Efficient destruction of bacteria with Ti(IV) and antibacterial ions in co-substituted hydroxyapatite films

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Abstract

Hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$: HAP) was co-substituted with Ti(IV) and antibacterial ions (Ag^+ , Cu^{2+} or Zn^{2+}) (HAPTiM), by coprecipitation and ion-exchange methods. Both HAPTiAg and HAPTiCu coated on porous spongy nickel film showed high efficiency for killing *Escherichia coli* and *Staphylococcus aureus* in the dark and under weak UVA irradiation, respectively. Moreover, their bactericidal activities were much higher than that of P25-TiO₂ film. The studies of ESR revealed that not only $\text{O}_2^{\bullet-}$ was formed on HAPTiM, HAPTi, HAP and P25-TiO₂ films under weak UVA irradiation, but also at ambient temperature without light $\text{O}_2^{\bullet-}$ was generated on HAPTiCu, HAPTiAg, and HAPTi. The redox couples of $\text{Cu}^0/\text{Cu}^{2+}$ and Ag^0/Ag^+ in the structure of HAPTiCu (Ag) caused the transfer of electron leading to the $\text{O}_2^{\bullet-}$ generation under the above conditions. The higher bactericidal activities of HAPTiM were due to the synergy of the oxidation role of the $\text{O}_2^{\bullet-}$ and the bacteriostatic action of antibacterial ions. The process of the damage of the cell wall and the cell membrane was directly observed by TEM, and further confirmed by the determination of potassium ion (K^+) leakage from the killed bacteria.

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1. Introduction

In 1985, Matsunaga et al. reported for the first time that TiO₂ photocatalyst could kill bacterial cells in water [1]. Since then, numerous studies related to the bactericidal effect of TiO₂ photocatalyst have been reported [2–5]. TiO₂ photocatalytic disinfection seems to be a promising technique. However, this technique has disadvantages in practical application due to the recovery of TiO₂ powder and the high cost of light. Only a few works have reported the use of TiO₂ films coated on various substances under weak UV light [6–10]. Moreover, these films showed lower bactericidal activity under weak UV light, which is not effective in real purification. The bactericidal process on TiO₂ was investigated by generating decay curves of survival, examining changes in the concentrations of cell envelope components, and taking atomic force microscopy measurements. These data suggest that the bactericidal process consists

of two steps [4,11]. Disordering and partially decomposing of the outer membrane in the cell envelope occurs; this is followed by the disordering and decomposing of the inner membrane. To carry out the above reaction, relatively strong UV light is necessary to produce various reactive species from photo-excited TiO₂. On the other hand, the antibacterial process using metal ions includes the following steps [6]: first, inorganic ions are taken into the cell through the outer of membrane of the cell; then they make the bacteria inactive by simple bacteriostatic action. For cells having little porous protein in the outer membrane, the bactericidal function of inorganic ions would be disabled [12–14]. It is well known that microorganisms are resistant to many environmental circumstances, and inorganic ions-resistant bacteria arise easily under inorganic ion antibacterial conditions. Therefore, the antibacterial activity of inorganic ions is selective; in some cases, they do not function well. To obtain higher bactericidal activity under weak UV intensity, TiO₂ films deposited with antibacterial metals such as copper and silver have been developed [15].

Calcium hydroxyapatite $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ (HAP) is commonly used in the field of bioceramics and as an absorbent for

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liquid chromatography. It has thermal catalytic and photocatalytic properties [16]. Suzuki coworkers have found that Ca(II) of HAP can be exchanged with various metal ions in aqueous media [17–19]. Some studies have been reported that Ag, Cu or Zn ions were incorporated in HAP and showed obvious antimicrobial effect [20,21]. Wakamura et al. [22] prepared HAP with Ti(IV) by the coprecipitation method, and investigated the photocatalytic activity of this material for the destruction of bacteria and acetaldehyde. However, the bactericidal activity of the material is low; only 1 log bacteria was inactivated. Moreover, the mechanism of the reaction was not proven.

In this work, HAP modified with Ti(IV) (HAPTi) was obtained by coprecipitation method, and inorganic ions (Ag^+ , Cu^{2+} and Zn^{2+}) were incorporated in HAPTi (HAPTiM) by ion-exchange method. The HAPTiAg (Cu) coated on porous spumous nickel meshwork showed higher bactericidal activity for *Escherichia coli* and *Staphylococcus aureus* in water under weak UV irradiation or in the dark. Electron spin resonance (ESR) was used to detect the reactive oxygen species (ROS) involved in the reactions. For the first time, it was proven that HAPTi, HAPTiAg and HAPTiCu could generate $\text{O}_2^{\bullet-}$ at ambient temperatures in the dark in addition to under weak UV irradiation. The mechanisms of killing bacteria were discussed.

2. Experimental section

2.1. Materials

Titanium P-25 (TiO_2 ; ca. 80% anatase, 20% rutile; BET area, ca. $50 \text{ m}^2 \text{ g}^{-1}$) was purchased from Degussa Co. The reagent 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), used as the spin trapping agent in ESR studies, was purchased from the Sigma Chemical Co. and stored at -20°C in a freezer. Superoxide dismutase (SOD) (2500–7000 U mg^{-1} , Sigma Chemical Co.) was used. *E. coli* (DH 4 α) and *S. aureus* (ATCC 6538) were purchased from the Institute of Microbiology, Chinese Academy of Sciences. All other chemicals were analytical grade. Deionized and doubly distilled water was used throughout this study.

2.2. Preparation of materials

HAP particles doped with Ti(IV) were prepared by coprecipitation. $\text{Ca}(\text{NO}_3)_2$ and $\text{Ti}(\text{SO}_4)_2$ were dissolved in 1 l deionized-distilled water free from CO_2 at atomic ratios Ti/(Ca + Ti): 0 and 0.1. The total amount of Ca and Ti in the solution was held at 0.1 mol; 0.060 mol of H_3PO_4 was added to the solutions and the solution pH was adjusted to 9 by adding a 15 M NH_4OH solution. The suspension was aged in a capped Teflon vessel at 100°C for 6 h. The resulting precipitates were filtered off, washed with 5 l deionized-distilled water, and finally dried in an air oven at 70°C . The HAPTi(IV) ($X_{\text{Ti}} = 0.1$) particles doped with various inorganic ions including Ag^+ , Cu^{2+} and Zn^{2+} (HAPTiM), were prepared by ion-exchange method. 0.5 g samples of the HAPTi particles were immersed in 90 ml of aqueous solutions comprising the metal nitrates at 0.01 M.

The suspensions were left at ambient temperature for 24 h, and then the particles were separated by filtration, washed with 1000–1500 ml of deionized-distilled water and dried in an air oven at 70°C . By ICP-AES analysis, the amounts of Ag^+ , Cu^{2+} and Zn^{2+} incorporated in HAPTi were 10.23, 6.12 and 5.27 wt.%, respectively. The bactericidal films were prepared by the following method. Suspensions of 0.2 g powders homogeneously dispersed in silica sols were coated on a round porous spumous nickel meshwork by dip-coating method. The films were dried in an air oven at 100°C for 2 h. As a reference, 0.2 g P25- TiO_2 power was supported on spumous nickel meshwork by the same process.

2.3. Characterization

The series of prepared materials were characterized by various conventional methods as follows. The X-ray diffraction (XRD) patterns were taken by a powder method using a Scintag-XDS-2000 X-ray diffractometer Cu K α 400 mA, 40 kV. The X-ray photoelectron spectroscopy (XPS) data were taken on an AXIS-Ultra instrument from Kratos using monochromatic Al K α radiation (225 W, 15 mA, 15 kV) and low-energy electron flooding for charge compensation. To compensate for surface charges effects, binding energies were calibrated using C1s hydrocarbon peak at 284.80 eV. Electron spin resonance (ESR) spectra were obtained using a Bruker model ESP 300 E electron paramagnetic resonance spectrometer. For the determination of ROS from irradiated samples, a quanta-ray Nd:YAG laser system as the irradiation light source ($\lambda = 355 \text{ nm}$) was used. The settings were center field, 3480.00 G; microwave frequency, 9.79 GHz; power, 10 mW. For ICP-AES analysis, the powders were dissolved in dilute HNO_3 solution.

2.4. Bacterial experiments

2.4.1. Bacterial cultures

Two types of bacteria, *S. aureus*, a Gram-positive bacterium, and *E. coli*, a Gram-negative bacterium, were used as model bacteria in this study. Luria–Bertani (LB) and nutrient agar were used as sources for culturing bacteria at 37°C with shaking for 18 h. The culture was washed with a 0.9% saline solution by centrifugation at 4000 rpm. The treated cells were then re-suspended and diluted to $\sim 1 \times 10^7$ colony forming units (cfu ml^{-1}) with 0.9% saline.

2.4.2. Bacterial activity of different films

The bactericidal experiments were performed in an open round glass dish, with an illuminated area of 64 cm^2 using 4 W black light (365 nm) with 0.2 mW cm^{-2} (UV R-365). Meanwhile, the same experiments were also carried out in the dark. The model bacteria included *E. coli* and *S. aureus*. The diluted cell suspension 30 ml and films were added to the glass plate with a cover. Control experiments included a bacterial suspension without catalyst irradiated by UV, and the bacterial activity of Ag^+ , Cu^{2+} , and Zn^{2+} released from HATiM films. Before and during the light irradiation, an aliquot of the reaction solution was immediately diluted with saline and an

appropriate dilution of the sample was incubated at 37 °C for 24 h on nutrient LB medium, and then the colonies were counted to determine the number of viable cells. For the measurement of K^+ leakage from the inactive bacteria, at every time interval, 1 ml of the illuminated bacterial suspension was centrifuged and the supernatant for inductively coupled plasma optical emission spectrometry (ICP-OES) analysis was conducted on an OPTIMA 2000 apparatus (Perkin-Elmer Co.). All the above experiments were repeated three times. In the above experiments, Ag^+ , Cu^{2+} and Zn^{2+} released from HAPTiM films were measured by ICP-OES.

2.4.3. Transmission electron microscopy (TEM)

In this experiment, the HAPTiM powder were used because HAPTiM films and HAPTiM powder have same damage for the *E. coli*, while the HAPTiM power exhibited higher activity than HAPTiM film due to the surface area decrease. In order to check the morphology changes, the high concentration *E. coli*, 10^8 cfu ml⁻¹ cells were mixed with 0.2 g l⁻¹ of the prepared powders and the suspension was irradiated. At given time intervals, the cell suspensions were collected and centrifuged down to pellets. For the TEM analysis of *E. coli*, all samples were prepared according to the following standard procedures [23]. The bacteria pellets were pre-fixed in 2.5% glutaraldehyde at 4 °C for 12 h, and then were washed two times with 0.1 M phosphate buffer (PBS) (pH 7.2). After washing with PBS, the specimens were rendered by mixing with 2% Na₂H₅[P(W₂O₇)₆] aqueous solution at a volume ratio of 1:1 for 2 h. Then the mixed suspensions were dropped onto copper grids with holey carbon film. The grids were dried under natural conditions and examined using a TEM Hitachi H-7500. The same experiments were repeated three times.

3. Results and discussion

3.1. Structure of different prepared materials

Fig. 1 shows the XRD patterns of different samples. Compared with the characteristic patterns of HAP, HAPTi

Fig. 1. XRD patterns of different samples.

Fig. 2. Diffuse reflection UV-vis spectra of different samples (a) HAP, (b) HAPTi and (c) P25-TiO₂. The inset shows UV-vis diffuse reflectance spectra of HAPTiM (Ag, Cu and Zn).

($X_{Ti} = 0.1$) retained well-crystallized HAP. Moreover, the incorporated Zn^{2+} , Cu^{2+} and Ag^+ in HAPTi did not significantly lower the crystallinity of the HAP. XRD patterns showed no significant Zn^{2+} and Cu^{2+} in this study. Four new peaks at 20.7°, 33.4°, 36.7° and 38.5° appeared in the XRD patterns of HAPTiAg, assigned to AgCaP (JCPDS No. 71-1304). A new peaks at 18.2° appeared in the XRD patterns of HAPTiZn, assigned to Ca₃Ti₂O₇ (JCPDS). Fig. 2 illustrates the diffuse reflectance absorption UV-vis spectra of different samples. The UV absorption of HAPTi above 370 nm is observed, which was similar to that of P-25TiO₂, but was not observed in the spectrum of the HAP. This result clearly implied that the surface of HAP particles was modified by substitution with Ti(IV). The inset shows the diffuse reflectance absorption UV-vis spectra of HAPTi with different metal ions. The additional absorption peaks appear at 400–500 nm in the spectrum of HAPTiAg, at 500–800 nm in the spectrum of HAPTiCu, while there is not new peak in the spectrum of HAPTiZn. According to chemical analysis, the atomic ratio of Ti(IV) to Ca(II) in HAPTi was around 0.11—approximately the dosage of Ti(IV) ($X_{Ti} = 0.1$). This result means that the Ti(IV) substituted the position of Ca(II), and was present as a divalent ion leading to oxygen vacancy in the crystal of HAP. With the addition of Ag^+ , Cu^{2+} and Zn^{2+} in the crystal of HAPTi, the Ti(IV) content (1.10 mmol g^{-1}) did not change, but the Ca(II) content decreased from 8.9 mmol g^{-1} Ca(II) in HAPTi to 5.2, 7.4 and 6.8 mmol g^{-1} Ca(II) in HAPTiAg, HAPTiCu and HAPTiZn, respectively, indicating that these ions replaced mainly the Ca(II) of the HAPTi.

3.2. Bactericidal activity of various films

The bactericidal activities of the samples were evaluated by the inactivation of *E. coli* in water. Fig. 3 shows the inactivation of *E. coli* under different control conditions. Clearly, neither HAP film nor HAPTi film under weak UVA irradiation showed any bactericidal effects on *E. coli*. Additionally, no significant inactivation of *E. coli* was observed under UVA irradiation with no catalyst. *E. coli* inactivation of 1.8 log occurred at 180 min of irradiation on P25-TiO₂ films. In contrast, as shown in

Fig. 3. Temporal course of the *E. coli* inactivation (5×10^6 cfu ml⁻¹, 30 ml) in aqueous solution under UVA irradiation (several control experiments) (a) only UVA irradiation, (b) HAP film, (c) HAPTi film and (d) P25TiO₂ film.

Fig. 4A, *E. coli* was almost completely killed within 150 min on HAPTiAg films under weak UVA irradiation and in the dark. Complete *E. coli* inactivation of 6.7 log occurred at 150 min with or without UVA irradiation. The maximum concentration

Fig. 4. Temporal course of the *E. coli* inactivation (5×10^6 cfu ml⁻¹, 30 ml) in different aqueous solutions: (A) HAPTiAg film, (B) HAPTiCu film, and (C) HAPTiZn film.

of Ag⁺ ions leached out into aqueous solution from the HAPTiAg film was about 0.6 mg l⁻¹, which did not show any bactericidal effects on *E. coli*. Similarly, in the presence of HAPTiCu film, 6.6 log *E. coli* were inactivated at 180 min irradiation with UVA light (0.2 mW cm⁻²) (Fig. 4B), while 3.6 log *E. coli* were removed at the same time period in darkness. The maximum Cu²⁺ ions release was 0.012 mg l⁻¹ from HAPTiCu film with or without UV irradiation. The bactericidal effect of the Cu²⁺ concentration was not observed, while HAPTiZn showed different performance from HAPTiAg and HAPTiCu. In the presence of HAPTiZn film (Fig. 4C), 3.28 log *E. coli* were inactivated at 180 min irradiation. Only 0.668 log *E. coli* were removed in the dark, and the released Zn²⁺ ions (0.02 mg l⁻¹) also did not show any bactericidal

Fig. 5. DMPO spin-trapping ESR spectra recorded at ambient temperature in methanol dispersion under UVA irradiation or in dark, (A) HAPTiAg, (B) HAPTiCu and (C) HAPTi, control: DMPO with UVA irradiation.

effect on *E. coli*. Compared with all the above results, the bactericidal activity of HAPTiAg and HAPTiCu films in both UVA and darkness was much higher than that of P25-TiO₂ film with UVA irradiation, while HAPTiZn showed higher activity only under UVA irradiation. Moreover, the single bacteriostatic action of silver, copper and zinc ions was not observed under otherwise identical conditions. In addition, in the experiment, we found that these HAPTiAg (or Cu) films did not show much higher activity under visible light ($\lambda > 400$ nm) than in the dark, indicating HAPTiAg (or Cu) did not visible light-driven activity although they had adsorption of visible light which was mainly contributed to the plasmon resonance of metal. In our another work [24], it has been proved that the adsorption of visible light could not generated reactive oxygen species.

Fig. 6. DMPO spin-trapping ESR spectra recorded at ambient temperature in methanol dispersion under UVA irradiation or in dark (A) P25-TiO₂, (B) HAP and (C) HAPTiZn, control: DMPO with UVA irradiation.

All the above results indicated that it is quite possible that the *E. coli* was killed by the synergy of the decomposition role of ROS and the bacteriostatic action of these antibacterial ions. To confirm this conjecture, the ESR spin-trap technique (with DMPO) was used to obtain information on the ROS involved in different reactions. Since O₂^{•−} radicals in water are very unstable and undergo facile disproportionation rather than slow reaction with DMPO [25,26], the involvement of O₂^{•−} radicals in different reaction systems was examined in methanol with an Nd:YAG laser (355 nm) irradiation source or in the dark. In HAPTiAg, HAPTiCu and HAPTi systems with and without UVA irradiation, six characteristic peaks of the DMPO-O₂^{•−} adducts were observed (Fig. 5A–C), while in the control system, DMPO with methanol did not exhibit any signal. Furthermore, another control experiment was carried out. Superoxide dismutase (SOD) was added into HAPTiAg, HAPTiCu and HAPTi methanol suspensions in the dark under

Fig. 7. Temporal course of the *S. aureus* inactivation (5×10^6 cfu ml^{−1}, 30 ml) in different aqueous solutions. (A) HAPTiAg film, (B) HAPTiCu film and (C) HAPTiZn film.

otherwise identical conditions. Obviously, the signal of DMPO- $O_2^{\bullet-}$ adducts were not observed in the control experiment since SOD scavenged $O_2^{\bullet-}$. The results confirmed that $O_2^{\bullet-}$ radicals were generated from HAPTiAg, HAPTiCu and HAPTi systems in the dark. For the P25-TiO₂, HAP, and HAPTiZn systems (Fig. 6A–C), six characteristic peaks of the DMPO- $O_2^{\bullet-}$ adducts were observed under UV irradiation, but no such signals were detected in the dark. The results verified that HAPTiAg, HAPTiCu, and HAPTi can generate $O_2^{\bullet-}$ both under UVA irradiation and in the dark, while other materials have to be excited by UV light and then $O_2^{\bullet-}$ is generated. Therefore, in the dark, HAP and P25-TiO₂ films had no bactericidal activity, whereas HAPTi did not show any obvious effect either due to the little intensity of the DMPO- $O_2^{\bullet-}$. HAPTiZn film only had bacteriostatic action in this case, causing no significant inactivation of *E. coli*. However, in both the HAPTiAg and HAPTiCu film systems, *E. coli* not

only was decomposed by $O_2^{\bullet-}$, but also was inhibited by silver or copper ions, causing faster inactivation in the dark. Similarly, under UV irradiation, both HAPTiAg and HAPTiCu films had the same role as that in the dark, and the more $O_2^{\bullet-}$ was formed due to UV irradiation, the more inactivation of *E. coli* occurred. In the same case, HAPTiZn film also had both oxidation and antibacterial activity, causing higher inactivation of *E. coli*. In contrast, HAP, HAPTi and P-25TiO₂ only had the function of decomposing bacteria under UVA irradiation. Since the tested UVA intensity was relatively weak, the amount of $O_2^{\bullet-}$ formed from HAP and HAPTi was too small to lead to any obvious *E. coli* inactivation. However, the intensity of $O_2^{\bullet-}$ from P-25TiO₂ was stronger than that from HAP and HAPTi, and some *E. coli* inactivation was observed. Nevertheless, the bactericidal activity of P-25TiO₂ was still much lower than that of HAPTiM. The results demonstrated that the synergistic effect

Fig. 8. (A) *E. coli*-not treated, (B and C) TEM micrographs of *E. coli* in UVA illuminated HAPTiAg suspension for 2 h and (D and E) TEM micrographs in HAPTiAg suspension in the dark for 5 h.

of the oxidation reaction and antibacterial reaction was much greater than that of their sum. As shown in Fig. 7A–C, *S. aureus* was also killed efficiently on HAPTiAg and HAPTiCu films, respectively, under UVA irradiation or in the dark. The complete *S. aureus* inactivation of 6.7 log and 7.3 log occurred at 120 and 150 min on HAPTiAg films with and without UVA irradiation, while 4.65 log *S. aureus* and 3.2 log *S. aureus* were inactivated at 180 min on HAPTiCu film with or without UVA, respectively. 1.2 log *S. aureus* were inactivated at 180 min on HAPTiZn film in the dark due to the bacteriostatic action of zinc ions, while 3.2 log *S. aureus* inactivation occurred on HAPTiZn film with UVA irradiation for the same time period (Fig. 7C). As expected, HAP and HAPTi films still did not exhibit any significant bactericidal activity for *S. aureus*, while only about 1 log *S. aureus* was inactivated on P25-TiO₂ film under weak UVA irradiation (data not shown here).

3.3. A possible mechanism of HAPTiAg(Cu) generating O₂^{•−} in the dark

It has been reported [27,28] that the radical O₂^{•−} could be formed on HAP by heat treatment or UV irradiation. The main mechanism for radical O₂^{•−} formation on HAP has been proposed. The UV irradiation or heat treatment causes the changes of the surface PO₄ group, probably the formation of an oxygen vacancy, which traps the electrons, leading to the formation of the O₂^{•−} species [28]. In the present study, HAPTi HAPTiAg(Cu) could generate O₂^{•−} species at room temperature without UV irradiation. Moreover, the intensity of O₂^{•−} signals formed in HAPTiAg(Cu) was stronger than that in HAPTi. Particularly, the highest intensity of O₂^{•−} signals was observed in HAPTiCu. Based on the structure characterization of HAPTi and HAPTiAg(Cu), Ti(IV), Ag⁺, Cu²⁺ and Zn²⁺ all substituted the Ca(II) in the crystal of HAP. It is quite possible

Fig. 9. (A) *E. coli*-not treated, (B and C) TEM micrographs of *E. coli* in UVA illuminated HAPTiCu suspension for 2 h and (D and E) TEM micrographs in HAPTiCu suspension without light for 5 h.

that the substitution of Ti(IV) caused the oxygen vacancy in the crystal of HAP because the valency of Ti(IV) is higher than that of Ca(II). The formation of the oxygen vacancy was attributed to the possible formation of $O_2^{\bullet-}$ at ambient temperature. Furthermore, HAPTiAg and HAPTiCu were characterized by XPS. The Cu2p3 binding energy (BE) was 932.73 eV assigned to Cu^0 , 934.49 eV assigned to Cu^{2+} , respectively. Also the silver species mainly exists as Ag^0 (BE: 367.82 eV) and Ag^+ (BE: 367.55 eV). Thus, the redox couples Cu^0/Cu^{2+} and Ag^0/Ag^+ were formed in the structure of HAPTiCu and HAPTiAg, respectively. In the presence of oxygen, the $O_2^{\bullet-}$ could be generated by the electron transfer of the redox couple, which is agreed with the reported work [29]. No Zn^0 was formed in the structure of HAPTiZn by XPS analysis, the redox couple Zn^0/Zn^{2+} did not occur. Therefore, the $O_2^{\bullet-}$ could not be generated in HAPTiZn in the dark.

3.4. Cell damage mechanism

Fig. 8 shows the transmission electron microscopy (TEM) findings for *E. coli* after different treatment of HAPTiAg films with or without UVA irradiation. Fig. 8A shows the appearance of *E. coli* before the reaction. The characteristics of the *E. coli* are a well-defined cell wall as well as the rendered interior of the cell, which corresponds to the presence of proteins and DNA. Great changes had taken place to the morphology of *E. coli* that had been illuminated for 150 min (Fig. 8B–C). The cell wall was decomposed and the rendered interior of the cell became white, indicating that the outer membrane of the cell was damaged leading to leakage of the interior component. Similarly, the cell wall of *E. coli* was also destructed resulting in leakage of the interior component (Fig. 8D–E) in the dark with HAPTiAg film. Fig. 9A–E also shows the cell damage process of the *E. coli* on HAPTiCu film with or without UVA irradiation, indicating the *E. coli* also was killed and disrupted on HAPTiCu film due to the synergistic effect of the oxidation of $O_2^{\bullet-}$ and the antibacterial action of copper ions. Based on the TEM investigation, the cell wall, the peptidoglycan layer and the cell membrane of the bacteria were decomposed by $O_2^{\bullet-}$. In order to determine the change in cell membrane permeability, K^+ leakage from *E. coli* cells was examined. Under only UVA irradiation or in the presence of 0.6 mg l^{-1} silver ions (Fig. 10A), the K^+ leakage was slow. After the addition of HAPTiAg film with or without UVA irradiation, K^+ immediately started to leak from the *E. coli* cells and the leakage gradually increased with reaction time, paralleling the loss of cell viability. The resultant K^+ concentrations were much higher than that of the control experiments. Moreover, the amount of the K^+ leakage was almost equal under the two conditions, indicating that the cell membrane was damaged to the same extent with or without UVA irradiation. The K^+ leakage was also determined for HAPTiCu film with and without UVA (Fig. 10B). At 0.012 mg l^{-1} copper ions, the K^+ leaked from the cells slowly, and much less K^+ leaked compared to the HAPTiCu film system, where K^+ immediately leaked out and the leakage increased in parallel with the inactivation of *E. coli* with reaction time. Also, there was

Fig. 10. Leakage of K^+ from *E. coli* cells in different aqueous solutions (A) HAPTiAg film and (B) HAPTiCu film.

nearly same K^+ leakage from *E. coli* cells in both UVA and in the dark. These results demonstrate that the K^+ leakage was consistent with the disruption of the cell wall and the cell membrane by the oxidation and antibacterial action. In the bactericidal process of HAPTiAg and HAPTiCu, Ti(IV) and Ag^+ (Cu^{2+}) ions assist each other. On the one hand, the outer membrane of the cell is attacked by $O_2^{\bullet-}$ produced from HAPTiAg(Cu). Successively, Ag^+ (Cu^{2+}) ions are effectively taken into the cytoplasmic membrane by the partially decomposed outer membrane. Finally, the bacteria are inactivated by the bacteriostatic action of Ag^+ or Cu^{2+} . On the other hand, the bacteriostatic action of these ions enhances the efficiency of $O_2^{\bullet-}$ in killing bacteria.

4. Conclusions

Ti(IV) and antibacterial ions co-substituted hydroxyapatite (HAPTiM) was prepared by a coprecipitation and ion-exchange methods. HAPTiAg and HAPTiCu coated on porous spumous nickel film showed high efficiency for killing of *E. coli* and *S. aureus* in dark and under weak UVA irradiation. For the first time, the studies of ESR proved that $O_2^{\bullet-}$ radicals were formed on HAPTiCu, HAPTiAg and HAPTi in the dark at ambient temperature. XPS results indicated that the interactions between the redox couple of Cu^0/Cu^{2+} or Ag^0/Ag^+ and O_2 possibly resulted in the $O_2^{\bullet-}$ formation. Moreover, HAPTiM, HAPTi, HAP and P25-TiO₂ also generated $O_2^{\bullet-}$ radicals under weak UVA irradiation. The high bactericidal activities of HAPTiM were due to the synergy of the oxidation role of the $O_2^{\bullet-}$ and the bacteriostatic action of antibacterial ions. Furthermore, The damage process of the cell was shown by TEM and the determination of K^+ leakage.

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