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Antimicrobial effects of metal ions (Ag^+, Cu^{2+}, Zn^{2+}) in hydroxyapatite

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The antimicrobial ceramics (AC) based on hydroxyapatite (HA) were made in a wet chemical process with additions of $AgNO_3$, $Cu(NO_3)_2 \cdot 3H_2O$ and $Zn(NO_3)_2 \cdot 6H_2O$. The ACs were composed of metal-ion substituted hydroxyapatite and nitrate–apatite, which was identified by X-ray diffraction. The viable count and turbidity measurement was adopted to observe the antimicrobial effects of the various ACs. The aerobic *Escherichia coli* was used in the study. An obvious antimicrobial effect against *E. coli* was observed in Ag^+ AC. In contrast to Ag^+ AC, it was difficult to ascertain any bactericidal effect in the case of Cu^{2+} and Zn^{2+} AC. The bactericidal effect of Ag^+ was observed using a dialysis tube experiment. This suggests that Ag^+ dissolved out and reacted with *E. coli*, thus inhibiting its growth.

1. Introduction

Antimicrobial ceramics (ACs) are becoming increasingly important because of their wide range of applications, including fabrics, building materials, cosmetics, electrical appliances, etc. It is known that certain metal ions penetrate into bacteria and inactivate their enzymes, or some metal ions can generate hydrogen peroxide, thus killing bacteria [1]. However, the mechanism is not clearly understood. The AC consist of substrate and metal ions, and they are classified as zeolite AC, calcium phosphate AC, amorphous silica AC, etc., according to the substrate [1]. The applications of ACs are closely related to human lifestyle, such as fabrics, cosmetics, electrical appliances, which require good biocompatibility.

It is well known that hydroxyapatite (HA) has good biocompatibility and it is used as an implant biomaterial because it possesses similar structure to the major mineral constituent of human hard bone [2–4]. In addition to its good biocompatibility, the cation exchange rate of HA is very high with heavy metals or harmful ions such as Pb^{2+}, Cd^{2+}, Cu^{2+}, Mn^{2+}, Ag^+, Co^{2+}, etc. [5, 6]. Thus, metal ion-substituted HA was prepared to study the antimicrobial effects against bacteria or fungi [7–9].

Several metal ions have been used in antimicrobial ceramics, such as Ag^+, Cu^{2+}, etc. [1, 10–13]. Among these, three metal ions were studied in the present work: Ag^+, Cu^{2+} and Zn^{2+} [1, 10–13]. The AC was prepared via the wet chemical method [7–9] and its powder was analysed by X-ray diffraction (XRD). The antimicrobial effects of the metal ion ACs against *Escherichia coli* were tested with spread plate method and photographed. The growth rate of *E. coli* treated with various ACs was observed in order to determine the antimicrobial effects.

A dialysis tube was used for the selective penetration of Ag^+ ions into the antimicrobial test.

2. Materials and methods

2.1. Antimicrobial ceramics

Three types of AC based on HA with known compositions (Table I) were made by wet chemical process.

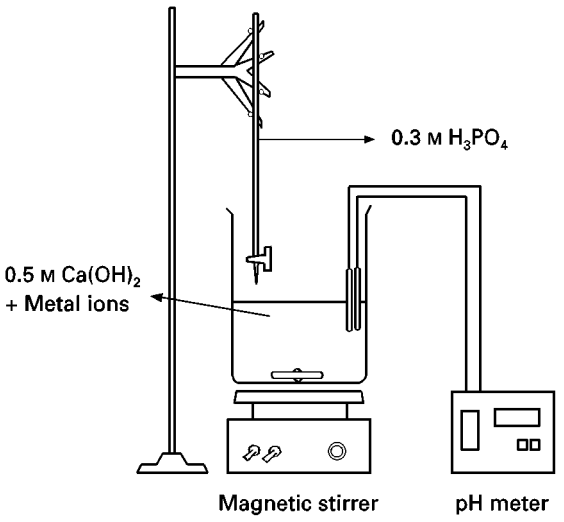

Figure 1 Schematic drawing of the chemical reaction apparatus for hydroxyapatite-based antimicrobial ceramics.

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TABLE I The chemical compositions of three types of antimicrobial ceramics

Sample	$AgNO_3$ (mol)	$Cu(NO_3)_23H_2O$ (mol)	$Zn(NO_3)_26H_2O$ (mol)	$Ca(OH)_2$ (mol)	H_3PO_4 (mol)
A – 1	0.001			0.167	0.1
A – 2	0.01			0.167	0.1
A – 3	0.0001			0.167	0.1
B – 1		0.001		0.167	0.1
B – 2		0.01		0.167	0.1
B – 3		0.0001		0.167	0.1
C – 1			0.001	0.167	0.1
C – 2			0.01	0.167	0.1
C – 3			0.0001	0.167	0.1

The metal ion chemical reagent was completely dissolved in an exact amount of distilled water and 0.5 M $Ca(OH)_2$ was suspended in the solution. 0.3 M H_3PO_4 was slowly dropped into 0.5 M $Ca(OH)_2$ suspension and the solution was stirred with a magnetic bar during the chemical reaction (as shown in Fig. 1). The pH was monitored and the reaction was terminated at pH 9.1, which could produce the stoichiometric HA with a Ca/P ratio of 1.67 [7]. The precipitate formed during the reaction was filtered and dried in an oven at 120 °C. After drying, the AC was grounded with a mortar and pestle in order to obtain a fine powder. The powder was investigated using XRD (Rigaku D/max-RB, Japan), which was operated at 40 keV and 80 mA with CuK_α.

2.2. Antimicrobial test

2.2.1. Viable count (spread plate method)

An amount (1 mg) of each AC was mixed in a test tube with 1 ml phosphate buffer saline solution (PBS), (Table II), which contained about 1×10^5 cells/ml *E. coli*; each tube was shaken at 30 °C, 200 r.p.m. for 24 h.

The 0.1 ml of shaken AC solutions was inoculated on to the 20 ml LB agar plate. These were incubated at 37 °C for 24 h and the number of colonies was counted and photographed. All glassware was sterilized in the autoclave at 121 °C for 30 min before each experiment.

2.2.2. The antimicrobial effects of the Ag^+ ion

The complete depletion of Ag^+ ions was realized by adding more NaCl to PBS in order to investigate the antimicrobial effects of the Ag^+ ion. Sample A2 and 2 mg $AgNO_3$ were reacted with 2 mg NaCl in 1 ml PBS solution in a 10 ml test tube (containing 1×10^5 cells/ml *E. coli*). The tube was shaken at 30 °C, 200 r.p.m. for 20 h and the 0.1 ml solution was inoculated and grown on the 20 ml LB agar plate at 37 °C for 15 h. Then the number of colonies was counted.

TABLE II The compositions of the phosphte buffer saline solution

NaCl	Na_2HPO_4	NaH_2PO_4	H_2O	pH
8.5 g	2.2 g	0.2 g	1000 ml	7.4

Ag^+ ions can selectively penetrate the dialysis tubes with molecular sizes ranging from 6000–8000. A schematic drawing of the experimental procedure is shown in Fig. 2. (a) 0.1 ml PBS containing 1×10^5 *E. coli* cells/ml and 0.9 ml H_2O were mixed with 1 mg $AgNO_3$ in a 10 ml test tube, and (b) 1 ml H_2O and 1 mg $AgNO_3$ were mixed in a 10 ml test tube. Both tubes were shaken at 37 °C, 150 r.p.m. for 24 h and then 1 ml of the solution from each tube was mixed in the dialysis tube. The dialysis tubes were immersed in the solution containing 19 ml H_2O and 1 ml PBS (1×10^5 *E. coli* cells/ml) in the flasks. The flasks were incubated at 30 °C, 200 r.p.m. for 24 h. After shaking, 0.1 ml of each solution from the flasks was inoculated into the 20 ml of LB agar plate. The LB agar plates were subsequently cultured in the 30 °C incubator for 24 h and the number of the colonies was counted.

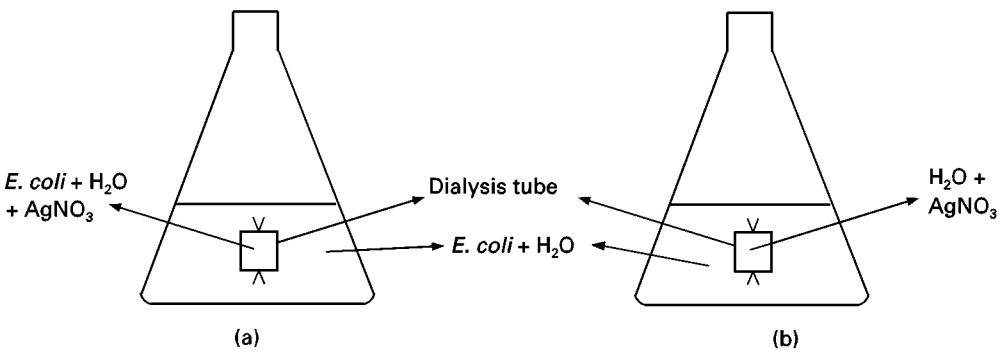

Figure 2 Schematic illustration of the Ag^+ ion selective penetration experiment using a dialysis tube.

TABLE III The compositions of LB liquid nutrient

Peptone	Yeast extract	NaCl	H_2O	pH
2 g	1 g	2 g	200 ml	7.5

2.2.3. Turbidity measurement

The antimicrobial effect of ACs was observed with a spectrophotometer in an LB liquid nutrient. The growth rate of *E. coli* in samples A and B was examined in two different ways.

(a) 1 mg of ACs, 1 ml PBS (which contained 1×10^5 cells) and 50 ml LB liquid nutrient were mixed in 500 ml shaking flasks and the flasks were shaken at 37 °C, 150 r.p.m. for 15 h. Then 0.5 ml of the solution from the flasks was diluted with 2 ml water and the absorbance was measured at 420 nm every 1 or 2 h during shaking.

(b) 1 mg ACs, 1 ml PBS (containing 1×10^5 cells) were mixed in 10 ml test tubes and the tubes were shaken at 37 °C, 150 r.p.m. for 15 h. Then 0.1 ml solution from the tubes was inoculated in 20 ml of LB liquid nutrient in 500 ml shaking flasks and the flasks were shaken at 37 °C, 150 r.p.m. for 15 h. The 0.5 ml of the solution from the flask was diluted with 2 ml water and the absorbance was measured at 420 nm at an interval of 1 or 2 h. The compositions of LB liquid nutrient are listed in Table III.

3. Results and discussion

3.1. The analysis of antimicrobial ceramics

The constituent phases of the ACs were analysed by X-ray diffraction (XRD) (Fig. 3). The AC powder consisted of hydroxyapatite (HA) and nitrate–apatite; $(Ca, M)_{10}(PO_4)_6(OH)_2$ and $Ca_5(PO_4, NO_3)_3(OH)$ (M: metal ion), respectively. It is suggested that the Ca^{2+} ions in hydroxyapatite can be substituted by Ag^+, Cu^{2+}, Zn^{2+} ions, thus providing the substituted HA with effectiveness. It was not necessary to eliminate the $Ca_5(PO_4, NO_3)_3(OH)$. Thus, the two compounds were used at the same time with the AC in this study.

3.2. The antimicrobial effects of the various ACs

The Ag^+ HA (A − 1, A − 2, A − 3) showed complete inhibition of the growth of *E. coli* after 20 h incubation (Table IV, Fig. 4). However, 92–232 colonies were seen after 30 h. In contrast to Ag^+ HA, the Cu^{2+} and Zn^{2+} HA did not show any antimicrobial effect in this experiment. This implied that the Ag^+ HA plays an important role in the inhibition of the growth of *E. coli.*

It is important to study Ag^+ HA to understand its inhibition of the growth of *E. coli.* When the 1 mg $AgNO_3$ was mixed in 1 ml PBS, the total amount of Ag^+ ions was reacted with NaCl in PBS during shaking in a tube, and produced the AgCl precipitate. The white AgCl precipitate was easily observed when 1 mg of $AgNO_3$ was added to the 1 ml PBS. The precipitation amount is shown in Table V. The amount of Cl^- was about 25 times more than that of Ag^+ (Table V). Thus almost all of the Ag^+ was precipitated with Cl^- as AgCl. In spite of the precipitation of AgCl, the antimicrobial effect of $AgNO_3$ was evident (Table IV, Fig. 4).

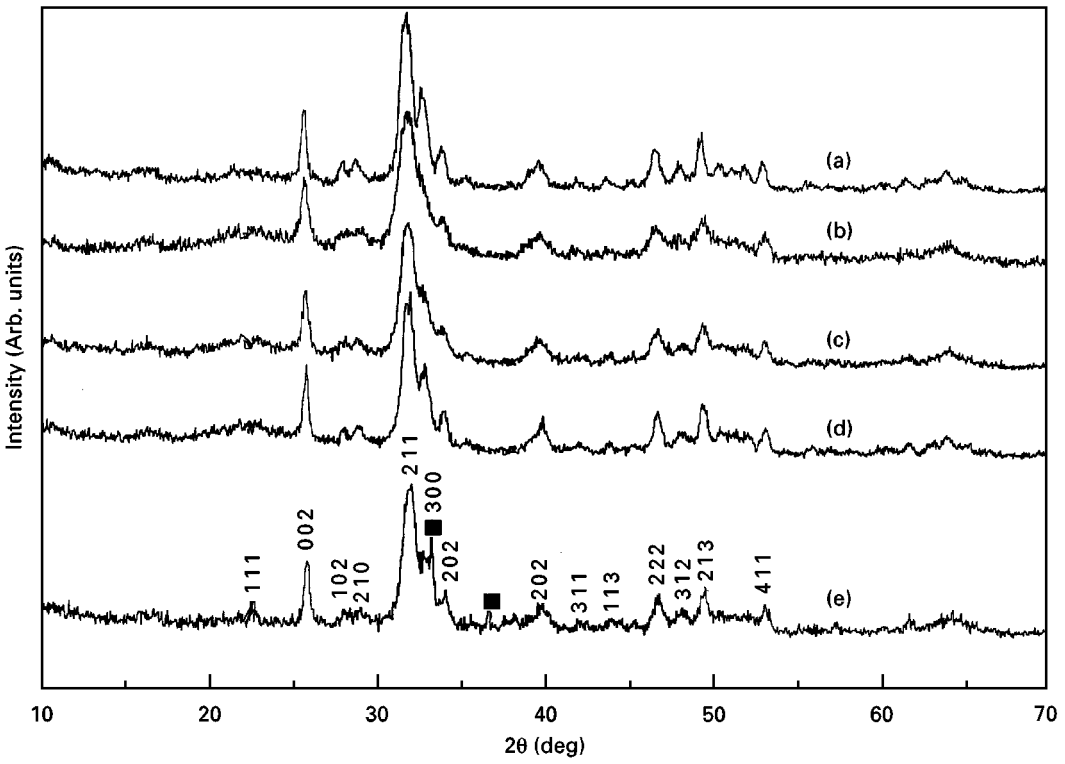

Figure 3 The X-ray diffraction results of various hydroxyapatites with metal ions. (a) Pure HA, (b) HA + $AgNO_3$ (0.01 mol), (c) HA + $Cu(No_3)_2$ (0.01 mol), (d) HA + $Zn(NO_3)_2$ (0.01 mol), (e) HA + $AgNO_3$ (0.1 mol). (■) nitrate-apatite.

TABLE IV Results of viable cell counts in the various antimicrobial ceramics

Sample	Number of Colonies (20 h)	Number of colonies (30 h)
Control	32 200	Too many
Hydroxyapatite	26 700	Too many
$AgNO_3$	0	52
$Cu(NO_3)_2$	17 900	Too many
$Zn(NO_3)_2$	24 500	Too many
A − 1	0	232
A − 2	0	92
A − 3	0	150
B − 1	21 500	Too many
B − 2	18 400	Too many
B − 3	26 500	Too many
C − 1	15 500	Too many
C − 2	18 000	Too many
C − 3	21 700	Too many

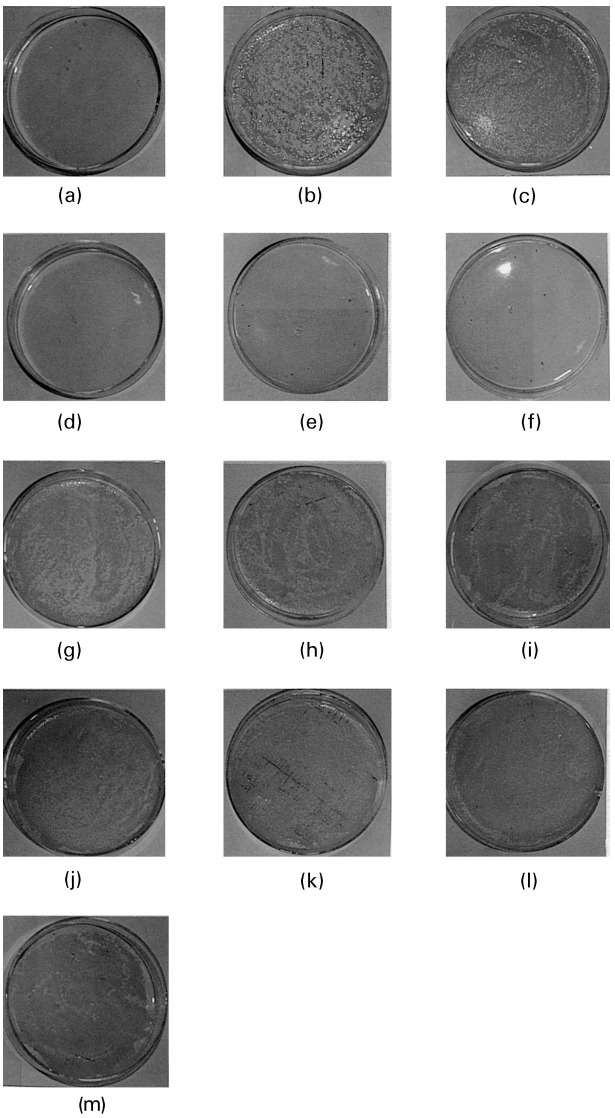

Figure 4 The spread plate test results of antimicrobial effects of the various ACs. (a) $AgNO_3$, (b) $Cu(NO_3)_2$, (c) $Zn(NO_3)_2$, (d) A − 1, (e) A − 2, (f) A − 3, (g) B − 1, (h) B − 2, (i) B − 3, (j) C − 1, (k) C − 2, (l) C − 3, (m) control.

In order to confirm the complete depletion of Ag^+ due to the precipitation of AgCl, 2 mg NaCl was added to 2 mg A − 2 sample and 2 mg $AgNO_3$, respectively, as described in Section 2.2.2. Despite the complete removal of Ag^+ ions, no colonies were observed in either LB-petri dish. This suggests that the trace Ag^+ or the nitrate (NO_3^-) influences the antimicrobial effect. When compared with the antimicrobial effects of $Cu(NO_3)_2$, $Zn(NO_3)_2$ and samples of B − 1, B − 2, B − 3, C − 1, C − 2, C − 3, it was evident that the nitrate did not have an antimicrobial effect (Table IV). This implied that the trace Ag^+ plays an important role in inhibiting growth of bacteria. It seems that Ag^+ blocked the metabolism in *E. coli*.

In order to investigate the interaction between *E. coli* cells and Ag^+, Ag^+ aqueous solution was added with 1×10^5 cells and kept inside the dialysis tube, as described in Section 2.2.2. When Ag^+ ions interacted with *E. coli*, these ions did not diffuse out of the dialysis tube and it did not inactivate *E. coli* in the shaking flask (Fig. 2a). This was indicated by the large amount of cell colonies observed after cultivation in the spread agar plate. This fact was clearly observed (Table VI). The cell-treated plate showed 64 colonies while the untreated plate had many colonies (Fig. 2a, b).

TABLE V Comparison of the amount precipitated between 1 ml PBS and 1 mg $AgNO_3$

1 ml PBS			1 mg $AgNO_3$	
Amount of NaCl (g)	0.85×10^{-2}		Amount of $AgNO_3$ (mg)	1
1 mol NaCl (g)	58.5	$AgNO_3$ (g)	1 mol 1.698	
Cl^- (mol)	1.45×10^{-4}		Ag^+ (mol)	5.9×10^{-6}
Amount of AgCl: (mol)		5.9×10^{-6}		
(g)		8.455×10^{-4}		

TABLE VI Results of the viable cell count of *E. coli* with the dialysis tube cell treatment

Dialysis solution	Number of colonies
H_2O + cells + 1 mg $AgNO_3$	64
H_2O + 1 mg $AgNO_3$	Too many

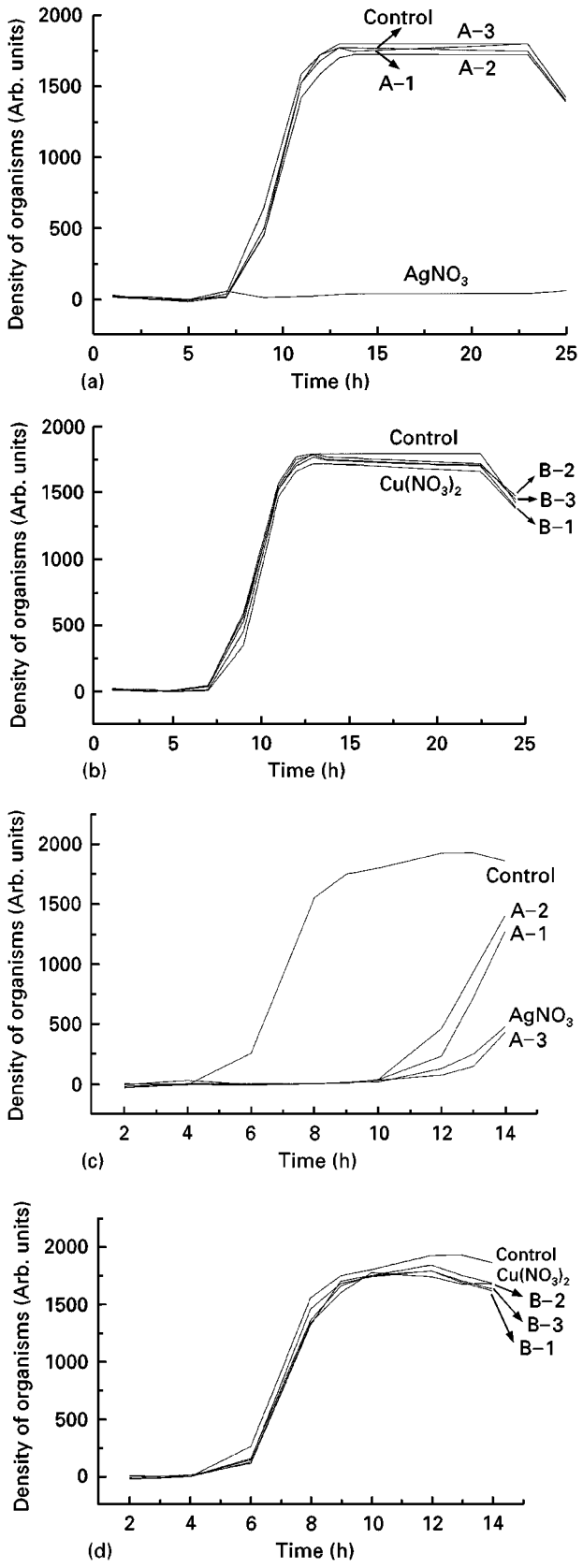

Figure 5 The growth curve of *E. coli*, treated with various kinds of ACs. (a) 50 ml liquid LB nutrient + 1 mg AC + 1 ml PBS (1×10^5 cells) without the shaking procedure in a tube: control, $AgNO_3$. A − 1, A − 2, A − 3; (b) 50 ml liquid LB nutrient + 1 mg AC + 1 ml PBS (1×10^5 cells) without the shaking procedure in a tube: control, $Cu(NO_3)_2$, B − 1, B − 2, B − 3; (c) 20 ml liquid LB nutrient + 0.1 ml PBS with the shaking procedure in a tube: control, $AgNO_3$, A − 1, A − 2, A − 3; (d) 20 ml liquid LB nutrient + 0.1 ml PBS with the shaking procedure in a tube: control, $Cu(NO_3)_2$, B − 1, B − 2, B − 3.

By comparison with Tables IV and VI, it is evident that Ag^+ inactivated the *E. coli* significantly. This fact was very consistent with that anti-infective properties of heavy metals which last for centuries, including silver [12] and the antimicrobial effect of silver sulphadiazine due to the production of small amounts of free Ag^+ by dissociation [13, 14].

3.3. Turbidity measurements of *E. coli* from various treated ACs

The growth rate of *E. coli* treated with various ACs was observed at 420 nm in a spectrophotometer (Fig. 5).

The Ag^+ HA without the shaking procedures in a tube (Section 2.2.3) did not have any effect on the inhibition of the growth of *E. coli*. However the complete antimicrobial effect was observed in $AgNO_3$, in which the Ag^+ easily diffused out in the solution. In contrast to $AgNO_3$, there was no antimicrobial effect in Cu^{2+} HA (Fig. 5b).

When the pretreatment of ACs and *E. coli* was carried out before *E. coli* inoculation in a liquid LB nutrient, as described in Section 2.2.3, the antimicrobial effect was evident in $AgNO_3$ and Ag^+HA (Fig. 5c). The exponential growth of *E. coli* in the control experiment began after 4 h. In contrast to the control, the exponential growth of *E. coli* started after 10 h incubation in $AgNO_3$ and Ag^+HA samples. The result was very consistent with the increasing number of colonies in Ag^+HA samples after 30 h cultivation in the spread plate test (Table IV). This suggested that the pretreatment of Ag^+HA in PBS with *E. coli* was necessary to dissociate Ag^+ ions.

The Cu^{2+}HA samples did not show any antimicrobial effect (Fig. 5d). Thus, this indicates the inability of Cu^{2+} and Zn^{2+} ions to inhibit the growth of *E. coli* in this study.

When the antimicrobial effects of A − 1, A − 2 and A − 3 were compared, low concentration of Ag^+ showed a higher effectiveness in this study. However, the mechanism still requires further study.

4. Conclusions

1. The antimicrobial ceramics (ACs) based on hydroxyapatite (HA) were composed of hydroxyapatite and nitrate–apatite. The HA was substituted with metal ions such as Ag^+, Cu^{2+} and Zn^{2+} in the chemical wet process.

2. The Ag^+ HA demonstrated an obvious antimicrobial effect. However, the Cu^{2+} and Zn^{2+}HA did not show any antimicrobial effects in this study.

3. It was concluded that Ag^+ ions reacted with *E. coli* and inactivated the *E. coli* metabolism, thus inhibiting its growth.

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