Synthesis, characterization, and antibacterial activities of a novel nanohydroxyapatite/zinc oxide complex

Gang Zhou,^{1,2} Yubao Li,¹ Wei Xiao,³ Li Zhang,¹ Yi Zuo,¹ Jing Xue,⁴ John A. Jansen⁵

¹Research Center for Nano-Biomaterials, Analytical and Testing Center, Sichuan University, Chengdu 610064, China

²College of Chemistry, Sichuan University, Chengdu 610064, China

³College of Life Science, Sichuan University, Chengdu 610064, China

⁴Key Lab of Oral Biomedical Engineering, Sichuan University, Chengdu 610041, China

⁵Department of Periodontology and Biomaterials, Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands

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Abstract: Nanohydroxyapatite (n-HA)/zinc oxide (ZnO) complex was synthesized by a direct precipitation method, and the antibacterial capability and antibacterial mechanism of this complex were investigated in this article. Transmission electron microscope (TEM), Fourier transform infrared, X-ray photoelectronic spectroscopy (XPS), and X-ray diffraction (XRD) were used to analyze the materials. In addition, the antibacterial capacity of n-HA/ZnO complex was examined by bacteriolytic plate, inhibition effect, and antibacterial rate assays under light or without light. Furthermore, agarose gel electrophoresis of plasmid DNA (pUC18) was used to study biocidal action of this complex. The results of TEM observation revealed that the complex's rods had a single crystalline obelisk-like hexagonal wurtzite structure and the crystal retained the nanometer size. XRD analysis indicated that the phase of ZnO appeared and the lattice parameters of

n-HA and ZnO changed. XPS spectra showed that the bonding energy of Ca, P, and O atoms changed in the complex. The analyses also showed that the two phases of ZnO and n-HA combined closely. Further, the results of the antibacterial test revealed that this complex possessed strong antibacterial capability; the antibacterial rate was 99.45% to *S. aureus* and 95.65% to *E. coli* under light, respectively. The antibacterial activity of this complex under light was better than without light, which was attributed to the generation of $\cdot\text{OH}$ under light. From the agarose gel electrophoresis, the dissolving solution of this complex could catalyze the cleavage of pUC18. © 2007 Wiley Periodicals, Inc. *J Biomed Mater Res* 85A: 929–937, 2008

Key words: nanohydroxyapatite; zinc oxide; antibacterial capability; antibacterial mechanism

INTRODUCTION

Clinical success of implants depends upon the integration of a biomaterial device into the surrounding body tissues. In addition to the encouragement of the cellular interactions, the biomaterial surface has to discourage adhesion of infectious bacteria, which are a common cause of implant failure.^{1,2} Polymethylmetacrylate (PMMA) loaded with antimicrobial agents is used for treatment and prevention of orthopedic infections.³ Unfortunately, PMMA bone cement is associated with several disadvantages, like reducing biocompatibility with bone, short duration of release, and thermal damage to the antibiotics.⁴

Therefore, in recent years, the use of inorganic antibacterial biomaterials is highlighted for the control of bacteria.⁵ The advantages of inorganic biomaterials are their safety, durability, and stability, compared with organic antibacterial biomaterials.⁶

Zinc oxide (ZnO) is an inorganic material, which processes unique properties such as nontoxicity and antiseptic effect.^{7–10} ZnO has already been studied extensively. Song et al. found that ZnO exhibited antibacterial activity of *E. coli*.¹¹ Another inorganic material that has been used widely as biomaterial is hydroxyapatite (HA) ceramic. This material is known for its good biocompatibility and bioactive bone behavior.^{12–15} Our intention is to prepare a new anti-inflammatory biomaterial that combines the favorable biological characteristics of HA and possess antimicrobial activity as expressed by ZnO.

In this article, a direct precipitation method was used to prepare a ZnO bearing n-HA slurry with a

Correspondence to: Y. Li; e-mail: nic7504@scu.edu.cn

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nanometer-sized crystal size. The properties of this complex were characterized and analyzed, using XPS, IR, TEM, and XRD. Bacteriolytic plate, inhibition effect, and antibacterial rate assays were used to measure the antibacterial activities of materials against *E. coli* and *S. aureus*, and to discuss the antibacterial mechanism.

MATERIALS AND METHODS

Preparation of the materials

n-HA slurry was synthesized by a precipitation method.¹⁶ Zinc nitrate [$\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$], PEG-400, and ammonia (25 wt %), purchased from Chengdu Chemical Reagent Company, were of analytical grade. Firstly, $\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ and PEG-400 powders were dissolved into aqueous solution, and then ammonia was dropped slowly into the solution with vigorous stirring. The dropping speed was near 4 mL min^{-1} and the reaction was carried out in ambient condition. The rotation speed of stirrer was adjusted to 1000 rpm. After titration, the precursor of $\text{Zn}(\text{OH})_2$ was introduced into n-HA slurry according to the n-HA/ZnO weight ratio of 9:1, and then kept stirring for 2 h. Then the obtained slurry was shaken in ultrasonic instrument for 2 h and aged for another 24 h. Finally, the white precipitate was fully washed with deionized water and ethanol, respectively, and dried at 80°C . The obtained powder was calcined at 300°C for 2 h.

Characterization of materials

The content of Zn element in the n-HA/ZnO complex was analyzed by Atom adsorption spectrometer (AAS, VARIAN Spectr AA 220FS).

X-ray photoelectron spectroscopy (XPS, XSAM 800) was utilized to analyze the surface area of the powder.

Chemical analysis of the complex was carried out by a Fourier transform infrared (FTIR) spectrophotometer (Thermo Nicolet 170SX) in the range from 4000 to 400 cm^{-1} at 2 cm^{-1} resolution averaging 100 scans.

The microstructure of the n-HA/ZnO complex was observed with a transmission electron microscope (TEM, JME-100CX).

XRD was used to determine the structure of the material. XRD analysis was performed with a DX-1000 analyzer (Cu-K α). The samples were measured in the 2θ range from 10° to 70° (scan speed of 0.02° per second).

Antibacterial test

All antibacterial tests were performed with bacteria JM no. 109 *Escherichia coli* (*E. coli*), ATCC no. 26003 *Staphylococcus aureus* (*S. aureus*), supplied by Microbial Laboratory of College of Life Science, Sichuan University, China.

Bacteriolytic plate assay

First, an inoculum with this strain was grown overnight in 2 mL NB media (10 g/L Peptone, 5 g/L NaCl, 3 g/L Beef extract) at 37°C with shaking. Then, a soft agar-inoculation mixture was made containing 15 mL of 0.8% agar previously melted at 45°C with 50 μL of the bacterial cell inoculums. The n-HA and n-HA/ZnO powder was put into the shape of a disc ($\phi 12.5 \times 2 \text{ mm}^2$) under UV light and without light irritation, then putting them on the soft-agar layer. Finally, the plates were incubated at 37°C for 24 h under light and without light, respectively. After incubation, the inhibition zones were visually inspected along the edge of "discs" and the plates were photographed. In this assay, all the data were the means of three parallel experiments ($d = 3$).

Inhibition effects assay

To determine the antibacterial activity, 0.1 mL diluted bacterial suspension was cultured in 5 mL liquid NB medium containing n-HA/ZnO complex for different periods. The inoculated medium was maintained at 37°C with shaking. The number of bacteria was counted by ultra-violet absorption of the cultured medium at 260 nm (A_{260}). All the data were the means of three parallel experiments ($d = 3$) of which the discrepancies were less than 5%.

Antibacterial rate assay

The antibacterial rate was calculated by the method of counting lawn.¹⁷ Hundred microliters of an overnight culture of *E. coli* and *S. aureus* was diluted into 10 mL NB liquid cultures, respectively. Then the liquid is transferred to the sterile tube containing 1 g of specimen's powder. The tubes were incubated 24 h at 37°C and the bacterial growth was measured by optical density at 260 nm.

Interaction between dissolving solution of n-HA/ZnO complex and plasmid DNA

Plasmid DNA (pUC18) was extracted from *E. coli* (JM no. 109) by alkaline lyses method.¹⁸ Ten microliters of diluted pUC18 suspension was cultured in EP. Then, 10 μL of the dissolved n-HA/ZnO complex was transferred into EP and incubated at 37°C without light. After 1, 5, 10, 15, 30, 60, and 300 min of incubation, 3 μL suspension was extracted and assayed by agarose gel electrophoresis.

RESULTS AND DISCUSSION

XPS analysis

Figure 1 shows the XPS spectra of n-HA and n-HA/ZnO complex. Figure 2 shows the XPS spectrum of Zn2P in n-HA/ZnO complex. From Figures

Figure 1. XPS spectra of n-HA (a) and n-HA/ZnO complex (b) powder calcined at 300°C.

1(b) and 2, it can be deduced that the binding energy peak of Zn is at 1022.2 eV, which belongs to ZnO according to Hoogewijs et al.,¹⁹ and Gaarenstroom and Winograd.²⁰ Besides, the binding energy of Ca, P, and O atoms has some differences between n-HA (Ca: 345.5 eV; O: 530.2 eV; P: 132.5 eV) and the complex (Ca: 347.2 eV; O: 531.2 eV; P: 133.3 eV). The values of elements in the complex are higher than those of n-HA, which implies that some interaction or chemical bonding may be formed between the two phases, such as between Ca^{2+} , OH^- , PO_4^{3-} , and Zn^{2+} in n-HA/ZnO complex.

Figure 2. XPS spectrum of Zn 2P in the n-HA/ZnO complex calcined at 300°C.

IR analysis

IR spectra of n-HA and n-HA/ZnO complex powder are shown in Figure 3(a,b). It can be seen that the major bands of HA associated with PO_4^{3-} (1094, 1036, 963, 604, and 566 cm^{-1}) and OH^- (3568 and 628 cm^{-1}) appear in Figure 3(b). The bands attributed to CO_3^{2-} (1350–1450 cm^{-1}) and HPO_4^- (873 cm^{-1}) also appeared in the spectra in Figure 3(a,b). Kumar et al.²¹ report that B-type (CO_3^{2-} for PO_4^{3-})-substituted HA is present. IR spectra also show that there are some changes in the frequencies of PO_4^{3-} and OH^- , shifting to the low wave numbers in the complex, which is in agreement with XPS analysis. Meanwhile, this movement indicates that the positive end of the OH^- dipole approaches the negative Ca vacancies (Vca),²² which contributes to the wave numbers shift. Besides, the sorption of Zn from ZnO is probably favored by suitable O–O separation between PO_4^{3-} groups of HA,²³ which is the bidentate coordination of Zn with PO_4^{3-} tetrahedral, as shown in Figure 4.

TEM observation

As shown in Figure 5, TEM micrograph illustrates that the n-HA/ZnO complex possesses a uniform and ultra fine microstructure with an average grain size of 80–90 nm in length and 15–30 nm in diameter, with no glassy or amorphous interface along the grain boundaries. Also, the ZnO grains combined

Figure 3. IR spectra of n-HA (a) and n-HA/ZnO complex (b) powder calcined at 300°C.

Figure 4. Schematic model illustrating possible coordination bonding. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

with n-HA crystals without phase-separation. It can also be seen that the complex is composed of well-defined crystals with an obelisk-like hexagonal wurtzite structure.

XRD analysis

According to XRD data calculated by Jade 6 software, the mean size of n-HA is about 58.66 ± 0.4 nm, and ZnO crystal is about 28.36 ± 0.6 nm, which is in agreement with the TEM observations. This indicates also that the complex is a nanosized material.

Figure 5. TEM photo of n-HA/ZnO complex.

Figure 6. XRD patterns of n-HA (a) and n-HA/ZnO complex (b) powder calcined at 300°C.

Figure 6 shows the appearance of the characteristic ZnO peaks at 34.13° , 38.48° , 47.68° , and 56.70° corresponding to the (100), (002), (102), and (110) crystal plane, which is comparative with the XRD pattern of ZnO powder (JCPDS no. 36-1451). This suggests that a ZnO phase forms in the complex.

On the other hand, the characteristic diffraction peaks of n-HA located at 25.92° , 31.94° , 40.12° , 47.01° , and 48.73° [as shown in Fig. 6(a)] suggest that HA retains its hexagonal structure. However, the wide peaks of n-HA account to its poor crystallization. Further, the (100) peak of ZnO is overlapped by the (121) peak of n-HA.

The results of XRD data (Table I) indicate a decrease in the a axis and an increase in the c axis of the HA unit cell after compounding. Moreover, the 2θ degree of n-HA/ZnO decrease in the (004), (002) crystal plain and increase in the (200), (300) plane compared to those of n-HA. According to the Smith–Lehrt formula, the same conclusion can be made, i.e. the trends in the lattice parameters can be largely attributed to the ion exchange. Moreover, the content of ZnO is up to 7.54 wt % by AAS analysis, whereas the theoretical value of ZnO is 10 wt %. This difference implies that part of Zn^{2+} ion has not formed $\text{Zn}(\text{OH})_2$, and enters into n-HA lattice. The diameter of Zn^{2+} is smaller than that of Ca^{2+} ,* and so it causes a contraction in the a -axis and expansion in c -axis dimensions in the complex compared to n-HA. As we know, HA belongs to the hexagonal system with a space group of P_63/m . For B-type, the charge compensation can take place by $-\text{OH}$ and

*Determined from the database JADE (Materials Data Inc.).

TABLE I
The Lattice Parameter and Crystal Size of n-HA, ZnO^a and n-HA/ZnO Composites

Sample	n-HA	ZnO ^a	n-HA/ZnO Composite
Lattice parameter (Å)	$a = b = 9.4179$, $c = 6.8853$	$a = b = 3.2498$, $c = 5.2066$	$a = b = 9.4077$, $c = 6.8855$
d -space (Å)	$d(002) = 3.4476$ $d(004) = 1.7217$ $d(300) = 2.7217$ $d(200) = 4.0703$	$d(002) = 2.6033$ $d(101) = 2.4759$ $d(102) = 1.9111$ $d(110) = 1.6247$	$d(002) = 3.4479$ $d(004) = 1.7227$ $d(300) = 2.7186$ $d(200) = 3.8904$ $d(002) = 2.6064$ $d(101) = 2.4781$ $d(102) = 1.9126$ $d(110) = 1.6258$

^aFrom JCPDC database (No.: 89-0511).

Ca vacancies.^{22,24} When Zn is substituted for Ca of the n-HA, it may alter the balance of the charge defect for the compensation system. Thus the a , b lattice parameter was found to decrease by 0.11%, whereas the c parameter changed little, only increasing by 0.003% in the new system. Besides, the Zn of ZnO coordinates with the O in PO_4^{3-} of n-HA act as a bridge to PO_4^{3-} groups, which is another reason for the occurrence of the changes in the n-HA unit cell. On the other hand, some differences exist also in the lattice parameters of ZnO in the complex compared to the standard ZnO, which may also be assigned to ion exchange and the bidentate coordination bonding between the ZnO and PO_4^{3-} group.

Zn(II) interaction with HA had indicated different mechanisms. Xu et al.²⁵ and Lusvardi et al.²⁶ suggested that ion exchange was a possible mechanism for the Zn bearing HA. Based on the discussion above, it can be speculated that these lattice changes may result from ion-ion, ion-dipole, and dipole-dipole interaction, translation, rotation, and vibration. Accordingly, the possible effective geometry can be schematically depicted as Figure 7.

Antibacterial capability assays

Table II illustrates the results of the bacteriolytic plate assay. The results show that only the n-HA/ZnO complex has an antibacterial effect on *E. coli* and *S. aureus*.

Figures 8 and 9 show the inhibition effect of the various materials at different concentrations on the growth of *E. coli* and *S. aureus*. The absorbance at 260 nm (A_{260}) characterizes the number of microorganisms in the cultured medium.²⁷ Lower absorbance means a higher antibacterial effect of the material. It can be seen that control II is only slightly different from that of the control I material after 24 h, which have A_{260} values of 1.90 and 2.06, respectively. This suggests that the n-HA has no inhibiting

effect on *E. coli* and *S. aureus*, while the n-HA/ZnO complex shows high antibacterial activities against *S. aureus* and *E. coli* at 24 h.

The antibacterial rates of both control I and control II are zero suggesting no antibacterial activity. In contrast, the antibacterial rates of the n-HA/ZnO complex are evident, which is above 87% against both *E. coli* and *S. aureus* (Table III).

Antibacterial mechanism

As stated above, the n-HA/ZnO complex shows a good antibacterial activity, while pure n-HA has no effect on *E. coli* and *S. aureus*, which suggests that ZnO provides the complex with the antibacterial property. This difference may associate with the antibacterial mechanism of ZnO. Generally, there are two kinds of theories to explain the antibacterial mechanism of ZnO. The one holds that metal zinc can react with water and release Zn^{2+} ,²⁸ which subsequently decomposes the bacterium. The other theory is based on light-catabolism²⁹ and advocates that ZnO generates activated oxygen ($\cdot\text{OH}$) under light, especially under UV light. The $\cdot\text{OH}$ combines with sulfhydryl groups of the respiratory enzyme or the nucleic acids in bacteria and stop the breath of bacteria.

e^- and h^+ will appear after UV irradiation, and further form $\cdot\text{OH}$ and $\cdot\text{O}_2^-$.¹¹ When $\cdot\text{OH}$ comes in contact with the cell, it may destroy the cell wall and membrane, or interact with protein thiol groups, causing the cytoplasm to run off and oxidizing the cell nucleus, which finally induces the death of the bacteria.³⁰ $\cdot\text{O}_2^-$ ions are the feasible chemical species at the reduction of oxygen molecule. It is found that the reduced oxygen plays as the antibacterial chemical species. According to the antibacterial theory, it may be explained that the rates of the n-HA/ZnO complex under light are higher than those without light toward bacteria.

Figure 7. Schematic model of structures: (a) n-HA; (b) CO_3^{2-} , HPO_4^{2-} substituted n-HA; (c) ZnO occupying lattice sites of n-HA; (d) O—Zn—O coordination bonding between ZnO and n-HA. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Tables II and III show that the n-HA/ZnO complex is more effective for *S. aureus* whether under light or not. Accumulating evidence shows that the complex exerts a high antibacterial activity against gram-positive but not gram-negative bacteria. The outer membrane, covering the cytoplasmic membrane and peptidoglycan layer of gram-negative bacteria, is composed of lipopolysaccharide (LPS), which restricts antibacterial ions passing across the outer membrane of gram-negative bacteria. On the other hand, this complex is able to cause permeabilization of both *S. aureus* and *E. coli* cytoplasmic membranes.

However, the level of permeability of *S. aureus* cytoplasmic membrane is markedly higher than that of *E. coli*. This may be due to the outer membrane barrier of *E. coli*, which somewhat prohibits antibacterial ions accessing the cytoplasmic membrane. Consequently, the n-HA/ZnO complex is more effective for *S. aureus* than *E. coli*.

Besides, Figure 10 shows the effects of the n-HA/ZnO complex on the cleavage of pUC18 extracted from JM no. 109 after different incubation times.

TABLE II
Diameters of Bacterium Repress (in circle/mm)

	<i>E. coli</i>		<i>S. aureus</i>	
	Under Light	No Light	Under Light	No Light
n-HA/ZnO composite	14.50 ± 0.05	13.00 ± 0.1	16.00 ± 0.4	14.75 ± 0.15
Control I	0	0	0	0
Control II	0	0	0	0

A control I assay was carried out without material, while control II assay was only n-HA powder.

Figure 8. Antibacterial activities of samples against *E. coli* with the incubation time. A control I assay was carried out when no material was added to the peptone solution. A control II assay was carried out when only n-HA powder was added to the peptone solution. The concentration of the samples was 1 g/mL. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Form 2 does not appear in control I and control II, which indicates that n-HA cannot accelerate the plasmid DNA cleavage. On the other hand, DNA cleavage would efficiently be accomplished by dissolving Zn^{2+} of n-HA/ZnO complex. As shown in

Figure 9. Antibacterial activities of samples against *S. aureus* with the incubation time. A control I assay was carried out when no material was added to the peptone solution. A control II assay was carried out when only n-HA powder was added to the peptone solution. The concentration of the samples was 1 g/mL. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE III
Results of the Antibacterial Rates of the n-HA/ZnO Composite Against Bacteria

	Antibacterial Rate (%)	
	Under Light	Without Light
<i>E. coli</i> (2.1×10^6 cfu/mL)	95.65	87.48
<i>S. aureus</i> (1.9×10^6 cfu/mL)	99.45	91.32

Note: cfu denotes colony forming unit; 2.1×10^6 cfu/mL, 1.9×10^6 cfu/mL are the concentration of bacteria used in antibacterial test experiments.

Figure 10, it can be deduced that with a prolong reaction time, form 1 can be efficiently transformed to form 2 at 300 min. As shown in the study, it shows better DNA cleavage ability after combining to filtrate off the n-HA/ZnO complex. In this catalytic cycle, the complex first combined with DNA, ZnO reacts with H_2O , thus hydroxyl anion coordinated to the metal Zinc may serve as nucleophilic reagent,³¹ as well as the Zn^{2+} activates the central phosphorus atom. Then, it attacks the phosphorus anion to form a transition state. Meanwhile, the —OH group of n-HA acts also as a nucleophilic reagent and induces the complex to connect with other sides of pUC18. This cooperative effect of n-HA and ZnO catalyzes the hydrolysis of the 3,5-phosphate diester bond in the DNA sequence. Finally, it breaks up to complete the DNA cleavage (shown in Figure 11), inducing the changes of DNA and destroying the bacteria.

Figure 10. Agarose gel electrophoresis cleavage reaction of *E. coli*'s DNA by the dissolving solution of materials with different time (ethidium bromide staining). Lane 1: Control I, A control I assay was carried out without material; Lane 2: Control II, A control II assay was preformed when only n-HA contacted with pUC18 for 300 min; Lanes 3–9: n-HA/ZnO complex was contacted with pUC18 at 1, 5, 10, 15, 30, 60, and 300 min.

Figure 11. Proposed mechanism of DNA cleavage catalyzed by n-HA/ZnO complex. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

CONCLUSION

n-HA/ZnO complex possesses a nanometer size, and the size, composition as well as structure of n-HA change little during complexation. The lattice parameters, a and c , of both n-HA and ZnO change after compounding. Zn^{2+} ions of ZnO can enter the n-HA crystal lattice. The n-HA/ZnO complex has a good antibacterial activity, which is 98.65% and 99.45% against *E. coli* and *S. aureus* under light, respectively, and the antibacterial activity for *S. aureus* is stronger than that for *E. coli*. The occurrence of antibacterial capability is supposed to be due to the generation of active oxide from the surface of complex and Zn^{2+} or OH^- , which can cleave the 3',5-phosphate diester bond, inducing the decomposition of bacteria.

The approach as used in the current study produced a complex with good antibacterial activity properties without sacrificing biocompatibility. The

resulting HA-based system holds wide potential for application in clinic as an antibacterial biomaterial, as well as for dental filling and repair.

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