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In situ formation of recombinant humanlike collagen-hydroxyapatite nanohybrid through bionic approach

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Processing bone graft materials that mimic the chemical composition and structure of natural bone is a good option for the treatment of bone defects. This investigation reports a processing condition for the *in situ* formation of recombinant humanlike collagen-hydroxyapatite (CHA) nanohybrid with chemical composition and crystal structure quite similar to natural bone through bionic approach. The processed CHA was characterized by various physicochemical methods and the results suggested that HA crystals are nucleated on the collagen domain with an average size of 32 nm, relatively similar to that of biological apatite. The CHA has enhanced bioresorption than HA and mimics the natural bone in composition and structure; thereby it may be considered for bone applications. © *2006 American Institute of Physics*. [DOI: 10.1063/1.2202138]

There is a great interest in designing materials for bone grafting, which mimic the natural bone in composition and structure. Currently, a variety of biomaterials are available for bone grafting both in monolithic and composite forms. It is believed that the biomaterials used for bone grafting should satisfy not only the minimal clinical requirements but also have the structural and chemical compositions similar to natural bone so as to facilitate direct biochemical bonding with host tissues. Recently, nanophase biomaterials are perceived to be beneficial for bone grafting applications than conventional biomaterials because they stimulate tissue formation at the bone-implant interface much faster than conventional ones.[1–4]

Hydroxyapatite (HA) is one of the widely used biomaterials in bone grafting[5,6] and bone drug delivery[7,8] owing to its structural and chemical compositions similar to natural bone minerals. Several manufacturing techniques have also been developed to produce HA.[9–14] Although HA is a good candidate for bone grafting, its bioresorbability (dissolution of material through physiological means) is very low compared to other calcium phosphate biomaterials.[15,16] The bioresorbable characteristic is a major concern in bone grafting as it directly involves the formation of new bone tissues. The resorbability of HA can slightly be improved with the help of some ceramic oxides, ionic doping agents, and natural polymers,[17–19] which leads to the concept of composites.

It is worth mentioning that the bone is not monolithic but it is essentially a nanohybrid, which constitutes HA nanocrystals and collagen fibrils, wherein the crystals are well aligned parallel to the collagen fibrils.[20] In this regard, the bone graft material made of HA and collagen may be a good option for bone grafting not only due to their chemical composition and structural similarity to natural bone but also due to their enhanced bioresorption and mechanical strength than pure HA. Numerous studies investigated the potential of HA-collagen composites for bone grafting.[21–23] It is worth pointing out that most of the bone graft composites are conventionally processed by manual kneading or mechanical mixing of inorganic and organic phases; thereby disintegration of reinforced particles into the body fluid is often encountered upon implantation. In this investigation, in contrast to conventional composite processing methodologies, we report a possible *in situ* formation of recombinant humanlike collagen-hydroxyapatite (CHA) nanohybrid through bionic approach (a process that either mimics or inspires the biological systems) under controlled *p*H with the most desirable physicochemical and bioresorbable properties suitable for bone grafting.

The CHA nanohybrid was processed by a wet chemical method as described in our earlier report with some modifications.[13] Briefly, 0.3*M* aqueous solution of $(NH_4)_2HPO_4$ was mixed with water-solubilized collagen and then slowly added dropwise to 0.5*M* aqueous solution of $CaCl_2$ under a stirring condition of 1000 rpm. The *p*H of the reaction solution was adjusted to 9 by adding concentrated NH_4OH solution using a syringe and it was maintained at room temperature. After the sol-gel transition triggered by *p*H increases to 9, the hybrid seed particles started to precipitate in the reaction medium, which was aged for 48 h, filtered, washed with de-ionized water, and dried.

The processed CHA was subjected to Fourier transform infrared (FTIR) spectroscopic analysis using a system made by ThermoNicolet Avatar 360, USA, to confirm the chemical composition and the possible chemical interaction of HA and collagen constituted in the nanohybrid, using a potassium bromide (KBr) pellet method at a ratio of 1 mg sample per 300 mg KBr. The functional groups of CHA can be easily seen from the FTIR spectrum (Fig. 1). The result shows that all characteristic peaks pertain to the HA phase in addition to typical absorption peaks of the collagen macromolecules, which are labeled in the spectrum. A peak noticed at 1050 cm^{-1} can be attributed to the stretching vibrations of PO_4^{3-}, and two sharp peaks observed at 610 and 575 cm^{-1} can be assigned to the deformation vibration of PO_4^{3-}. An intense band noticed at 3450 cm^{-1} corresponds to the combination of OH^- group (HA phase) and N–H group (collagen phase). The peaks at 2900 and 900 cm^{-1} represent the stretching and vibrational modes, respectively, of the C–H

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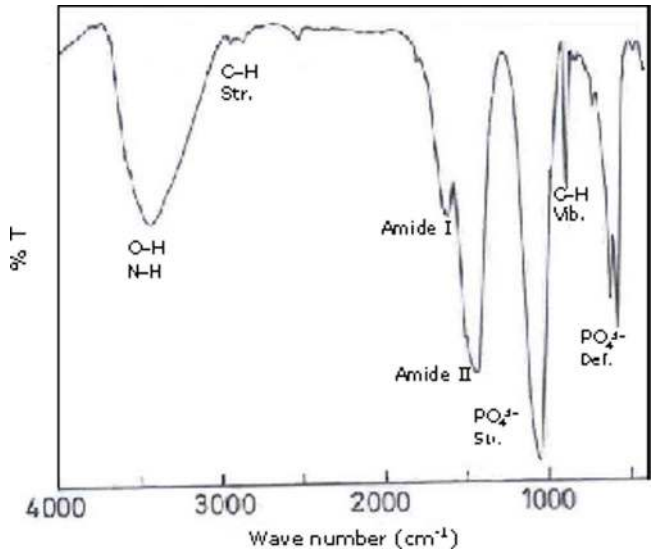

FIG. 1. Transmission IR spectrum of CHA nanohybrid, representing typical amide bands (collagen phase) and phosphate groups (HA phase) for the evidence of HA nucleation onto collagen domain.

groups of collagen macromolecules. Besides, the absorption peaks noticed around 1670 and 1500 cm^{-1} typically represent the amide I (C═O bend) and amide II (C–N bend) bands, respectively. It should be noted that the intensity of the amide I band is relatively lower than that of the amide II band, which is an indication that the anionic groups (typically, carbonyl groups) of the collagen initiate the nucleation of HA crystals and subsequently allow crystal growth on the collagen matrix, which is a good sign of bionic growth of HA that occurs in the natural bone during biomineralization.

The crystallographic structure of CHA can be assessed from its x-ray diffraction (XRD) pattern (Fig. 2), performed by Shimadzu XRD-600 powder diffractometer (Japan). The result shows all the diffracted peaks corresponding characteristically to HA phase. The diffracted peaks observed at 25.9° and 30°–35° (2θ) regions denote the uniqueness of the HA present in the CHA nanohybrid. The characteristic peaks of the HA are labeled (*) in the same figure with reference to its standard diffraction file.[24] The size of HA crystallites responsible for the Bragg reflection of (002) plane (2θ=25.9°) was determined by Scherrer's formula:[25] $d=k\lambda/b\cos\theta$, where k is shape constant (0.9), d is crystallite size (in nanometers), λ is wavelength of monochromatic x-ray beam (0.154 06 nm), b is full width at half maximum for the diffraction peaks (radian), and θ is Bragg angle of

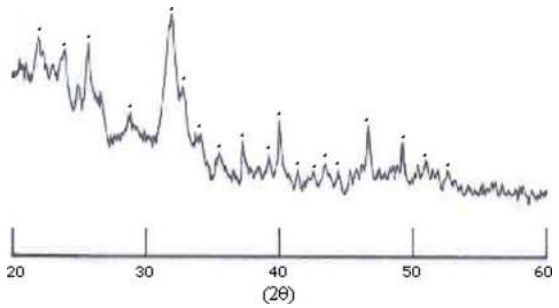

FIG. 2. X-ray diffraction pattern of CHA nanohybrid, showing Bragg peaks corresponding characteristically to nucleated HA (marked as *). Data collected at a scan rate of 1°/min over the range of 20°–60° (2θ) with Cu $K\alpha_1$ radiation.

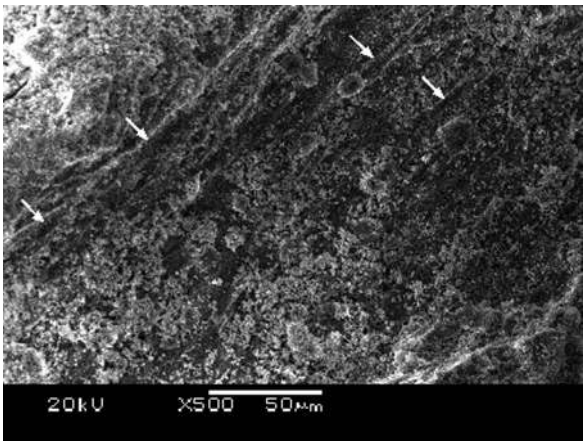

FIG. 3. SEM micrograph of CHA nanohybrid, indicating large amount of ultrafine HA crystallites embedded in the collagen domain.

diffraction (°). The calculated crystallite size of HA was 32 nm. As can be seen from Fig. 2, the overall diffracted pattern shows apparently low crystallinity owing to its low temperature processing. In addition, the presence of organic macromolecules associated with the collagen greatly influences the crystalline behavior of the CHA. However, the obtained results are comparable to the diffraction pattern of the biological apatite and are in agreement with our previous study.[11] These data confirmed the formation of HA crystals on the collagen domain *in situ*. Since the XRD pattern of CHA nanohybrid did not show any significant changes in the crystal structure of HA, it is believed that the presence of collagen macromolecules did not induce or alter the apatite's crystal lattice; thereby structural integrity of HA and collagen phases are confirmed.

In order to study the surface morphology of CHA, crystal orientation, and growth of HA on the collagen domain, a scanning electron microscopy (SEM) (JEOL 5600, Japan) was employed. The sample was gold coated to a thickness of 20–30 nm with an accelerating voltage of 20 kV. A representative micrograph is shown in Fig. 3, which provides a good evidence for the nucleation and bionic growth of HA on the collagen domain. The processed CHA nanohybrid possesses large amount of ultrafine crystals embedded in the collagen substrate along their axial direction. Majority of the crystals are spread-out on the substrate and a few of them get agglomerated owing to their high surface area and thus occasionally appear to be in clusterlike deposits. These results suggest the feasibility of *in situ* nucleation of HA crystals on the collagen domain in the axial direction and the greater influence of anionic functional groups of collagen macromolecules on the growth of HA crystals.

Bioresorption is one of the key characteristics of biomaterials, in particular, bone graft materials, as it controls their bioactivity and other biological functions. Therefore, in this study, *in vitro* bioresorbability of the CHA nanohybrid was evaluated in a Hank's medium (a buffer solution used for cell/tissue cultures) of *p*H of 7.4 at a ratio of 1 mg/ml in a thermostatic incubator at 37 °C. The obtained results are depicted in Fig. 4. The *p*H of the buffer solution without the sample was quite stable throughout the experimental period, while addition of the HA had significant effect on the *p*H of the medium. Since solubility depends on the buffering condition, all the tests are evaluated carefully keeping the resorbable nature of biphasic factors of HA and collagen. The

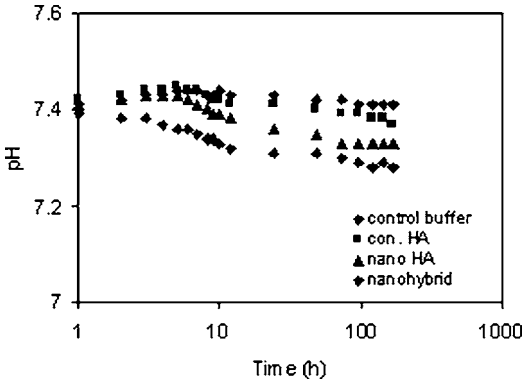

FIG. 4. Solubility of CHA nanohybrid in Hank's medium under *in vitro* physiological conditions.

*p*H of the buffer solution with pure HA was also determined and included in the same figure for comparison.[13] No significant changes were noticed in the *p*H level of conventional micro HA (con.HA), indicating its physiological stability during the period of study, whereas the nano HA relatively has soluble nature. CHA nanohybrid shows a drastic change in its *p*H due to their nanocharacteristic behavior, indicating an enhanced resorption than HA. The fact is nanophase materials always have increased number of atoms and crystal grains at their surfaces and possess larger surface area-to-volume ratio compared to microscale counterparts, which makes the nanohybrid more soluble under the controlled physiological conditions *in vitro*.

In summary, the experimental results of this investigation provided evidence for the feasibility of *in situ* formation of CHA nanohybrid through bionic approach with the most desirable physicochemical and bioresorbable characteristics. The physicochemical characteristics confirmed the nucleation and crystal growth of HA on the collagen domain. The structural integrity and chemical interaction of these two phases are also evident wherein anionic functional groups of collagen play a key role for the growth of HA crystals. The *in vitro* solubility evaluation of CHA performed under the physiological conditions has provided a proof for its resorbable nature. The overall results therefore suggest that the CHA nanohybrid may be a good choice for bone grafting applications.

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