MULTIFUNCTIONAL ANTIBACTERIAL BIOCOMPOSITE RICH OF TRACE ELEMENTS BASED ON BIOLOGICAL HYDROXYAPATITE AND ZnO NANOPARTICLES

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Abstract. An effective multifunctional biocomposite based on biological hydroxyapatite (B-HAp) and ZnO nanoparticles serving as bone implant, disinfectant and source of vital trace elements, was synthesized by cost effective method. Sterilized B-HAp powder rich of vital trace elements was extracted from cattle bones while ZnO nanoparticles were prepared using co-precipitation method. The prepared particles and composites were characterized by XRD, FT-IR, SEM and ICP-Ms techniques. XRD and FT-IR analyses confirmed synthesis of pure B-HAp powder, ZnO nanoparticles and B-HAp/ZnO composites. SEM data showed that the B-HAp particles have sizes in range of 1–2 μm while ZnO particles have sizes in range of 20–30 nm. The antibacterial activity of these composites was tested against *Escherichia coli*, gram negative strain, and *Staphylococcus aureus*, gram positive strain. The synthetic composites showed antibacterial activity to both of the tested micro-organisms. The results reveal the effectiveness of biocomposite based on B-HAp and ZnO nanoparticles as disinfectant and source of vital trace elements. The results indicated that B-HAp/ZnO composites are promising for medical applications such as bone repair and replacement.

Key words: biological hydroxyapatite, ZnO nanoparticles, trace elements, antibacterial activity.

INTRODUCTION

The harmful effects of antibiotic resistant bacteria generate an interest to develop multifunctional biocomposites that should exhibit excellent antibacterial activity for health care [16]. The biocomposites based on hydroxyapatite (HAp, $Ca_{10}(PO_4)_6(OH)_2$) are widely used in orthopedic applications as bone filler and bone implants [9]. Biological hydroxyapatite (B-HAp) is the main inorganic component of natural bone; it represents about 70% of bone weight and is rich of vital trace elements such as zinc, strontium and copper which have a role in bone functions [7]. It has various advantages comparing with other biomaterials such as

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non-toxicity, bioactivity, biocompatibility, bioresorbability, biodegradability, and high adsorption capacity [12]. Also, *in situ* reaction of HAp has not influenced physiological temperature or pH and has not released toxic monomers or solvents [5, 6]. However, using B-HAp has become a severe challenge due to the risk of developing post operatory infections due to its non-antibacterial activity [3, 6]. The most common types of antibacterial HAp powders have been prepared by mixing HAp powder, synthetic or biological HAp, with organic antibacterial drugs [11]. Unfortunately, most organic antibacterial drugs have many limitations due to their toxicity, ability to cause allergies and to develop antibiotic resistant bacteria [14]. For solving problems described with antibiotic resistant bacteria resulting from organic antibacterial agents, nanosized metal oxides based on zinc, silver, iron and copper have been tested as antimicrobial agents [17]. For instance, zinc oxide nanoparticles have shown excellent antimicrobial activity against both Gram-positive and Gram-negative bacteria and also the pathogenic bacteria have not yet developed resistance against it [15]. Since HAp powders have not antibacterial characteristic, the present study proposes using B-HAp/ZnO composite instead of synthetic HAp powder to develop multifunctional biocomposites. Zinc oxide nanopaticles can serve as antibacterial agent for a broad spectrum of bacteria and as a vital trace element [1, 17]. Recently, many studies have reported that nano-sized ZnO particles may be a promised solution for disinfectant due to their safety and strong antibacterial activity at low concentrations [4]. On other hand, zinc is one of the important vital trace elements because of its roles in function and metabolism of living systems. Zinc is essential for enzyme action, and structural integrity to many metabolically important proteins [8]. Clinical trials have reported that zinc strongly influences skeletal growth and inhibits postmenopausal bone [5, 10]. For these reasons, the aim of this study is to synthesize B-HAp/ZnO composite gel to enhance the antimicrobial properties of B-Hap, as disinfectant and source of vital trace element. Several studies have suggested antimicrobial activity of synthetic bone implants can be enhanced by incorporating Zn^{2+} ions or inorganic ZnO nanopaticles into HAp structures [2]. The main drawback of metal substituted HAp as antimicrobial agents is that their antibacterial activity strongly depends on their particle size that must be at nanosized range [13]. The present study aims to develop a multifunctional orthopedic composites based on B-HAp and ZnO nanopaticles; B-HAp acts as an alternate bone substitute and source of vital trace elements while ZnO nanoparticles act as an inorganic antimicrobial agent and as a source of zinc metal. Sterilized B-HAp powder was extracted from bovine bones while ZnO nanoparticles were prepared using the co-precipitation method. The prepared particles and composites were characterized by X-ray diffraction (XRD), Fourier transform infrared (FT-IR), scanning electron microscopy (SEM) and ICP-Ms techniques. In order to test the antibacterial activity of these compounds, *Escherichia coli* and *Staphylococcus aureus* are used.

MATERIALS AND METHODS

EXTRACTION OF B-HAp POWDER

In a typical procedure for the extraction of sterilized B-HAp powder, 500 g of fresh crushed bones were cleaned and boiled in 2.0 L of distilled water for 10 min to remove contamination. The boiled bones were filtered and added to 2.0 L of distilled water, and then placed in a commercial tightly sealed autoclave. To sterilize and soften the bone, the autoclave was heated at 130 °C for 3 h. The resulted soft bones were filtered, milled, and dried overnight at 80 °C then calcinated at 800 °C for 2 h.

SYNTHESIS OF ZnO NANOPARTICLES AND PREPARATION OF B-HAp/ZnO COMPOSITES

The ZnO nanoparticles were prepared *via* co-precipitation using analytical grade $Zn(NO_3)_2 \cdot 4H_2O$, NaOH, and H_2O_2 (30%) (Adwic, El-Nasr Chemical Co., Cairo, Egypt). A 0.1 M of $Zn(NO_3) \cdot 4H_2O$ solution was stirred constantly for 10 min by stirrer at 50 °C. Then 0.2 M of NaOH solution heated at 50 °C was added dropwise into the previous solution under high speed stirring. Then, 1.0 mol H_2O_2 was added to the suspension and the reactant was heated at 80 °C for 60 min. The precipitated ZnO gel was washed three times with deionized water and filtered. To prepare B-HAp/ZnO composites, the B-HAp was mixed with filtered ZnO gel at room temperature where the weight ratio of B-HAp:ZnO components was 20:1 and 20:2, respectively.

CHARACTERIZATION OF THE PREPARED B-HAp, ZnO AND THEIR COMPOSITES

The phase composition and crystalline structure of B-HAp, ZnO and their composites were characterized by XRD using a Philips PW 1840 diffractometer at 40 KV and 30 mA, using $Cu_{k\alpha}$ (λ = 1.54 Å) radiation as an X-ray source in the 2θ range from 10° to 65°. Peaks on the X-ray patterns recorded for the B-HAp and ZnO samples were labeled by referring to the XRD data obtained from the Powder Diffraction File (PDF) from Joint Committee on Powder Diffraction (JCPDS). FT-IR spectra were scanned on Perkin-Elmer-1600 using KBr pellet technique for the range 4,000 and 400 cm^{-1}. The microstructures of the samples were examined by scanning electron microscopy (SEM) using a JEOL 6400 electron microscope at 30 KV of electron acceleration voltage.

TRACE ELEMENT LEVEL MEASUREMENT

The B-HAp powder was treated by acid digestion prior to analysis. 500 mg of extracted B-HAp powder was transferred to Teflon beakers then both 15 mL and 3 mL of concentrated nitric acid (E-Merck, Germany) and perchloric acid (BDH, UK) respectively with three replicates. The mixture was heated at 80 °C for 10 min. Trace element analysis was carried out by using calibrated inductively coupled plasma mass spectrometry (ICP-MS: Finnigan element 2).

IN VITRO ANTIMICROBIAL EFFICACY OF B-HAp/ZnO COMPOSITES

Two of standard Gram-positive, *Staphylococcus aureus* ATCC 25923, and Gram-negative strains, *Escherichia coli* ATCC 25922, of bacteria were used for antibacterial tests. The minimum inhibitory concentration (MIC) of B-HAp/ZnO composites was used to investigate the antimicrobial activity of standard bacteria against serial concentrations of B-HAp-ZnO composites (0.00 – 10 μg/mL, 0.00 is the negative control) in Muller Hinton broth. In a typical procedure, 1.0 mL of bacterial suspension containing about 10^5 CFU/mL was transferred to 2.0 mL volumes of B-HAp/ZnO composites with different concentrations. All samples were incubated at 37°C on a shaker for 24 h and the growth of the bacteria was monitored by measuring the optical density at 630 nm then MIC was recorded for the B-HAp/ZnO composites against the test bacteria. The mean values of MIC were calculated from triplicates.

RESULTS AND DISCUSSION

X-RAY DIFFRACTION SPECTROSCOPY

The XRD patterns of extracted B-HAp, ZnO, and B-HAp/ZnO composites are shown in Figure 1(a–d). XRD pattern of B-HAp (Fig. 1a) exhibits its crystalline structure with pure HAp phase as compared to standard HAp structure [JCPDS card No. 74-0874]. The main peak sets at 2θ values of 31.7° was for reflection (211). The extracted B-HAp is pure where no peaks for other calcium phosphate or calcium carbonate compounds were detected. Fig. 1b shows the XRD pattern of the ZnO nanoparticles prepared by co-precipitation method. XRD pattern shows a pure single phase as compared to standard ZnO HAp structure [JCPDS card No. 36-1451]. The main peak sets at 2θ values of 36.3° was for reflection (101). Figure 1(c and d) shows the XRD patterns of B-HAp/ZnO composites prepared by mixing B-HAp and ZnO nanoparticles with a weight ratio of B-HAp:ZnO components at 20:1 and 20:2, respectively. Addition of ZnO has an effect on XRD patterns in

particular at 2θ values of 36.3° corresponding to the reflection (101) of ZnO phase. The intensity of peaks of ZnO phase was increased with increasing the ratio of ZnO phase in the composite.

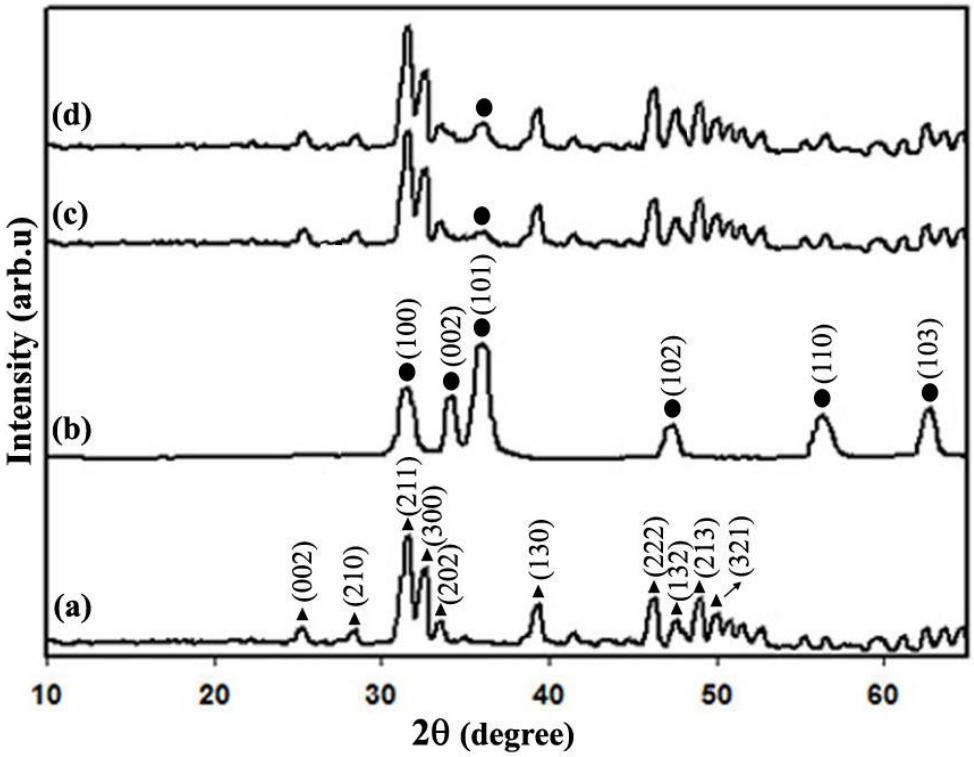

Fig. 1. XRD patterns of (a) B-HAp, (b) synthetic ZnO nanoparticles, (c) B-HAp/ZnO, 95:5, and (d) B-HAp/ZnO, 90:10.

FOURIER TRANSFORM INFRARED SPECTROSCOPY

FT-IR analysis (Fig. 2) was applied to investigate the main chemical groups that characterize B-HAp and ZnO structures such as PO_4^{-3}, $-OH^{-1}$, CO_3^{-2}, and ZnO. Fig. 2a shows FT-IR spectrum for B-HAp where there are three distinctive absorption bands for PO_4^{-3} ions that presented at 574, 603 and 1028 cm^{-1} and belonged B-HAp. FT-IR spectra showed a relative strong peak at 877, 1413, and 1463 cm^{-1} are the characteristic peaks of the carbonate C–O bonds belonging to the carbonate group of B-HAp. ZnO exhibits characteristic absorption bands for the

vibrational modes of Zn–O bond appeared at around 1028, 1419, and 1581 cm^{-1}. All FT-IR spectra showed a strong peak at 3466 and 3470 cm^{-1} which are the characteristic peak of H–O–H group.

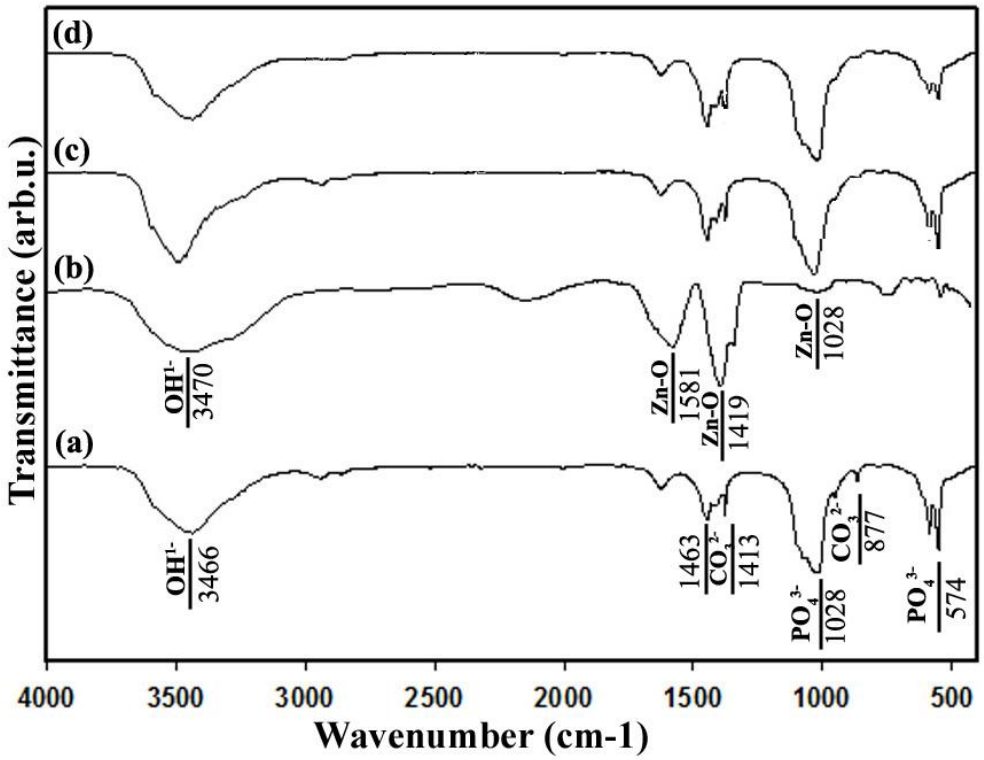

Fig. 2. FT-IR spectra of (a) B-HAp, (b) synthetic ZnO nanoparticles, (c) B-HAp/ZnO, 95:5, and (d) B-HAp/ZnO, 90:10.

SCANNING ELECTRON MICROSCOPY

The morphology of extracted B-HAp, synthetic ZnO, and B-HAp/ZnO composite powders were observed by SEM as shown in Figure 3(a–c). SEM image, Fig. 3a shows that B-HAp particles have irregular morphology with micro-sized structures in the range of 1–2 μm. Fig. 3b shows that ZnO particles have relative regular spherical shapes with nano-sized structures in range of 20–30 nm. The morphology of B-HAp/ZnO composite powder, Fig. 3c, shows that agglomeration of ZnO nanoparticles on the surfaces of B-HAp particles.

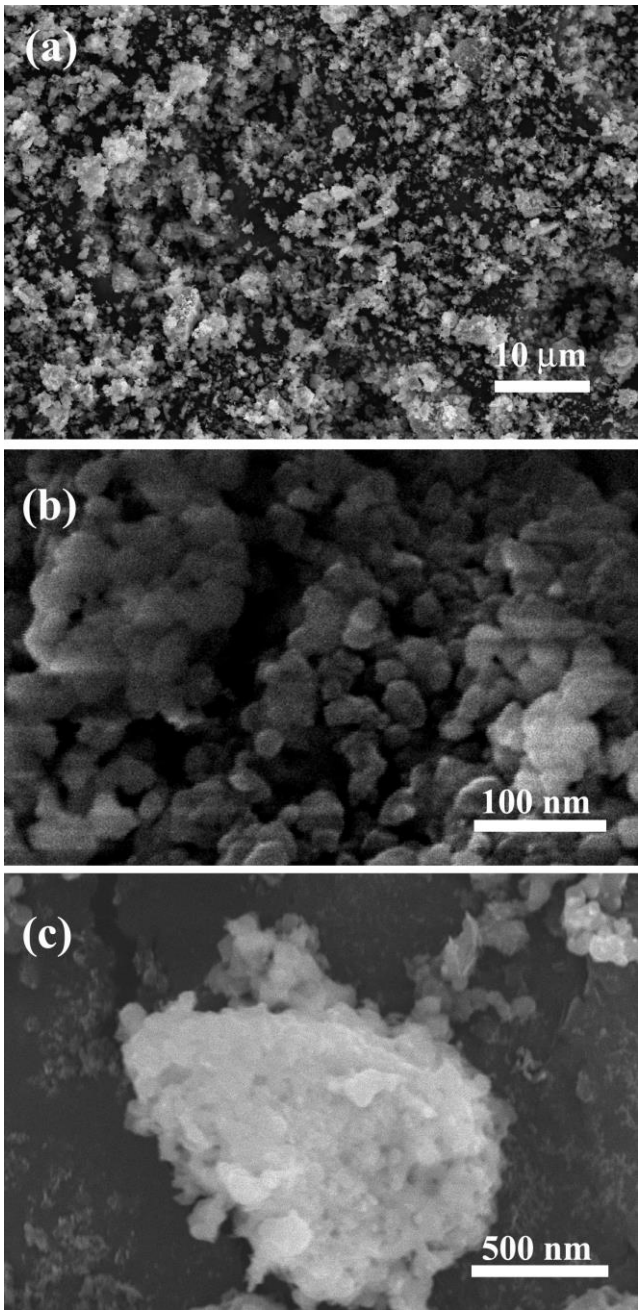

Fig. 3. SEM images of the surfaces of (a) B-HAp, (b) synthetic ZnO nanoparticles, (c) B-HAp/ZnO, 90:10.

DETERMINATION OF TRACE ELEMENTS IN EXTRACTED B-HAp POWDER

Total concentrations of trace elements in extracted B-HAp powder were measured by ICP-MS as shown in Table 1. The concentrations of zinc, strontium, and iron showed high levels of 166, 287, and 108 μg/g, respectively. Many studies have reported the important of Zn, Sr, Fe, and Cu for bone growth and metabolism [3]. The toxic trace elements such as Pb and Cd showed low concentrations.

Table 1

Concentrations of trace elements in extracted b-HAp powder

Element	Concentration (μg/g)
Zinc	166
Strontium	287
Iron	108
Copper	1.7
Lead	0.05
Cadmium	0.008

ANTIMICROBIAL ACTIVITY OF B-HAp/ZnO COMPOSITES AGAINST MICROORGANISMS

The data presented in Table 2 indicates the antimicrobial activity of B-HAp/ZnO composites with different weight ratio against the standard *E. coli* and *S. aureus.* The B-HAp/ZnO composite of weight ratio 90:10 shows higher antibacterial activity against *E. coli* and *S. aureus* with inhibition zones (mm) of 16±0.06 and 15±0.06, respectively. The MIC values of tested B-HAp/ZnO (90:10, wt%) composites gel against *E. coli* and *S. aureus* were recorded at 0.80 and 0.75 mg/mL, respectively. The different bacteria strains have different responds to B-HAp/ZnO composites.

Table 2

The effects of different concentrations of b-HAp/ZnO composites on the growth of bacteria

Bacteria	Composite ratio (b-HAp:ZnO, wt%)			
	95:5		90:10	
	Zone of inhibition (mm)	MIC (mg/mL)	Zone of inhibition (mm)	MIC (mg/mL)
E. coli	11 ± 0.2	0.80	16 ± 0.3	0.45
S. aureus	11 ± 0.2	0.75	15 ± 0.3	0.40

CONCLUSION

Pure B-HAp powder, rich of vital trace elements, was extracted from bovine bone and nanosized ZnO particles were prepared using co-precipitation method. This study has demonstrated that B-HAp/ZnO composites prepared by mixing extracted B-HAp with ZnO nanoparticles are of great promise as multifunctional antibacterial composites rich of vital trace elements. B-HAp/ZnO composites may be promised for bone implants and health care.

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