Research Article

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Preparation of CaO-SiO$_2$-CuO bioactive glasses-embedded anodic alumina with improved biological activities

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Abstract: To improve bone cell cytocompatibility properties of porous anodic alumina (PAA) and implement anti-bacterial properties, amorphous CaO-SiO$_2$-CuO materials were loaded into PAA nano-pores (termed CaO-SiO$_2$-CuO/PAA) by a facile ultrasonic-assisted sol-dipping strategy. The surface features and chemistry of the obtained CaO-SiO$_2$-CuO/PAA were investigated by a field emission scanning microscope (FESEM), an energy-dispersive X-ray spectrometer (EDS) and an X-ray photoelectron spectroscopy (XPS). The ability of the CaO-SiO$_2$-CuO/PAA specimens to form apatite via a bio-mineralization process was evaluated by soaking them in simulated body fluid (SBF) in vitro. The surface microstructure and chemical properties after soaking in SBF were characterized. The release of ions into the SBF was also measured. In addition, rat osteoblasts and two types of bacteria were cultured on the samples to determine their cytocompatibility and antibacterial properties. The results showed that the amorphous CaO-SiO$_2$-CuO materials were successfully decorated into PAA nano-pores and at the same time maintained their nano-featured surfaces. The CaO-SiO$_2$-CuO/PAA samples induced apatite-mineralization in SBF. Meanwhile, the CaO-SiO$_2$-CuO/PAA samples demonstrated great potential for promoting the proliferation of osteoblasts and inhibiting Escherichia coli (E. coli) as well as Staphylococcus aureus (S. aureus) growth. Specifically, there was an 86.5±4.1% reduction in E. coli, an 88.0±2.2% reduction in S. aureus for the CaO-SiO$_2$-CuO/PAA surfaces compared to PAA controls. The capability to promote osteoblast proliferation and better antibacterial activity of CaO-SiO$_2$-CuO/PAA may be attributed to the fact that Cu ions can be slowly and constantly released from the samples. Importantly, this was achieved without the use of antibiotics or any pharmaceutical agent. Ultimately, these results suggest that the CaO-SiO$_2$-CuO/PAA substrates possessed improved bone cell cytocompatibility and high antibacterial properties leading to a promising bioactive coating candidate for enhanced orthopedic applications.

Keywords: Porous anodic alumina, CaO-SiO$_2$, Copper

1 Introduction

In regenerative medicine approaches, the role of nanoporous bio-interfaces design in stimulating and guiding the tissue regeneration process has received a lot of attention [1]. Porous anodic alumina (PAA) is typically a self-organized material with ordered nano-porous structures and with promising applications to biotechnology [1, 2]. Recently, PAA has been shown to be a superior platform for cell-interface studies due to its excellent chemical stability, controllable dimensions as well as biocompatibility and orthopedic biomimetic properties [3–8]. Our research also demonstrates that the nano-pore structure and the pore size are important physical cues for osteoblasts and macrophages, which affect their spreading and cell shape, subsequently regulate their osteogenic functionalities [9, 10]. More importantly, PAA is not always used on its own but can be coated for promising medical applica-
tions, particularly orthopedic implants [11, 12]; PAA also has the potential to be used as improved medical scaffolds for numerous tissue engineering applications [13].

For orthopedic implantation, early bone formation and osseointegration are essential for the success of implants. However, insufficient bioactive properties leading to prolonged bone growth and improper tissue integration have been frequently observed [14]. PAA is composed of amorphous alumina and the chemical composition is generally considered biologically inert. Furthermore, bacterial infection is one of the common complications in orthopedics and early clinical failure of implants is often caused by the development of a bacterial biofilm and chronic infection at the site of surgery [15]. Therefore, in order to avoid infection, it is necessary to endow implants with self-antibacterial ability. Interestingly, not only does the pore structure of PAA make them attractive as nanoreservoirs to store a large amount of bioactive materials, but also their nano-featured surfaces make it feasible for selectively increasing desirable cell functions, thus, eliciting improved osteointegration and a better resistance to infection.

Previous studies have shown that Ca-Si based biomaterials may be used as potential materials for bone tissue regeneration due to their excellent bioactivity and biocompatibility [16]. The Ca and Si ions from biomaterials could stimulate the proliferation and osteogenic differentiation of several kinds of stem cells and osteoblasts [17]. This CaO-SiO$_2$ system, as a new family of biomaterials, has been recognized as the basis for many third generation tissue regeneration materials presently in development [18].

It is established that copper (Cu) is an essential trace element in human physiology, and Cu species play diverse roles in biological functions, such as enzyme activity, biomineralization and hormonal activity, while they also possess excellent antibacterial qualities [19, 20]. Recently, Cu has been incorporated into various biomaterials showing anti-bacterial and angiogenic characteristics, and a positive effect on cellular activity and the proliferation of endothelial cells and osteoblasts [20–24]. More importantly, it has been further demonstrated that Si and Cu ions had a synergistic effect on angiogenesis at suitable concentrations [25, 26]. Therefore, it is reasonable to assume that incorporating suitable quantities of beneficial Cu into the CaO-SiO$_2$ system and loading PAA with these bioinorganic CaO-SiO$_2$-CuO species may be an effective, unique and safe way to further enhance their biological performance.

Hence, in our study, CaO-SiO$_2$-CuO bioactive species were firstly decorated into PAA nano-pores by a facile ultrasonic-assisted sol-dipping method and secondly were characterized for their suitability to serve as a novel orthopedic implant coating.

2 Materials and Methods

2.1 Preparation of porous anodic alumina

The PAA specimens were fabricated as previously described [27]. Briefly, high-purity (99.999 wt.%) aluminum strips (Shtongren Co., Ltd, Shanghai, China) were subjected to annealing, grease removal and electrochemical polishing processes. Then, the pre-treated specimens were first anodized in 0.3 M oxalic acid (Sinopharm Chemical Reagent Co., Ltd, Shanghai, China) containing a volume mixture of ethanol and water at 100 V. The formed oxide layers were etched using a mixture of phosphoric acid and chromic acid (Sinopharm Chemical Reagent Co., Ltd, Shanghai, China). Then, the second anodization process was conducted in 5 wt.% phosphoric acid containing a volume mixture of ethanol and water at 100 V. After the second anodization step was completed, the samples were immersed in a phosphoric acid solution to enlarge the pores. Field emission scanning electron microscopy (FESEM, JSM-5600LV, JEOL, Japan) equipped with energy dispersive X-ray spectrometer (EDS) was employed for morphology characterization and elemental composition analysis.

2.2 Sol preparation and pore loading

The CaO-SiO$_2$-CuO/PAA samples were prepared by using an ultrasonic-assisted sol-dipping technique to fill CaO-SiO$_2$-CuO sol into the PAA nano-pores. Sol–gel method was used to prepare CaO-SiO$_2$-CuO sol and the raw materials used were tetraethyl orthosilicate (TEOS, Si(OC$_2$H$_5$)$_4$), copper nitrate hydrate (Cu(NO$_3$)$_2$·3H$_2$O) and calcium nitrate tetrahydrate (CaN, Ca(NO$_3$)$_2$·4H$_2$O) (all from Sinopharm Chemical Reagent Co., Ltd, Shanghai, China). Firstly, TEOS was hydrolyzed in deionized water catalyzed by 2M HNO$_3$ solution for 30 minutes under stirring. After hydrolysis, Ca(NO$_3$)$_2$·4H$_2$O and Cu(NO$_3$)$_2$ were added into TEOS sols for further reaction. CaO-SiO$_2$-CuO sol was of chemical composition CaO (53 wt.%) - SiO$_2$ (45 wt.%) - CuO (2 wt.%). Before pore loading, the viscosity of the sols was analyzed by an ARES (ARES-RFS) rheometer at a constant shear rate of 100 S$^{-1}$. Subsequently, PAA samples were placed in a vessel containing an appropriate amount of CaO-SiO$_2$-CuO precursor sol and ultrasonically treated.
for 20 min, followed by keeping them under vacuum condition for a period of 180 min. Finally, the samples were taken out, and gently washed with deionized water to remove the excess sol from the surfaces. Then, the PAA was first dried in air at room temperature and then dried at 100°C overnight and calcined at 500°C for 5 h to eliminate residual nitrates. The Cu-free samples (termed CaO-SiO$_2$/PAA) with 55CaO and 45 SiO$_2$ (wt.%) were also synthesized by adopting the same procedure to compare its biological properties with that of the Cu incorporated samples (CaO-SiO$_2$-CuO/PAA).

### 2.3 Characterization of pore loading

The specimens were characterized after loading using a field emission scanning electron microscope (FESEM, JSM-5600LV, JEOL, Japan), an energy dispersive spectroscopy (EDS, IE 300X, Oxford, England), and an X-ray photoelectron spectroscopy (XPS, AxisUltra DLD, Kratos, Japan). The Ca, Si and Cu content of the CaO-SiO$_2$-CuO/PAA were measured via inductively coupled plasma optical emission spectroscopy (ICP-OES, Prodigy, Leeman, USA). In addition, the weight of each samples before and after loading were accurately measured using a four-digit balance (BS124S, Sartorius, Germany). The weight increment was expressed as the percentage of the initial weight. Five samples for each of the materials were examined.

### 2.4 Apatite bio-mineralization in simulated body fluid and ion release

The apatite-forming ability of the CaO-SiO$_2$/PAA and CaO-SiO$_2$-CuO/PAA was carried out by immersing the samples in simulated body fluid (SBF) for various periods of time. The SBF solution with ionic concentrations nearly equal to human blood plasma was prepared according to standard ingredient [28]. Each specimen was immersed in SBF in sterilized plastic container and incubated for 1, 3 and 7 days at 37°C. After washing the bone fragments three times with PBS, the chips of the calvaria were digested into identical size pieces (5 mm × 5 mm) and were sterilized in an autoclave at 121°C for 40 min. About 50 µL of the cell culture medium containing 5×10$^5$ cells were seeded onto the top of the substrates, which were previously placed in 48-well culture plates. The cells were allowed to attach for 1 h to the substrates, then 2 mL of fresh culture medium was added to each well and the cells were incubated for 1, 3 and 5 days in a humidified atmosphere at 37°C and 5% CO$_2$. The cell morphology on the samples was observed using a field emission scanning electron microscope (FESEM, JSM-5600LV, JEOL, Japan), an energy dispersive X-ray spectrometer (EDS, IE 300X, Oxford, England) and a fourier transform infrared spectroscopy (FTIR, Thermo Nicolet FTIR Nexus 670, Thermo, America).

To examine the release behavior of Ca, Si and Cu ions from the CaO-SiO$_2$-CuO/PAA samples the immersion solutions were collected at every time period. After 6 h, 1, 3, 5 and 7 days of soaking, the concentrations of Ca, Si and Cu ions of SBF was measured by using inductively coupled plasma optical emission spectroscopy (ICP-OES, Prodigy, Leeman, USA). The pH behavior of SBF was recorded continuously for 7 days using a pH meter (pHS-3C, Jingke Leici Co., China). Five specimens were tested for each incubation time and each test was performed in triplicate.

### 2.5 Cell culture, attachment and proliferation

Healthy osteoblasts were isolated from neonatal rat calvaria via a sequential collagenase digestion method according to an established protocol [29]. Briefly, the rat calvaria were washed three times in phosphate-buffered saline (PBS, pH=7.4) and then minced into fragments about 1 mm in diameter. After washing the bone fragments three times with PBS, the chips of the calvaria were digested for 20 min at 37°C with a 0.25% (w/v) trypsin-EDTA solution (Gibco, USA) to maximize osteoblast release. The supernatants were centrifuged at 1000 rpm for 10 min, and then suspended in the Dulbecco’s modified Eagle’s medium (DMEM, Gibco, USA) containing 10% (v/v) heat-inactivated fetal calf serum with 50 µg/mL L-ascorbic acid, 1% glutamine, 50UI/mL penicillin/streptomycin, and incubated at 37°C under a humidified atmosphere consisting of 5% CO$_2$. Cell culture media were refreshed every 2 days. The cells used in our study were between the second and fourth passages number.

Prior to cell seeding, all of the test samples were cut into identical size pieces (5 mm × 5 mm) and were sterilized in an autoclave at 121°C for 40 min. About 50 µL of the cell culture medium containing 5×10$^5$ cells were seeded onto the top of the substrates, which were previously placed in 48-well culture plates. The cells were allowed to attach for 1 h to the substrates, then 2 mL of fresh culture medium was added to each well and the cells were incubated for 1, 3 and 5 days in a humidified atmosphere at 37°C and 5% CO$_2$. The cell morphology on the samples was observed using a field emission scanning electron microscope (FESEM, JSM-5600LV, JEOL, Japan), an energy dispersive X-ray spectrometer (EDS, IE 300X, Oxford, England) and a fourier transform infrared spectroscopy (FTIR, Thermo Nicolet FTIR Nexus 670, Thermo, America).

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Figure 1: FESEM images showing the microstructures of the three specimens. (a) and (b): surface structure and cross section of PAA; (d) and (e): surface structure and cross section of CaO-SiO$_2$/PAA; (g) and (h): surface structure and cross section of CaO-SiO$_2$-CuO/PAA. EDS analysis of PAA (c), CaO-SiO$_2$/PAA (f) and CaO-SiO$_2$-CuO/PAA (i).

specimens were glued onto copper specimen stubs, and sputter-coated with gold before observation.

Changes in the number of viable cells on the samples after 1, 3 and 5 days in culture were quantitatively assessed by an MTT test [29]. MTT (Sigma), (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) is a yellow tetrazolium salt, which can be enzymatically converted by living cells into a purple formazan product. The intensity of the color produced is therefore directly proportional to the number of viable cells in culture, and thus to their proliferation in vitro. In brief, substrates/cells constructs were placed in cell culture medium containing MTT and were incubated in a humidified atmosphere at 37°C for 4 h. After incubation, the MTT solution was removed and the insoluble formazan crystals were dissolved in dimethyl sulfoxide (DMSO). The absorbance of each well was then measured at 570 nm in a microplate reader (ELX 800, Bio-Tek, USA) with a reference wavelength at 620 nm. The PAA samples were used as controls. Five specimens for each material were tested for each incubation time period and each test was performed in triplicate. Results were reported as optical density units.

2.6 Antibacterial assays

Escherichia coli (E. coli, ATCC 8099) and Staphylococcus aureus (S. aureus, ATCC 6538) were employed to evaluate the antibacterial activity of different substrates according to the National Standard of China GB/T 21510-2008 protocols (Antimicrobial property detection methods for nano-inorganic materials). They were cultured in standard Luria-Bertani (LB) culture medium. Following incubation at 37°C for 24 h, each sample was moved into a conical tube with fresh PBS buffer and sonicated for 5 min. 10 mL of
the bacterial suspension was diluted to create subsequent dilutions. Following this, 100 µL of the different diluted bacterial suspensions were inoculated onto LB broth agar plates (Sangon, Shanghai, China). After overnight incubation, the viable bacterial in each group were counted by quantification of bacterial formation (CFU). The PAA sample served as a control.

The antibacterial rates (C) were calculated based on the following equation:

\[ C(\%) = (A - B)/A \times 100\% \]

Where \( A \) is CFUs of the control group; and \( B \) is CFUs of the experimental group. The test was carried out in triplicate for each group and five specimens of each group were tested.

### 2.7 Statistical analysis

All data are expressed as the mean ± standard deviation (SD) and were analyzed using One-Way ANOVA with a Post Hoc test. A \( p \)-value < 0.05 was considered statistically significant.

### 3 Results

#### 3.1 Sample characterization

Figure 1 shows the typical surface and cross-sectional FE-SEM images of the resultant PAA, CaO-SiO\(_2\)/PAA and CaO-SiO\(_2\)-CuO/PAA. The as-produced PAA exhibited an ideally hexagonal configuration, uniform pore size and highly ordered pore arrangement (Figure 1a). The average pore diameter was about 200 nm. Figure 1b shows the cross section of the prepared PAA. It can be observed that the nanopipes are parallel to each other. The results of pore loading (Figure 1d, 1e, 1g, 1h) indicated that filling of the pores with CaO-SiO\(_2\) and CaO-SiO\(_2\)-CuO materials can be achieved by the ultrasonic-assisted pressure-induced mechanism. In detail, it can be seen that the outlines of the PAA porous nano-structure were not covered by CaO-SiO\(_2\) or CaO-SiO\(_2\)-CuO nano-granules and they were fully loaded into the PAA nano-pores. The chemical compositions of PAA, CaO-SiO\(_2\)/PAA and CaO-SiO\(_2\)-CuO/PAA substrates were characterized by energy dispersive X-ray spectroscopy (EDS) (Figure 1c, 1f and 1i). EDS analysis shows that there are only Al and O elements the PAA samples, while the Ca, Si and Ca, Si, Cu elements were detected on the surfaces of the CaO-SiO\(_2\)/PAA and CaO-SiO\(_2\)-CuO/PAA samples. These results again confirmed that the bioactive species (CaO-SiO\(_2\), CaO-SiO\(_2\)-CuO) was successfully embedded into the PAA nano-pores. Meanwhile, the experimental composition of the CaO-SiO\(_2\)-CuO/PAA samples determined by ICP-OES analysis are reported in Table 1. For the CaO-SiO\(_2\)/PAA and CaO-SiO\(_2\)-CuO/PAA samples, the resulting weight increment was 5.3±0.6% and 5.7±0.4%, respectively.

<table>
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<tr>
<th>Sample</th>
<th>Elemental composition (µg/mL)</th>
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<td>Ca</td>
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<td>CaO-SiO(_2)-CuO/PAA</td>
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The XPS survey spectrum of the CaO-SiO\(_2\)-CuO/PAA samples was presented in Figure 2. Feature peaks of Ca, Si, Cu, O, C, N and Al were all detected. From the high-resolution spectra of samples (Figure 2a), the Al2p spec-
Figure 3: FESEM micrographs of CaO-SiO_2/PAA (a, c and e) and CaO-SiO_2-CuO/PAA (b, d and f) samples after soaking in SBF for 1 day (a, b), 3 days (c, d) and 7 days (e, f). The inserts in the top right-hand corner are the corresponding EDS analysis.

d (78.9 eV) corresponded to the typical binding energy from Al_2O_3 [9]. The Si2p peak of the CaO-SiO_2-CuO/PAA is at 102.8 eV, corresponding to Si2p in SiO_2^− [31]. The bending energy (BE) of the Cu2p peaks were discerned at 933.3 and 935.3 eV (Figure 2b). The higher BE peak at about 935.3 eV is assigned to Cu^{2+}, while the lower BE peak at about 933.3 eV suggests the presence of Cu^{+} or Cu^0 species [31]. Because Cu2p3/2 XPS cannot differentiate between Cu^{+} and Cu^0, Auger Cu LMM spectra were used to confirm the presence of Cu^{+} at BE about 570 eV [32].
3.2 Apatite bio-mineralization on the surfaces of the samples and ion release

The FESEM images of the CaO-SiO$_2$/PAA and CaO-SiO$_2$-CuO/PAA samples after the immersion in a SBF solution for 1, 3 and 7 days are displayed in Figure 3. A change in surface morphology is seen if compared with the initial surface of the samples. For the CaO-SiO$_2$/PAA samples, the FESEM micrographs demonstrate that spherical particles have partially covered the loading surface with variable shape and size after 1 and 3 days of soaking in SBF (Figure 3a and 3c). During the subsequent immersion for 7 days, the specimen surfaces were fully covered by a large number of tiny worm-like granules of about 10-20 nm in diameter, which is a typical characteristic of biological apatite (Figure 3e). For the CaO-SiO$_2$-CuO/PAA samples, with increasing immersion time, the granules grew in size and eventually formed a layer to cover the surface of the samples as it occurred on the CaO-SiO$_2$/PAA specimens. However, the morphology of the layer formed on the CaO-SiO$_2$-CuO/PAA samples was almost tiny globular clusters, which was not the same as the typical morphology for bone-like apatite (Figure 3b, 3d, 3f). The chemical composition of the growth particles was then analyzed by EDS (inset), which revealed the presence of Ca and P peaks. For the CaO-SiO$_2$-CuO/PAA samples, it is interesting to note that a small amount of Cu was also detected in the newly formed layer which suggests that Cu ions might be partially incorporated into Ca-P layer.

The formation of calcium phosphate materials was further validated by FTIR spectroscopy. Figure 4 shows the FTIR spectra of the CaO-SiO$_2$/PAA and CaO-SiO$_2$-CuO/PAA samples immersed in a SBF solution for 1, 3 and 7 days. The broad band in the 3000-3500 cm$^{-1}$ range and the peak at about 1640 cm$^{-1}$ are attributable to vibrational modes of adsorbed water. The triply degenerated asymmetric stretching modes of $\nu_3$ PO$_4$ bands at 1020 cm$^{-1}$ are well visible. The peak around 1490 cm$^{-1}$ indicates the CO$_3^{2-}$ vibrational modes, suggesting that the formed apatite is carbonated.

The changes in ion concentrations and pH value of SBF solutions during the immersion test were shown in Figure 5. It can be seen that calcium ion release increased with dissolution time but appeared to approach saturation at longer dissolution times. A calcium concentration of approximately 82.3 ppm was measured after immersing for 168 hours. The variation in Si ion concentration was similar to that of Ca, and its concentration reached 6.89 ppm after soaking for 168 hours. Figure 5b suggested the release pattern of Cu ions from the CaO-SiO$_2$-CuO/PAA sample measured in SBF solution from 0 to 168 hours. It indicated that the Cu ions were released rapidly in the first 6 hours. After that, the amount of Cu ions release was almost linearly correlated to the immersion time. So continuous Cu released from the as-prepared samples can be realized. At day 7, the cumulative amount of Cu ions released from the CaO-SiO$_2$-CuO/PAA samples were about 11.6%, of the total amount of Cu incorporated into the samples. Corresponding with the ion release, the pH value increased to 7.5 after 6h of soaking and then kept increasing at a relatively slow rate with prolonged soaking.
Figure 5: The variations in ion concentrations and pH value (a, b) in the SBF solution after soaking CaO-SiO$_2$-CuO/PAA specimens for 6 h as well as 1, 3, 5 and 7 days.

3.3 Cell morphology and proliferation on the surface of the samples

The initial adhesion and spreading of osteoblasts cultured on PAA, CaO-SiO$_2$/PAA and CaO-SiO$_2$-CuO/PAA surfaces for 4 and 24 h were observed by FESEM and typical images are displayed in Figure 6. At an early stage of culture (4 h after cell seeding), cells attached to the material surface and mainly presented a spherical morphology (Figure 6a, 6c, 6e). From high magnification FESEM micrographs, noticeable filopodia extensions were observed. After 24 h, the spreading was more obvious (Figure 6b, 6d, 6f). It is clear to see the cells had a close contact with the surfaces and adopted an irregular morphology, which indicated that all three sample groups were favorable for the adhesion and spreading of osteoblasts. However, there was no significant difference in cell morphology between the different surfaces.

Figure 6: FESEM morphology of rat osteoblasts cultured on the PAA (a, b), CaO-SiO$_2$/PAA (c, d) and CaO-SiO$_2$-CuO/PAA (e, f) substrates for 4 (a, c and e) and 24 h (b, d and f), respectively. The inserts in the top right-hand corner are the corresponding high magnification images. MTT assay results of rat osteoblasts cultured on the PAA, CaO-SiO$_2$/PAA and CaO-SiO$_2$-CuO/PAA samples for 1, 3 and 5 days (g). Data are expressed as the mean ± standard deviation (n=5); *p<0.05 compared to the PAA samples at the respective time.

To investigate the cytocompatibility of those substrates, the proliferation of rat osteoblasts on PAA, CaO-SiO$_2$/PAA and CaO-SiO$_2$-CuO/PAA surfaces were determined by MTT assays and Figure 6g shows the results of the cells cultured for 1, 3 and 5 days. At day 1, the OD values on PAA and CaO-SiO$_2$/PAA surfaces appeared similar, and cell attachment on CaO-SiO$_2$-CuO/PAA was higher than that on the PAA control and CaO-SiO$_2$/PAA. After being cultured for 3 and 5 days, the results also revealed that
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The antibacterial properties of the substrates were examined using gram-negative *E. coli* and gram-positive *S. aureus*. Figure 7a shows typical photographs of the bacterial colonies on the different specimens, combined with the bacteria counting results. Results showed that pure PAA presented no antibacterial activity. CaO-SiO\textsubscript{2}/PAA displayed significant antibacterial activity as compared with the blank control. There was a 40.0±4.1% reduction in *E. coli*, and a 48.2±3.2% reduction in *S. aureus*. After Cu incorporation, the CaO-SiO\textsubscript{2}-CuO/PAA presented the highest antibacterial effect (p<0.05) (Figure 7b). It can be seen that the percentage reductions in *E. coli* and *S. aureus* cultured on CaO-SiO\textsubscript{2}-CuO/PAA was 86.5±4.1% and 88.0±2.2%, respectively. Incredibly, this was all accomplished without using antibiotics.

### 4 Discussion

Owing to the large open volume, the PAA nano-porous structure has great potential as carriers for bioactive material loading and release, which offers a potential for the development of bioactive PAA coatings on various biomaterial substrates [7, 11].

In our previous work, CaO-SiO\textsubscript{2}-Ag\textsubscript{2}O/PAA possessed an enhanced apatite-forming ability and antibacterial properties were prepared using a sol-dipping method [33]. However, although antibacterial, silver species are quite toxic to mammalian cells. It has been suggested that diffusing silver may act as "rojan horses" by entering the cells and then releasing silver ions which damage intracellular functions [34, 35]. For its use in combination with Ti, the required Ag dose should be low (0.2 ppm), which may not be enough for the prolonged inhibition of bacterial growth [36]. Alternatively, as an important trace element in human physiology, Cu plays an important role in maintaining bone health. Cu deficiency in humans and animals could decrease bone strength, impair bone formation and growth and reduce bone mineralization [19]. Therefore, for better tissue regeneration, the combination of beneficial inorganic Cu ions may provide a more effective and safer strategy couple with a multitude of positive-functions as compared with Ag.

The modification of a material surface in one respect is always accompanied by significant changes in other surface properties. In this study, CaO-SiO\textsubscript{2}-CuO samples were successfully loaded into PAA nano-pores by an ultrasonic-assisted sol-dipping technique without apparently changing surface nano-topography. The work here suggested that the prepared precursor viscosity, vaccum atmosphere and pore size are important factors to determine the pore-filling degree. To fill inorganic particles into PAA nano-pores via this sol-gel-derived solution route, sufficient infiltration of precursor sols into the nanostructures must be achieved. The viscosity of precursor sol (SiO\textsubscript{2}-CaO or SiO\textsubscript{2}-CaO-CuO) in this study was optimized to ±0.02 Pa s which has good infiltration property into nano-pores. Meanwhile, it is difficult to achieve a uniform sol penetration into each pore of the PAA and local unfilled nano-
pores and uneven distribution of glass particles within the PAA pores was unavoidable.

It is well accepted that if the surfaces of biomaterials can be helpful for the formation of hydroxyapatite on surfaces in SBF; they are beneficial for the formation of new bone tissues in vivo as well as accelerating the bone integration [28, 37]. It is known that CaO-SiO$_2$ bio-glass or ceramic possess excellent apatite-forming ability in SBF. In this work, no negative impact of Cu incorporation on apatite formation on CaO-SiO$_2$-CuO/PAA was observed. It was found that apatite formed on the CaO-SiO$_2$-CuO/PAA substrate even for short soaking periods (1 day). In addition, it was noted that the morphology of the apatite which formed on the samples consisted of tiny spherical granule shapes, which was not the same as the typical morphology for bone-like apatite. One possible explanation is that Cu in the CaO-SiO$_2$-CuO glass system may have an effect on the process of apatite formation and change the morphology of apatite crystals. In particular, traces of Cu were found incorporated in the apatite layer formed on the CaO-SiO$_2$-CuO/PAA surface after immersion in SBF, which might influence the attachment and growth of osteoblasts.

In our work, Cu ions could be released from the sol-gel derived amorphous CaO-SiO$_2$-CuO/PAA materials in a sustained release profile. Previous studies confirmed that Cu$^{2+}$ (as well as Cu$^+$) predominantly work as network modifiers and are incorporated in the silicate glass matrix in octahedral coordination, which suggests that it was chelated by the silicate network [23]. Upon the dissolution of the inorganic matrix, Cu ions could be released continuously to the surrounding environment. At the same time, the formation of a Ca-P surface layer in the physiological environment may also act as a diffusion barrier to inhibit the fast release of ions. Even after 7 days, the accumulative release of Cu ion was approximately 11.6%, indicating the excellent slow-release behavior of Cu ions. More importantly, we observed a Cu level in the range from 0 ppm to 0.31 ppm released in SBF depending on the culturing time. The released concentration of Cu ions at day 7 was 0.31µg/mL (~0.31 ppm), which is within the physiological level of human blood plasma (~1.5 ppm) [38, 39].

MTT assay results show that the CaO-SiO$_2$-CuO/PAA substrate is favorable for the adhesion and proliferation of rat osteoblasts and shows better cytocompatibility than the CaO-SiO$_2$/PAA and PAA. Previous studies have shown that surface nano-structure and chemistry play crucial roles in cell-material interactions as they influence initial protein interactions [9, 40, 41]. Our results show that PAA is nano-porous, whereas CaO-SiO$_2$/PAA and CaO-SiO$_2$-CuO/PAA have similar nano-featured surfaces. It is known that Cu ions exhibit dose-dependent effects on human cells. A number of studies have proved that the incorporation of Cu into bioglass and Ca-Si ceramics with a certain dosage is beneficial to in vitro biological performance [22]. In this work, bio-mineralization studies in SBF revealed that Cu levels from 0 to 0.31 ppm were released from CaO-SiO$_2$-CuO/PAA and these ionic dissolution products can promote the adhesion and proliferation of rat osteoblasts. Considering the similar surface topography and phase compositions of the CaO-SiO$_2$/PAA CaO-SiO$_2$-CuO/PAA samples, the incorporation of Cu is thought to be the main reason for the enhanced attachment and proliferation of osteoblasts.

The attachment and initial growth of bacteria on an implant surface dictates the progression of infection. Treatment often requires aggressive antibiotics use, which has some drawbacks, such as toxicity and limited bioavailability [42]. Implant surface modification that prevent initial bacterial adhesion may offer a controlled inflammatory response. Previous studies have emphasized the possible fabrication of antimicrobial surfaces using nanotechnology [43]. In the present study, the antibacterial activity of the PAA loadings was examined using both E. coli (Gram-negative bacteria) and S. aureus (Gram-positive bacteria) colonies. CaO-SiO$_2$-CuO/PAA materials were significantly more effective at inhibiting both bacteria functions. This antibacterial effect is partly ascribed to the bacterial toxicity contributed by Cu$^{2+}$ release. Similar to other metallic antibacterial agents, the positive charge of copper ions can interact with the –SH$_2$ and –NH$_2$ regions of proteins on cell membranes that can induce bacteria death by interfering with cellular metabolism. Furthermore, the complexation of Cu ions with DNA molecules can destroy their helical structure thus leading to cell death [44]. Moreover, Cu can act as electron donor/acceptor by changing between Cu$^+$ and Cu$^{2+}$. This redox property can be potentially harmful by producing extremely reactive hydroxyl radicals that can oxidize proteins and lipids to have detrimental effects to the bacterial [45]. Interestingly, there was also a decrease in bacterial adhesion on the CaO-SiO$_2$/PAA surface. Previous studies have proved that the most likely antibacterial mechanism of CaO-SiO$_2$/PAA is increasing pH values, which is harmful to bacteria [33]. Results from this present study showed that the dissolution of CaO-SiO$_2$-CuO/PAA could also lead to a weak alkaline microenvironment with increased pH values. Therefore, it is reasonable to speculate that the antibacterial ability of the CaO-SiO$_2$-CuO/PAA may be attributed to released Cu ions and increased pH values.
5 Conclusions

In this study, amorphous CaO-SiO$_2$-CuO bioglasses were successfully decorated into PAA nano-pores while retaining its nano-featured surface by an ultrasonic-assisted sol-dipping method. The CaO-SiO$_2$-CuO/PAA induced a great amount of apatite formation with an excellent mineralization ability in SBF. Furthermore, the CaO-SiO$_2$-CuO/PAA system exhibited obvious biological activity in promoting the adhesion and proliferation of osteoblasts and was highly effective in reducing bacterial adhesion. The improved comprehensive biological performance of the presently formulated materials are due to the slow and constant release of copper ions. In summary, this study provides significant promise for the use of CaO-SiO$_2$-CuO/PAA as a bioactive surface coating for orthopedic implants due to their enhanced osteoblast growth and antibacterial properties without the use of antibiotics or other pharmaceutical agents.

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References


