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Antibacterial Property of Hydroxyapatite with Ag^+ Dopant Produced by Hydrothermal Process

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The antimicrobial powders of hydroxyapatite with Ag^+ dopant were one-step synthesized with $Ca(NO_3)_2{\cdot}4H_2O$ and $AgNO_3$, $(NH_4)_2HPO_4$ by hydrothermal method. It can be found that the concentration of Ag^+ ions in hydroxyapatite powder has good linear relationship with the dosage of $AgNO_3$. The analyses of XRD patterns show that the Ag-hydroxyapatite powder has the same crystal structure and functional group with hydroxyapatite powder, which indicates that the Ag-hydroxyapatite powder exhibits the structural properties of pure hydroxyapatite powder. Through calculations of EDS and XRF, it is known that the Ca^{2+} ions in crystal structure of hydroxyapatite powder can be substituted partially by Ag^+ ions in hydrothermal condition, which synthesize the final product $Ag_xCa_{10-x}(PO_4)_6(OH)_2$. The result of antibacterial experiment shows that the produced Ag-hydroxyapatite powder has good antibacterial property, the MIC ≤ 50 μg/mL (*Escherichia coli* and *Staphylococcus aureus*).

Key Words: Ag, Hydroxyapatite, Hydrothermal, Antibacterial property, Synthesis.

INTRODUCTION

Hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$ abbreviated as HA or HAp], as one of the most attractive kind of apatite which has the crystal structure of $M_{10}^{2+}(ZO_4)_6^{3-}X^{2-}$, is obtained by substituting the elements in the crystal structure of apatite. Due to the similar chemical component and structure with $Ca_3(PO_4)_2$ and the good bioactive and biocompatibility, hydroxyapatite, acts as a promising bioceramic material, becomes the hot spot in the medical material field[1,2]. Ag^+ ions have attracted more attention because of its good antibacterial properties[3,4]. It is thought that the compound with Ag or Ag^+ ion can block the breath and electron transition of microbial, baffle the synthesis of enzyme, destroy protein, damnify the cytomembrane and interfere the combination of DNA[5]. The antibacterial property of Ag^+ ions is better than Zn^{2+}, Cu^{2+} ions[6,7]. Ag^+ ions can substitute certain other elements and settle down in the crystal structure, which make the final product has no toxicity. So the antimicrobial with Ag dopant becomes the most important kind in the inorganic antibacterial material. The study on antimicrobial with Ag dopant is focus on the antimicrobial with the medium of silicate, phosphate and oxide[8], the HA powder as the medium is seldom mentioned. Now, the ion exchange process is mainly adopted to prepare the Ag-HA powder, in which the weak bonds of Ag^+ ions and the medium make the short antibacterial property with the quick dissociation of Ag^+ ions. Furthermore, the Ag^+ ions at initial stage have the deep concentration and toxicity, which make the ion exchange process unsuitable to prepare antimicrobial.

In this study, Ag-HA powder was synthesized with the hydrothermal method, which has not been mentioned before. The XRD, EDS, XRF and TEM measurements were carried out with the obtained powders and the antibacterial property was characterized by the bactericidal coefficient and the MIC.

EXPERIMENTAL

$Ca(NO_3)_2{\cdot}4H_2O$ (AR); $(NH_4)_2HPO_4$, $AgNO_3$, SDBSAR; Ammonia, φ = 50 %; absolute ethyl alcohol, *Escherichia coli*, ATCC 1818; *Staphylococcus aureus*, ATCC 1811.

Reaction kettle with PTEF lining, φ 50 mm × 100 mm; BS 214D electronic balance, Sartorius: used for preparation Ag-HA with hydrothermal method; BD86 X-ray diffractometer, Beijing university; Philips CM200 transmission electron microscope, FEI: used to characterize the structure and morphology of the produced powders; EDAXGenesis 2000 energy dispersive spectroscope, FEI: used to analyze the elements; X-ray fluorescence spectroscope, Spectro used to measure the weight percentage contents (wt %) of the elements.

Hydrothermal synthesis of Ag-HA powder: $Ca(NO_3)_2{\cdot}4H_2O$, $AgNO_3$ and SDBS were weighted and dissolved in distilled water with stirring. Ammonia was added to adjust the pH of the mixture above 10. This was the solution A. Solution B was obtained by dissolving certain $(NH_4)_2HPO_4$ in distilled water and the pH of the B was also above 10. Solution B was added

dropwise into solution A with stirring for 0.5 h, then the mixture of A and B was placed into the reaction kettle to synthesize Ag-HA powder. After suction filtration, the obtained powder was rinsed by distilled water two or three times, then by absolute ethyl alcohol two or three times. The powder was dried at 80-90 ºC and then grinded.

Repeat above steps with different ratios of raw materials at different hydrothermal conditions to get the different samples. All the obtained powders were carried out the below characterization measurements.

Measurement of the concentration of Ag^+ ions in Ag-HA powder: Volhard method was followed to measure the content of Ag^+ ions in the powder. After accurately weighting, certain powder was added into the mixture of 100 mL distilled water and 5 mL ammonia (φ = 50 %) and dissolved with heating. The cooled solution with 2 mL ferrous ammonium sulfate indicator was titrated by the NH_4SCN. The appearance of red $Fe(SCN)^{2+}$ which followed the white precipitation of AgSCN indicated the termination of the reaction.

The concentration of Ag^+ ions can be calculated with the formula below (the result show the percentage of Ag^+ ions which substitute the Ca^{2+} ions in the structure):

$$Ag\ (\%) = \frac{n_{Ag}}{N_{Ca}} = \frac{C_{NH_4SCN} V_{NH_4SCN} \times 10^{-3}}{\frac{m_s}{M_{Ca_5(PO_4)_3OH} \times 5}} \times 100$$

where c_{NH_4SCN}: the concentration of NH_4SCN, mol/L; V_{NH_4SCN}: the used volume of NH_4SCN, mL; m_s: the weighted powder, g; $M_{Ca_5(PO_4)_3OH}$: the relative molecular mass of $Ca_5(PO_4)_3OH$, 502.37 g/mol.

Antibacterial study of Ag-HA powder: The antibacterial property of Ag-HA powder was characterized by the bactericidal coefficient. The cultures used in this experiment were *Escherichia coli* and *Staphylococcus aureus*.

Solution-I: Certain Ag-HA powder was put into the 25 mL LB agar medium and sterilized.

Solution II: The 25 μL culture solution with bacterial was added into aseptic culture dish with transfer liquid gun in the aseptic condition.

The solution I was dissolved and cooled to 50 ºC, then mixed with the solution II in the culture dish which was put on the desk and shaked by swirling rapidly to make the solution II, culture medium and Ag-HA powder mix evenly. The plates which obtained after condensation were cultured at 37 ºC for 24-28 h, then observed, the left number of colony and the total number of colony in the mixture can be used to calculate the bactericidal coeifficient.

$$\text{Bactericidal coefficient}\ (\%) = \frac{A - B}{A} \times 100$$

where A: the total no. of colony B: the left no. of colony after reaction.

RESULTS AND DISCUSSION

Analysis of the concentration of Ag^+ ions in Ag-HA powder and XRD: Through the contrast tests, the dosage of SDBS which act as surfactant was 0.10 g, the synthesis lasted for 5 h at 180 ºC. The dosages of $(NH_4)_2HPO_4$, $Ca(NO_3)_2{\cdot}4H_2O$ were kept 6.36 g, 2.13 g to make Ca/P as constant 1.67.

Fig. 1 shows the variance of the concentration of Ag^+ ions (wt %) with the different dosages of $AgNO_3$ (g). The concentration of Ag^+ ions (y) in the Ag-HA powder were 0.19, 0.24, 0.35, 0.55 and 0.65 % corresponding to the dosages of $AgNO_3$ (x) 0.01, 0.02, 0.04, 0.08 and 0.10 g. The relationship between x and y is linear, the equation is y = 5.1167x + 0.1402, correlation coefficient R^2 = 0.9998 which indicates that a little Ag^+ ions remains in the mother solution.

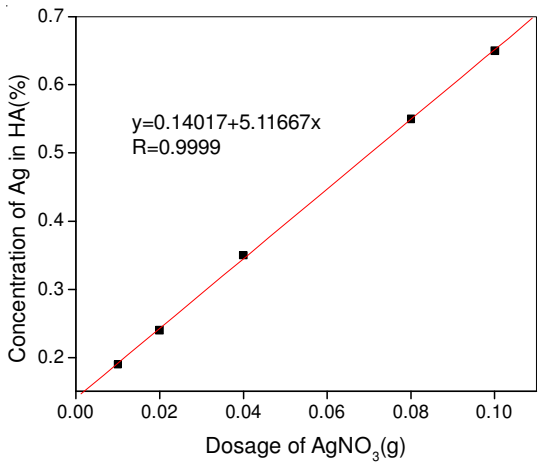

Fig. 1. Linear relationship of concertation of Ag^+ in hydroxyapatite (HA) and dosage of $AgNO_3$

In Fig. 2, the XRD patterns for HA-powder and Ag-HA powders with different concentration of Ag^+ ions (0.35, 0.65 and 0.80 %) are presented.

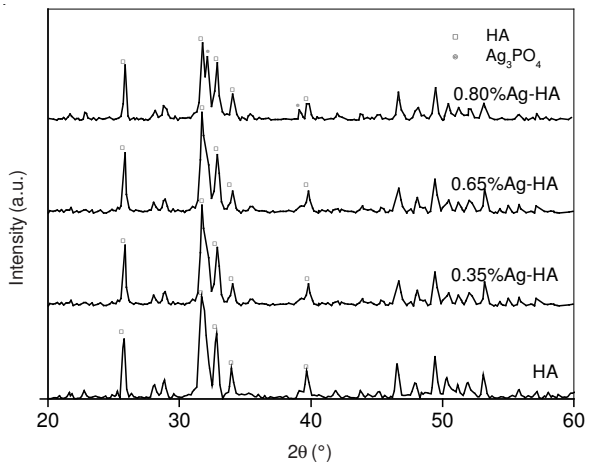

Fig. 2. XRD patterns of the hydroxyapatite (HA) and Ag-HA powder

It is clear that the 4 peaks at 2θ = 25.80, 31.68, 32.84 and 33.96º which are the characteristic peaks of HA (indicated with % in Fig. 2) can be found in the XRD patterns of Ag-HA powders. The diffraction peak of Ag_3PO_4 (indicated with % in Fig. 2) arises in the XRD pattern of the 0.80 % Ag-HA powder. It can be thought that the crystal structure of Ag-HA powder with certain concentration of Ag^+ ions (< 0.80, wt %) is same with HA (JCPDS 09-432), too many Ag^+ ions (≥ 0.80 %) can form Ag_3PO_4. It can be assumed that Ag^+ ions substitute Ca^{2+} partially in the crystal lattice at hydrothermal condition, which forms sosoloid. In fact, comparing the Ag-HA powders, it can be found that the colour of 0.36 % Ag-HA and 0.65 % powders

TABLE-1
SPACING D AND PEAK LOCATION OF THE HYDROXYAPATITE (HA) POWDER AND Ag-HA POWDERS

Sample	HA	0.35 % Ag-HA	0.55 % Ag-HA	0.65 % Ag-HA
Spacing d and peak location of (002) interface	3.4530/25.800	3.442/25.880	3.442/25.880	3.442/25.880
Spacing d and peak location of (211) interface	2.824/31.680	2.821/31.720	2.817/31.760	2.810/31.840

is same with HA powder, no faint yellow or brownish black precipitation which indicates Ag_3PO_4 and Ag_2O, respectively while it of the 0.80 % Ag-HA powder is with a little faint yellow. These facts have proved the deduction from the analysis of XRD patterns.

The spacing d and the locations of the characteristic peaks of the powder can be obtained through the XRD test as listed in the Table-1.

It can be seen that the d of (002) along c axis decreases to 3.442 and keeps this value with the increasing of the dosage of Ag ions. However, the d of (211) decreases while θ increases with the increasing of the dosage of Ag ions. According to the Bragg formula, the variety of d indicates the existence of sosoloid. After substituting Ca^{2+} ions in crystal structure, the Ag ions have the radius which varies with its coordination number in the crystal with the value of 0.115 nm due to the higher coordination number. The Ag ions with bigger radius than Ca^{2+} ions whose radius is 0.106 nm make the aberrance of the crystal lattice.

Analysis of EDS and XRF for Ag-HA powder: Fig. 3 shows the analysis of elements and Table-2 shows the weight percentage concentrations (wt %) of the elements.

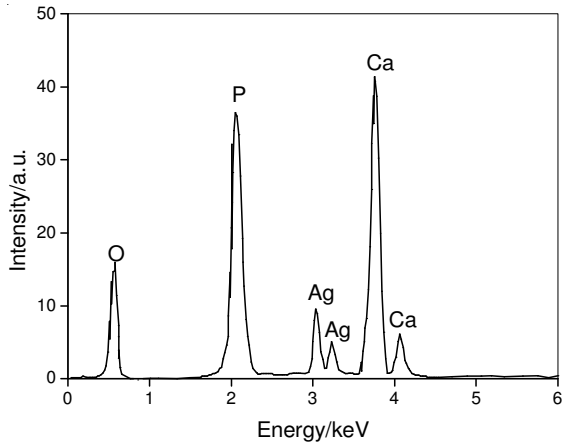

Fig. 3. EDS of the 0.65 % Ag-HA powder

TABLE-2
X-RAY FLUORESCENCE SPECTRUM
OF 0.65 %Ag-HA POWDER

Element	O	P	Ca	Ag
wt %	39.67	17.71	35.74	6.69

As mentioned before, the ratio between Ca atoms and P atoms is 1.67 in pure HA powder, while through the calculations based on the Fig. 3 and T2, the ratio is 1.56 in 0.65 % Ag-HA powder, which is smaller. Furthermore, the calculation shows that the ratio between (Ca, Ag) and P in the Ag-HA powder is just 1.67, which indicates that the Ag^+ ions have substituted Ca^{2+} ions partially in the crystal structure of HA powder at the hydrothermal condition and synthesized $Ag_xCa_{10-x}(PO_4)_6(OH)_2$ as ionic sosoloid.

Analysis of the TEM pictures: The morphology of hydroxyapatite and Ag-HA powder can be seen through the TEM pictures. The different pictures with different dosages of $AgNO_3$ have listed in Fig. 4.

It is clear that all the powders are clavate particles with uniform granularity and dispersion. There is no significant change of the diameters of the particles with the increasing of Ag^+ ions, but the lengths of the particles increase. HA powder belongs to hexagonal system, P63/m space group. In this structure, the OH^- groups stand at the four corners of the cell, all the ten Ca^{2+} ions occupy two different kind sites: four of the ten Ca^{2+} ions are in the centers of the Ca-O octahedrons consisted with 6 O ions, Ca (I); the rest 6 Ca^{2+} ions occupy the centers of triligand consisted with 3 O ions, Ca(II). In the crystal structure of HA powder, there are channels parallel with the c axis, which make the Ca^{2+} ions easier to be substituted by metal ions, such as Ag^+, Zn^{2+}, Cu^{2+}, to form the more completed structure. Due to the bigger radius of Ag ions (0.115 nm) than it of Ca^{2+} ions (0.106 nm), the substitution ratio may be 1:1 and the Ag ions enter the cell along the c axis to substitute Ca^{2+} ions to form $Ag_xCa_{10-x}(PO_4)_6(OH)_2$. And all the above sequiturs have been proved with the analysis of EDS and XRF. The unsaturated field stress due to the difference between the valences of Ag^+ ion and Ca^{2+} ion make the crystal deform during

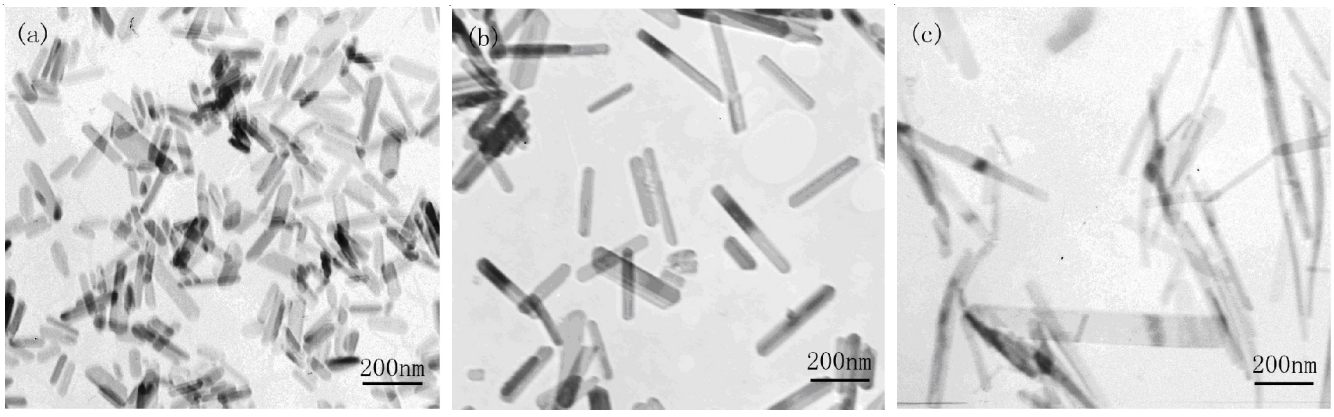

Fig. 4. TEM pictures of HA powder and Ag-HA powders. (a) HA powder (b) 0.35 % Ag-HA powder (c) 0.65 Ag-HA powder

TABLE-3
ANTIBACTERIAL ACTIVITY OF SAMPLES

Microorganism species	Concentration without HA (cfu/mL)	Concentration and bactericidal coeifficient with HA	Concentration and bactericidal coeifficient with 0.35 %Ag-HA	Concentration and bactericidal coeifficient with 0.65 %Ag-HA
Escherichia coli	1.39×10^7	1.38×10^7 cfu/mL 0 %	960 cfu/mL > 99.9 %	400 cfu/mL > 99.9 %
Staphylococcus aureus	4.32×10^6	4.22×10^6 cfu/mL 0 %	320 cfu/mL > 99.9 %	0 cfu/mL > 99.9 %

growth. The data summarized in Table-1 exhibit the decrease of the spacings of (002) and (211) along c axis in the substitution of Ca^{2+} by Ag^+ ions, *i.e*., the cells grow bigger with significant increase of the size along c axis while the size along a axis keeps stable. So the crystal of HA grows along the crystal axis and the lengths of the particles increase with the increasing of Ag^+ ions.

Antibacterial property of Ag-HA: The results of antibacterial property for the Ag-HA powder are given in Table-3.

After reacting with HA powder and Ag-HA powder at 37 ºC for 24 h, respectively, the solutions with *Escherichia coli* and *Staphylococcus aureus* show the different results: the number of colony of the two bacteria has no significant variety in the solutions reacted with HA powder, while the number of colony of the two bacteria decreased in the solution reacted with Ag-HA powder with the increasing of Ag ions, the bacterial coefficient is equal or greater than 99.9 %, the MIC of the powder is 50 μg/mL. The results show that HA powder has no antibacterial property, while the Ag-HA powder has good antibacterial property. The Ag^+ ions with positive charges can attract the bacterial with negative charges and form the stable covalent bonds with the -SH groups in protein which inactivate the enzymes. The bacterial died rapidly due to the destroyed structure. Furthermore, considering the concentration of the left bacterial in the solution after 24 h, it can be deduced that the effect of antibacterial property increases with the increasing of Ag dopant.

Figs. 5 and 6 are the actual pictures of the 0.65 % Ag-HA powder reacted with *Escherichia coli* and *Staphylococcus aureus*. It is clear that the quantity of the two bacterial becomes smaller with the addition of Ag-HA powder.

Conclusion

The antimicrobial powders of hydroxyapatite with Ag^+ dopant were synthesized with $Ca(NO^3)_2{\cdot}4H_2O$, $AgNO_3$, $(NH_4)_2HPO_4$ by hydrothermal method. The result of experiment shows that: When the dosage of $AgNO_3$ (x) is between 0.01 and 0.10 g, it has good linear relationship with the concentration of Ag^+ ions in the powder (y), $y = 5.1167x + 0.1402$. A little of Ag ions remain in the mother solution which make the y smaller than x. The analysis of XRD shows that the Ag-HA powder has the same crystal structure and functional group with HA powder, the Ag-HA powder exhibits the same structural properties with the pure HA powder. The results of EDS and XRF indicate that $Ag_xCa_{10-x}(PO_4)_6(OH)_2$ can be synthesized as ionic sosoloid by the substitution of Ca^{2+} ions with Ag^+ ions in the structure of HA powder. The synthesized powder has good antibacterial property, MIC ≤ 50 μg/mL (*Escherichia coli* and *Staphylococcus aureus*).

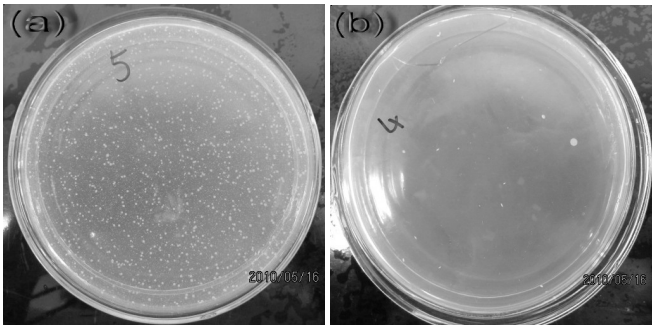

Fig. 5. Antibacterial effect of 0.65 % Ag-HA on *Escherichia coli*. (a) Without 0.65 % Ag-HA bwith 0.65 % Ag-HA

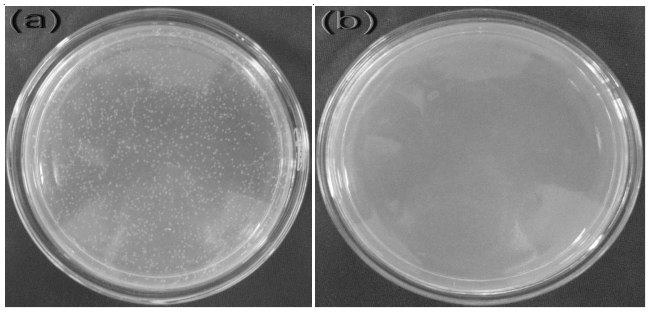

Fig. 6. Antibacterial effect of 0.65 % Ag-HA on *Staphylococcus aureus* (a) Without 0.65 % Ag-HA (b) with 0.65 % Ag-HA

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