CS, 80 MPa, 3.9 GPa, and much higher than that of CS/HA prepared by blending method, 68 MPa, 3.2 GPa, respectively, tensile modulus was not mentioned. Compared with the human bone mechanical strength (bending strength 50–160 MPa, tensile

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modulus 1–18 GPa), different kinds of artificial composite materials for bone tissue can not meet the material toughness and strength requirements at the same time. It is realized that the perfect mechanical strength of natural bone is not only related to the type of materials and components, but also to the fine structure and microstructure which were formed in the growth of the biological process. This fine structure could hardly be prepared by artificial methods.

Biological template synthesis and biomorphic synthesis are a possible approach to obtain the fine biological structure. Natural selection provides a tool by which nature can process, improve, and refine biologically based organisms over millions of years. Scientists can learn from these evolutionary refinements and develop technologies based on natural designs. Kenneth S et al. successfully converted the shells of conch and coral into hydroxyapatite composite materials by a hydrothermal method. Calcium carbonate crystals in Conch and coral were converted in situ into hydroxyapatite, and the fine microstructure was preserved [17, 18].This process also maintained the high mechanical properties. Taking Conch for example, the fracture stress of the conversion composite materials has 137–218 MPa, close to the mechanical properties of human bone; and their applications in bone tissue engineering have been widely reported. Professor Zhang Di et al.'s [19] work has proved that the fine structure of natural organisms can be copied down via the appropriate preparation method. In the natural world, shells in rich source from sea crustacea such as shrimp and crab are a typical fine structure of biological chitin/calcium carbonate composite material. In many studies, calcium carbonate as calcium source for the synthesis of hydroxyapatite [5, 20, 21]. Therefore, by choosing a suitable synthetic method, it is entirely possible to convert crab or shrimp shell's calcium carbonate into hydroxyapatite, while preserving the biological fine micro-structure and structural organization of original shells, which make us able to obtain a fine structure chitosan/hydroxyapatite composites, which with excellent properties. In this paper we adopt a kind of more moderate conditions of the hydrothermal method for calcium carbonate in shells into hydroxyapatite, a detailed comparison of the shells' fine structure before and after hydrothermal treatment has been discussed, and we have tested the tensile modulus of this chitosan/hydroxyapatite composite.

## 2 Materials and methods

### 2.1 Pretreatment of crabshell

Integrity shells were taken from crabs (Portunid from the East China Sea) as indicated in Fig. 1a. The crab shells were washed with distilled water, and treated in a 10 wt% NaOH aqueous solution to eliminate proteins. Finally, after soaking with alcohol, the shells were cleaned by ionized water and cut into rectangular pieces (as shown in Fig. 1b), stand-drying.

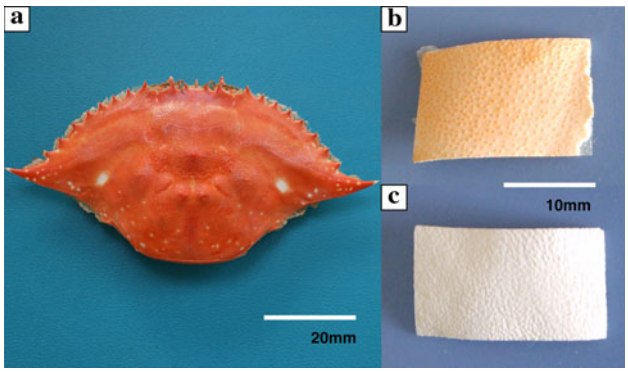


**Fig. 1** The whole crab's shell (**a**); a piece of pretreated original crabshell sample (**b**); the chitosan/hydroxyapatite composite sample (**c**)

### 2.2 Preparation of chitosan/hydroxyapatite composite

Pretreated raw shells were set into the Teflon autoclave, with appropriate amount of $NH_4H_2PO_4{\cdot}(NH_4)_2HPO_4$ and deionized water. After 1 min stirring, the Teflon autoclave was put in an air-blowing oven, under 120°C for 10 days. After reaction, the product was washed several times with distilled water to completely remove the buffer before characterization. Mark name as HC.

### 2.3 Characterization

X-ray diffraction (D/max-2550V Cu K radiation) was utilized to identify the phase inside and outside of the shells and the HC. Fourier transform infrared absorption spectra (FTIR) were determined by using a EQUINOX 55 (Bruker Co., Germany) spectroscopy. The apatite and calcium carbonate compositions were determined by thermogravimetic analyzer (TGA; DSC 2910, TA instruments) and differential thermal analyses (DTA; DTA 1600, TA instruments)were carried out in air at 25–800°C at a heating rate of 5°C/min. The tensile modulus were determined by a dynamic mechanical analysis machine (DMA, PerkinElmer DMA 6e).The specimens were shaped into 3 mm × 10 mm × 0.5 mm pieces, and all the specimens were tested in dry and wet condition respectively;the tests were performed from 10 to 80°C.

## 3 Results and discussion

Figure 1c is the photograph of a sample after hydrothermal reaction. Comparing with Fig. 1b, we find that there is no

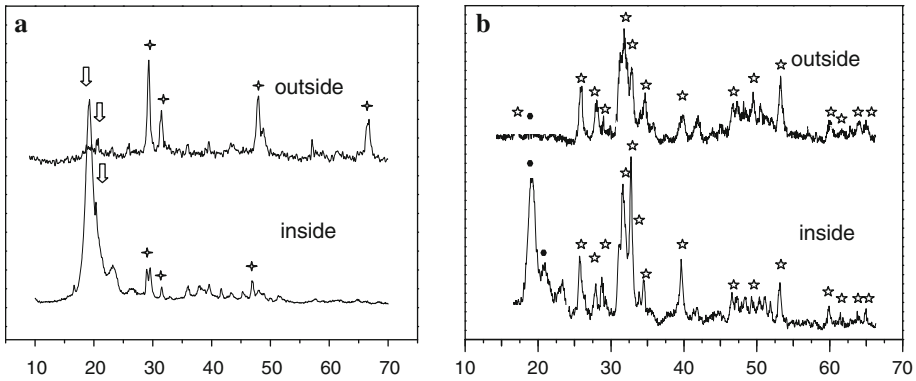


**Fig. 2** XRD spectrum of crabshell (⇩) chitosan/chitin, (✦). XRD spectrum of HC composite. $CaCO_3$ (●) chitosan/chitin, (☆) HAP

distinct difference in appearance except the color of the upper side. The HC samples seem whiter in appearance than original shells.

Figure 2 shows XRD profiles of the crabshells inside and outside surfaces. We can see that the outer surface and inner surface contain both calcium carbonate crystals and chitin. But the content of chitin is lower in the outer surface, and the content of calcium carbonate crystals is lower in the inner surface. That may mean a gradient distribution of the ratio between calcium carbonate and chitin along the vertical direction. After hydrothermal treatment, as shown in Fig. 3, the calcium carbonate crystal peaks disappear in the pattern, and the emergence of hydroxyapatite crystals peaks means that the calcium carbonate may have been successfully transformed into hydroxyapatite.

Chitin's XRD peaks and peak shape almost do not change. Chitosan, the deacetylated form of chitin, has the similar structure with chitin. IR spectra can help us determine whether deacetylation happened [13, 22, 23]. As shown in Fig. 4, Chitin's amide group of the characteristic peaks are at 1,658 and 1,556 $cm^{-1}$. Compared with the

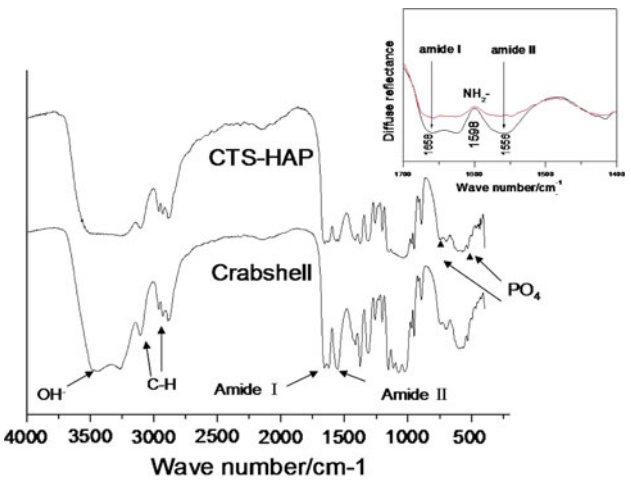


**Fig. 3** The IR spectra of crabshells and chitosan/hydroxyapatite composite

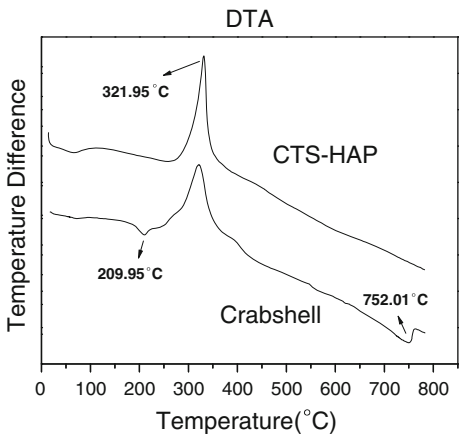


**Fig. 4** DTA of crabshells and CTS/HAP

spectra of the crabshell, these two peaks are weaker in the spectra of sample after treatment. As the figure amplified in the upper-right corner showed, the location between the two peaks has the trend to be filled. That means a new peak may be generated between two peaks. During deacetylation reaction, the amine in the vicinity of 1,598 $cm^{-1}$ infrared vibration should have a peak, so we can determine the deacetylation reaction did occur, but only to a low extent. The amine relative signal strength of the reaction product is still very weak, and therefore, a separate peak can not be found in the vicinity of 1,598 $cm^{-1}$. The emergence of amide bond strengthens the N–H vibration, resulting in the obvious changes of the peak shape and intensity in the 3,150–3,550 $cm^{-1}$ range. This region was apparently widened, showing the unique form of N–H bond. Based on the analysis above, we believe that after hydrothermal reaction the calcium carbonate in shells was converted into hydroxyapatite, while chitin occurred to deacetylation and was transformed into a low deacetylation degree of chitosan. The bands at 592 and 721 $cm^{-1}$ corresponded to different vibration modes of phosphate group in HA, while the bands at 3,571 and 642 $cm^{-1}$ were assigned to hydroxyl group as stretching and bending vibration. [24].

It should be noted that in the IR spectra of the original shells, there is no obvious peak of calcium carbonate, but the existence bands corresponded to different vibration modes of phosphate group in hydroxyapatite (592 $cm^{-1}$, 721 $cm^{-1}$, $PO_4^{3-}$). A. Heredia et al. [25] in the article (references marked) studied the IR spectra of the shrimp shell, and reported that there was a strong interaction between the calcium and chitin in the shell. Such a strong interaction may also exist in crab shell leading to the vibration of carbonate being limited to be not able to show its characteristic peaks. Crab itself also contains a little phosphate, which emerged in its IR spectra with the similar peaks of hydroxyapatite.

As well as the crabshells, the chitosan/hydroxyapatite composite (CTS-HAP) obtained by hydrothermal transformation also have a different amount of the organic component (chitosan) in external and internal surfaces. The distribution of chitosan inside and outside the surface is almost the same to that of chitin in crabshell. This phenomenon is reasonable because the chitosan was formed by the partial deacetylation of chitin in the crabshell, and its distribution in the crabshell would not be affected during the treatment. But the distribution of inorganic components inside and outside the surface of CTS-HAP was different from that of crabshell. We found that the proportion of the original calcium carbonate is very low in the inner surface, but after the transformation the proportion of hydroxyapatite increased significantly. We believe that this phenomenon indicates that chitin or chitosan has a very strong attraction for metal ions. During the hydrothermal conversion process, with the gradual dissolution of calcium carbonate, the ionized calcium is form and spread out. By the interaction with polar groups on the molecular chain of chitin in the environment, the ionized calcium combined with chitin, resulting in higher concentrations of calcium ions in the surface of organic matter [25]. In solution, hydrogen oxygen ions and phosphate ions around these locations could react with calcium ions to form hydroxyapatite of low solubility. Therefore, during the hydrothermal conversion process, the mechanism of organic matter's bio-mineralization also played an important role, resulting in the growth of a lot of hydroxyapatite in the inner surface of the shells (Figs. 4 and 5).

The thermal analysis was taken on the samples before and after treatment. As Fig. 4 shows, two exothermic peaks are located in the vicinity of 594.95 and 1,025 K in the DTA curve of the original crabshells. Combined with TG curve, it can be known that these peaks belong to the thermal decomposition of chitin and calcium carbonate. DTA curves of the conversion products are only in the emergence of a peak at 594.95 K; accordingly, its TG curves appear only one peak. Clearly the raw material of calcium carbonate was completely converted into hydroxyapatite with higher

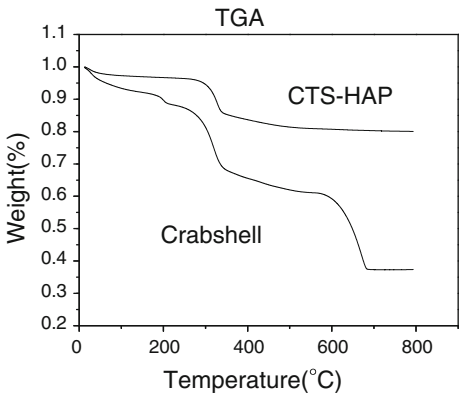


**Fig. 5** TG of crabshells and CTS/HAP

melting point and decomposition temperature than calcium carbonate. On the other hand, we note that the samples before and after reaction have the similar exothermic peak position and shape, indicating the microstructure was changed seldom in this conversion. Chitin's original crystallinity and micro-structure have been copied very well.

From the TGA diagram it can be found: The original crabshell samples have a significant weight loss from 600 to 700°C, and this section obviously has an endothermic peak. This is mainly derived from the decomposition of calcium carbonate, in which, carbonates are thermally decomposed by the elimination of $CO_2$ and the formation of the calcium oxide, the reaction formula is as follows:

$$CaCO_3 \rightarrow CaO + CO_2$$

Correspondingly, we can not find the weight loss section in the TGA diagram of CTS-HAP composite. It's just another proof of transformation: the calcium carbonate of original crabshell has been completely transformed into hydroxyapatite, which has a better thermal stability.

The structure of crabshell is very complicated. As Fig. 6a shows, the crabshell's outer surface is multi-layer structure; under the first uniform dense outer layer, there is a particle layer regular arranged by several rows of circular pie-like structure with the diameter of about 8 μm. From further amplification observed (6c), we find that the surface of each circular particle is dotted with dozens of nano-holes, and there are some fibrous materials between particles, which are estimated to be chitin fibers. The inner surface of the crabshell consists of the chitin fibers which are woven tightly. These woven structures are surrounded by large pores, and the pores' size is not same (Fig. 6b, d). Generally, the fine structures of biological shells include the following features: the overall structures are layered composite; the basic matrix phase is a porous weave structure composed of chitin fiber; and near to outer surface, there is composite structure of a kind of the cylindrical porous particles being uniformly embedded in chitin fibers.

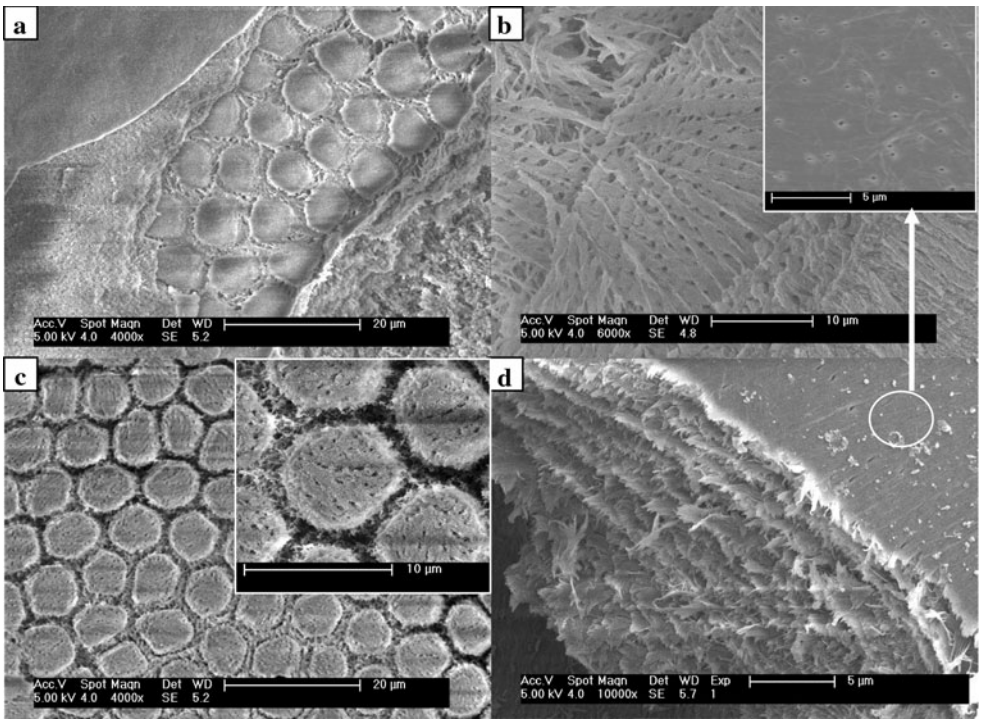


**Fig. 6** SEM images of original crabshells: the outer surface's multi-layer structure (**a**); the inner surface's porous woven structure (**b**); the rows of circular pie-like structure (**c**); the breaking face of woven fibers' layers (**d**)

Figure 7 shows the microstructure of the reaction product. Obviously, it is also multi-layer composite material. Figure 7a and c show the structure of the outer surface. The outer surface layer is hard and dense, and the microstructure of surface is relatively smooth. By opening up the outer surface, It can be seen that there are neatly arranged by 7–8 μm circular ball-like aggregations, which are composed of 200–300 nm spherical particles and fibers. As the subsurface structure reinforcements, the spherical particles are embedded in a large number of woven fibers (about 20 nm diameter). Between the spherical particles, they are filled with nano-fibers. Figure 7b clearly shows the organic fiber-woven structure of the inner surface. There is also a number of the pore structures staggered between the complex woven structures. Figure 7d showed the fracture face of the sample. The three-tier structure can be clearly observed. 1× is the innermost layer; under small magnification, the surface is smooth, and enlarged morphology is showed in 7b. 1x, 2× are two layers of fibers as shown in amplified SEM photo in the upper right corner of Fig. 7d. Summarily, along the thickness direction, structure and morphology of each layer are changed in different positions of these composite materials.

Compared with the morphology of the samples before and after reaction, no matter the surface layer structure, and the overall composite structure, the fine composite structure below the surface layer, all have striking similarities. The only exception is the circular mosaic reinforcement between the composite layers. During the treatment, about 8 μm diameter porous inorganic reinforcements are converted into a diameter of 200–300 nm spherical aggregated small particles. This phenomenon is reasonable. In the hydrothermal conversion process, the calcium carbonate in the original porous crabshells dissolves and ionized calcium is generated. Because of the highest concentration of calcium ions in the surface of porous calcium carbonate and the surrounding of chitin fibers, the phosphate and the hydroxyl ions in solution are most likely near the surface of the particles to reach saturation and the precipitation of hydroxyapatite nucleation and further growth of a spherical particle. The formation of nucleation rate and growth rate determine the final aggregations' morphology and particle size. According to the micro-morphology of converted product, it is estimated that the original circular mosaic matters prefer to composite materials of chitin fibers and calcium carbonate crystals rather than pure calcium carbonate crystals.

There are some holes for the adoption of ions in the chitin layers, while the structure of products as well. Pores in layers are the key channel for transportation of raw materials during the formation of the crabshells. It can be seen that the pores are all reserved after the hydrothermal process. On the one hand, according to the result, it can be inferred that during the reaction, the required ions can be transported through the original pores into the growing

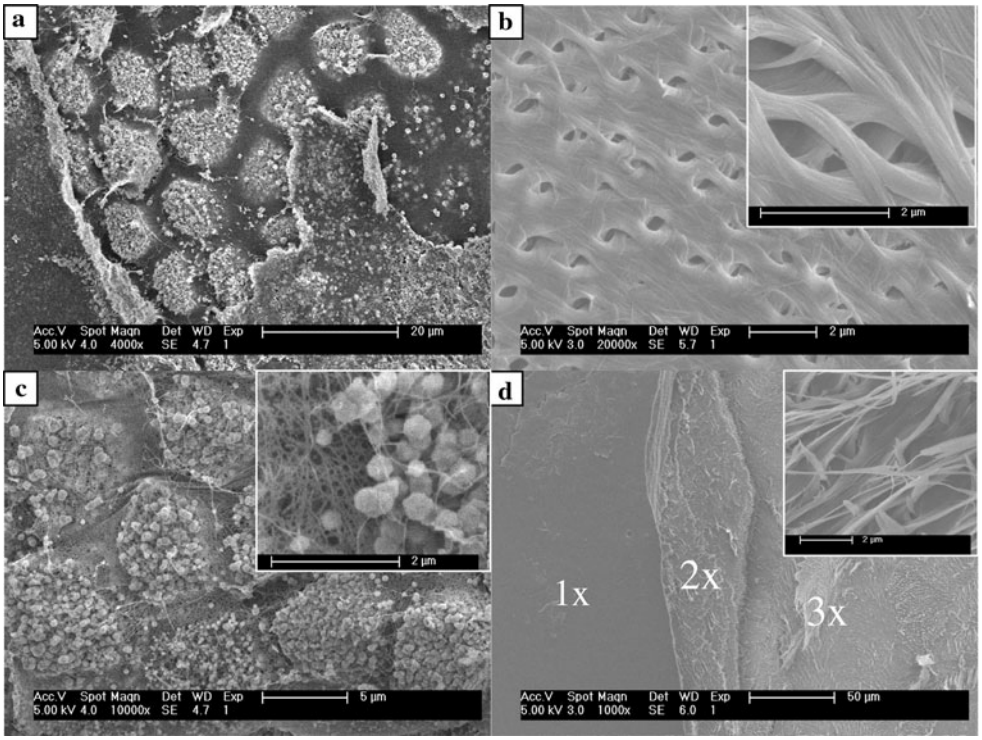


**Fig. 7** SEM images of CTS-HAP: the outer surfaces' structures (**a**); the inner surfaces' structures (**b**); the circular ball-like aggregations (**c**); the breaking faces of inner layers (**d**)

points of hydroxyapatite and into the vicinity of the reaction, so the pores are critical to promote the process. On the other hand, the reservation of such porous structures provides a positive factor for future bone tissue engineering applications, especially for the growth of bone cells in the HC.

In summary, by hydrothermal conversion, we have successfully converted the crabshell into chitosan-hydroxyapatite composite materials. In this composite material, the original crabshell's special layered composite structure, chitin's complex woven structures, the fine connection structure between inorganic phase and the chitin fibers have been all copied perfectly.

Fine natural structure is the result of long-term natural selection and evolution, and physical properties of biological tissue are closely related to the structure. Crabshell has a very good tensile modulus, can achieve 1 GPa which is far more than the value of previously reported hydroxyapatite-chitosan composite materials. In Fig. 8, the tensile modulus of natural shells and prepared hydroxyapatite-chitosan composite materials in two cases of wet and dry condition were compared. It was shown that the value of HC tensile modulus in the dry state is even slightly higher than that of crabshells. In dry and wet conditions, the values' difference is not significant. The results increase an order of magnitude comparing to the previous

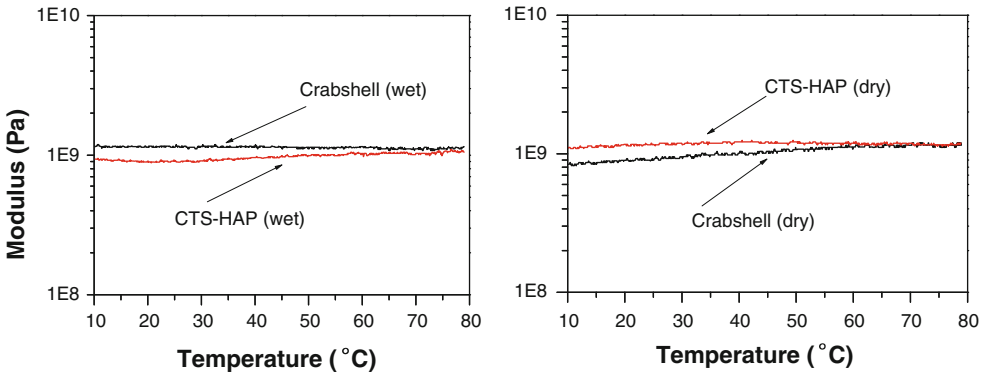


**Fig. 8** The DMA tensile modulus results of crabshells and CTS-HAP composite in wet and dry conditions

value of the tensile modulus in this type composite materials (mentioned in the literature, the value of CTS/HAP composite tensile modulus are 10–100 MPa. Yamaguchi et al. [15] whose studies on CTS/HAP composite, reported that their measured tensile modulus achieved 235 MPa). From 10 to 80°C, the value of the tensile modulus is very stable. The product with high value of tensile modulus shows that the composite material is expected to find significant applications in biomedical field, while also proves that the fine structure of the bio-medical material is important to the biological properties. According to the experimental results, we can expect to produce biological tissue-engineering material satisfied different strength requirements by choosing the appropriate organism of corresponding mechanical properties as raw materials, such as different parts of crustaceans.

## 4 Conclusion

In this paper, mild hydrothermal reaction achieved the biomorphic mineralization synthesis of chitosan-hydroxyapatite composite from crabshell. In this reaction, calcium carbonate crystals in the crab shell were converted into hydroxyapatite crystals, at the same time the crabshell's chitin is partially deacetylated into chitosan. The fine composite structure of the crab shell was almost exactly reproduced. Its tensile modulus under dry and wet state reached 1 GPa. This preparation method is a new route to prepare for high-performance chitosan-hydroxyapatite composite materials.

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