J Mater Sci: Mater Med (2008) 19:3603–3609
DOI 10.1007/s10856-008-3529-8

Anti-bacterial and cytotoxic properties of plasma sprayed silver-containing HA coatings

Yikai Chen · Xuebin Zheng · Youtao Xie · Chuanxian Ding · Hongjiang Ruan · Cunyi Fan

Received: 29 April 2008 / Accepted: 25 June 2008 / Published online: 19 July 2008
© Springer Science+Business Media, LLC 2008

Abstract Silver-containing hydroxyapatite (HA) coatings have been prepared on titanium substrate by vacuum plasma spraying (VPS) method and anti-bacterial properties of the coatings were examined. Three types of bacteria stains, *Escherichia coli*, *Pseudomonas aeruginosa,* and *Staphylococcus aureus*, were employed in this test. The results showed that the silver-containing HA coatings exhibited significant anti-bacterial effects against the three bacteria with anti-bacterial ratios higher than 95%. The release of silver ions in the physiological environment ensured excellent anti-bacterial properties of the silver-containing HA coatings. International standard ISO 10993-12 was adopted for cytotoxicity evaluation using fibroblast cell line L929, and it was found that the cytotoxicity for the coatings ranked 0 that showed no cytotoxicity for the coatings. Hemolysis test was processed according to ASTM F 756 standard with anti-coagulated rabbit blood, and the hemolysis ratios of the coatings were below 0.4%, indicating of non-hemolysis for the coatings.

1 Introduction

Hydroxyapatite (HA) coating is usually applied to titanium and titanium alloy implants by plasma spraying to improve the bonding of the implant with bone [1]. However, bacterial infections are usually caused by the adherence and colonization of bacteria on the coated implants [2, 3]. In order to reduce the incidence of postoperative infection, research has been carried out on anti-bacterial material containing various substances such as lactoferin, viologen, silicon, silver, copper, zinc, etc. [4–8].

Silver and silver ions have long been known to have strong inhibitory and bactericidal effects as well as a broad spectrum of anti-microbial activities [9–14]. There are different ways to introduce silver into coating materials, such as sol–gel method, ion implantation, ion exchange and sputtering [15–19]. Some studies reported the anti-microbial effect of silver-containing HA coatings and films [7, 18, 20] and showed that these coatings and films exhibited good anti-microbial effect. However, as widely clinical applied plasma sprayed HA coating, the potential of its anti-bacterial property with the addition of silver has not been reported.

In this paper, the anti-bacterial and biological properties of the plasma sprayed silver-containing HA coatings were studied. Three types of bacteria stains, *Escherichia coli*, *Pseudomonas aeruginosa,* and *Staphylococcus aureus* were chosen to evaluate bacterial adhesion on silver-containing HA coatings. Cytotoxicity examination and hemolysis test were carried out on the coatings as well.

2 Materials and methods

2.1 Preparation of coatings

HA powder with the particle size ranged from 15 to 50 μm was obtained from Sulzer Metco Co. Ltd, Switzerland. Silver powders with 99.9% purity and a typical size of 40–100 μm were used as an anti-bacterial additive in this

Y. Chen · X. Zheng (✉) · Y. Xie · C. Ding
Shanghai Institute of Ceramics, Chinese Academy of Sciences, 1295 Dingxi Road, Shanghai 200050, China
e-mail: xbzheng@mail.sic.ac.cn

Y. Chen
e-mail: chenyikai7@hotmail.com

H. Ruan · C. Fan
Shanghai Sixth People's Hospital, Shanghai Jiaotong University, 600 Yishan Road, Shanghai 200233, China

Table 1 Compositions of the HA and silver powders for the coatings preparation

Coating	HA	HA1	HA3	HA5
HA wt%	100	99	97	95
Ag wt%	0	1	3	5

Table 2 Spray parameters

Plasma gas Ar	40 slpm
Plasma gas H_2	10 slpm
Spray distance	300 mm
Chamber pressure	100 mbar
Powder carrier gas Ar	2.0 slpm
Current	650 A
Voltage	68 V

slpm: standard liter per minute

study. HA and silver powders with the compositions tabulated in Table 1 were ball-milled for 2 h to be applied to prepare coatings.

Titanium plates of $10 \times 30 \times 2$ mm^3 were used as substrates. A vacuum plasma spraying (VPS) system (F4-VB, Sulzer Metco, Switzerland) was applied to fabricate HA and silver-containing HA coatings with the spray parameters shown in Table 2. The phase compositions of the coatings were examined by X-ray diffraction (XRD, D/Max 2550 V, Rigaku, Tokyo, Japan). The silver concentration on the coatings was analyzed by inductively coupled plasma atomic emission spectroscopy (ICP-AES, AX, ICP-OES, Varian, USA).

2.2 Anti-bacterial test

The plate-counting method was used to evaluate anti-bacterial performance against the bacteria. The following bacteria were used in the study: *E. coli*, *P. aeruginosa* and *S. aureus*, among which *E. coli* and *P. aeruginosa* are gram negative, whereas *S. aureus* is gram positive. The bacteria were chosen because gram-negative bacteria are responsible for more than 80% of all infections, with *E. coli* being responsible for more infections than all other genera combined [7].

About 0.1 ml strain of 1×10^6 colony-forming units (CFU)/ml was inoculated onto coating samples, and were covered with a PE film, then placed in a sterilized glass dish for 24 h at 37°C with RH >90%. After the incubation, the inoculated strain was harvested into a sterilized Petri dish by 2 ml sterilized physiological saline solution washing, and then the harvested inoculums were diluted in a sterilized physiological saline solution at volume ratios of 10^{-1}, 10^{-2}, 10^{-3}. These dilution series were then inoculated onto nutrient agar plates and cultured at 37°C for a period of 48 h. The number of colonies formed on the agar was counted to obtain the number of each strain that existed on the surface of the sample after the primary culture. The anti-bacterial ratio *K* was calculated by the following formula:

$$K = (A - B)/A \times 100\%$$

where A and B are the average number of the bacteria for the control and the testing samples respectively. The obtained value represented an average of three test data.

2.3 Release of silver ions from the coatings

To examine the release of silver ions from the silver-containing coatings in simulated body fluid (SBF), whose ion concentrations were tabulated elsewhere [21], the specimens were immersed in 50 ml of SBF at 36.5°C for 1, 4, 7, 14, 21, and 49 days and the amount of released silver ions was examined by ICP-AES (ICP-AES, AX, ICP-OES, Varian, USA).

2.4 Cytotoxicity test

The cytotoxicity test was processed in accordance with ISO 10993-5 standards. The murine fibroblast cell line L929 with a concentration of 5×10^3 cells/ml was used to evaluate the cytotoxicity of the coatings. Extracts were prepared using a culture medium with calf serum as the extraction medium. The extracts of the medical stainless steel were used as negative controls and phenol solution as the positive controls.

Two hundred microliter of cells were seeded into each well of a 96-well flat-bottomed cell culture plates and incubated for 24 h at 37°C in a fully humidified air atmosphere containing 5% CO_2. After being incubated for 24 h, the plates were then washed twice with phosphate-buffered saline, and then the extracts and phenol were added to the 96-well plates with 200 μl/well. The cells were incubated at 37°C for 2, 4, and 7 days and the cell morphology was observed by inverted microscope after the extracts and phenol were removed from the 96-well plates. And then MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] solution was added with 20 μl/well, and incubated for 4 h at 37°C. At the end, DMSO (150 ml) was added to each well and shaken for 10 min. The optical absorbance at 490 nm was read on an ELISA. Cell relative rate (RGR) was estimated by the following formula:

$$\text{RGR} = 100 \times \left(\text{average absorbance of samples}/\text{average absorbance of negative controls}\right)$$

When the RGR is ≥100, 75–99, 50–74, 25–49, 1–24, and 0, correspondingly, the cytotoxicity rank is 0, 1, 2, 3, 4, and 5.

2.5 Hemolysis test

The hemolysis test was processed in accordance with ASTM F 756-00. The samples were immersed in 6 ml extraction medium of sterilized physiological saline and incubated at 37°C for 30 min under static conditions. The negative controls were sterilized physiological saline, and the positive controls were distilled water.

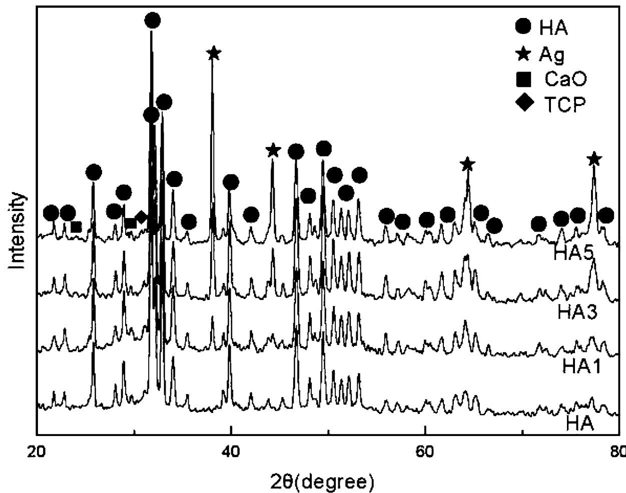

Fig. 1 XRD patterns of silver-containing HA coatings and HA coating

The anti-coagulated rabbit blood was diluted by sterilized physiological saline. The absorbance value of 6 ml distilled water with 0.2 ml diluted anti-coagulated rabbit blood, which was measured at 545 nm, was 1.031. The diluted anti-coagulated rabbit blood was added to extract of each sample with 0.2 ml, and incubated for 60 min in 37°C water bath. At the end of the incubation time, each tube was centrifuged at 750*g* for 5 min in a standard clinical centrifuge, and then the absorbance of the supernate was read with a spectrophotometer at a wavelength of 545 nm. Hemolysis ratio was calculated by following formula:

$$Z = (D_t - D_{nc})/(D_{pc} - D_{nc}) \times 100\%$$

where Z is hemolysis ratio, and D_t, D_{nc} and D_{pc} are average absorbance of samples, negative controls and positive controls. When Z is >5, 2–5, and 0–2, correspondly, the hemolytic grade is hemolytic, slightly hemolytic, and nonhemolytic.

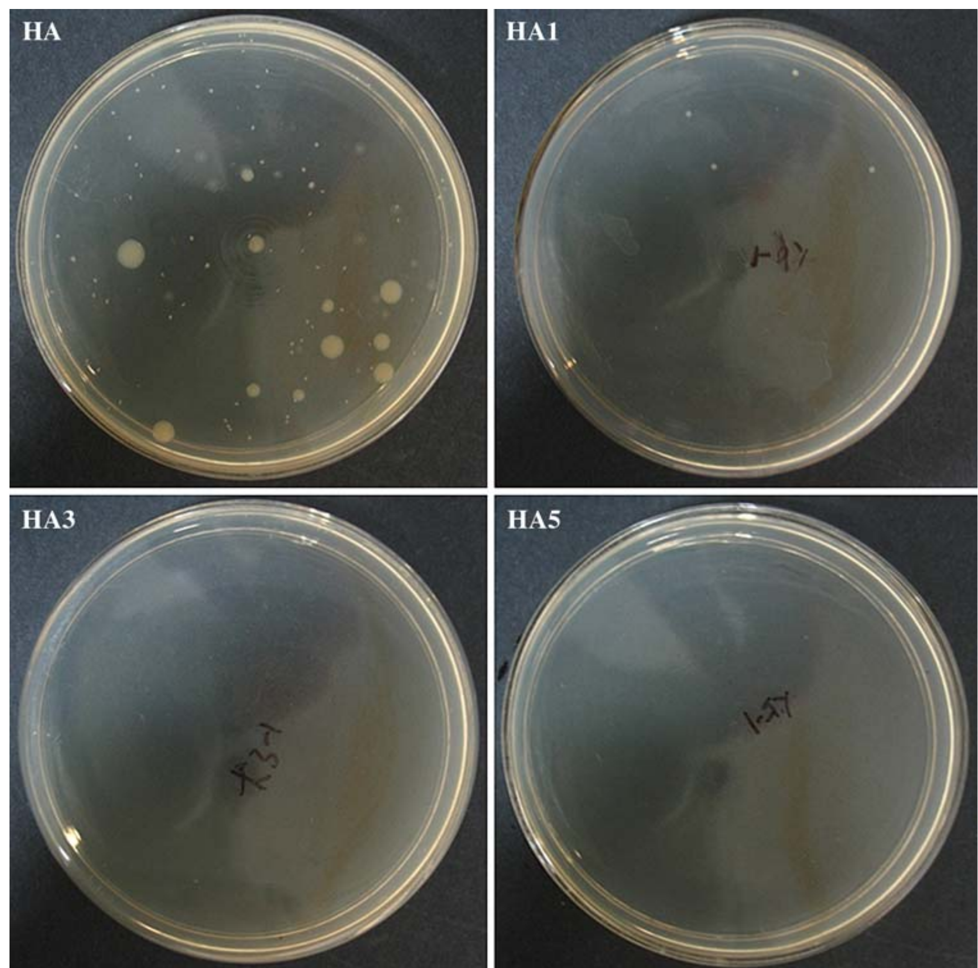

Fig. 2 Anti-microbial effect of the silver-containing HA coatings against *E. coli*

3 Results and discussion

The XRD patterns of the as-sprayed HA, HA1, HA3, and HA5 coatings are shown in Fig. 1, which shows that silver-containing HA coatings are composed of HA, TCP, CaO, and Ag. The peaks of TCP and CaO indicate the HA was decomposed during plasma spraying. It can be also observed from Fig. 1 that the intensities of the peaks of Ag increase obviously from HA1 to HA5, with increase in Ag content in the coatings. According to the results of ICP-AES, The Ag concentration on HA1, HA3, and HA5 coatings was 0.64%, 2.44%, and 4.09%, respectively, after plasma spraying.

The anti-microbial effects of the silver-containing HA coatings against *E. coli*, *P. aeruginosa* and *S. aureus* are shown in Figs. 2–4. From Fig. 2, it can be observed that the colony number of *E. coli* for HA1 coating obviously reduces as compared with the HA coating. Almost no distinct *E. coli* colonies are found in the test when the silver contents in the coatings are equal to or more than 3%. Similar results are obtained for *P. aeruginosa* and *S. aureus*, as shown in Figs. 3 and 4. The calculated anti-bacterial ratios of the silver-containing HA coatings are shown in Table 3, which reveals that the anti-bacterial ratios are more than 95% for all of the three bacteria. It is clear that the plasma sprayed silver-containing HA coatings in this study exhibit excellent anti-bacterial ability. Moreover, the anti-bacterial ratios of the samples increase gradually with increasing silver content.

It is obvious silver plays a determining role in the anti-bacterial effect for the coating, however, the mechanism of inhibitory action of silver ions on bacteria is only partially known. Generally, it is believed that silver ions interact with thiol groups in protein, which induce the inactivation of the bacterial proteins. Another possibility is that silver ions affect DNA by destroying its ability to replicate [9, 14, 22].

The silver ions release in SBF solution as a function of time is illustrated in Fig. 5. The silver ions concentrations of HA5 coating are significantly higher than those of HA3 and HA1 coatings. For all of the three coatings, silver ions released quickly from the coatings in the first several days,

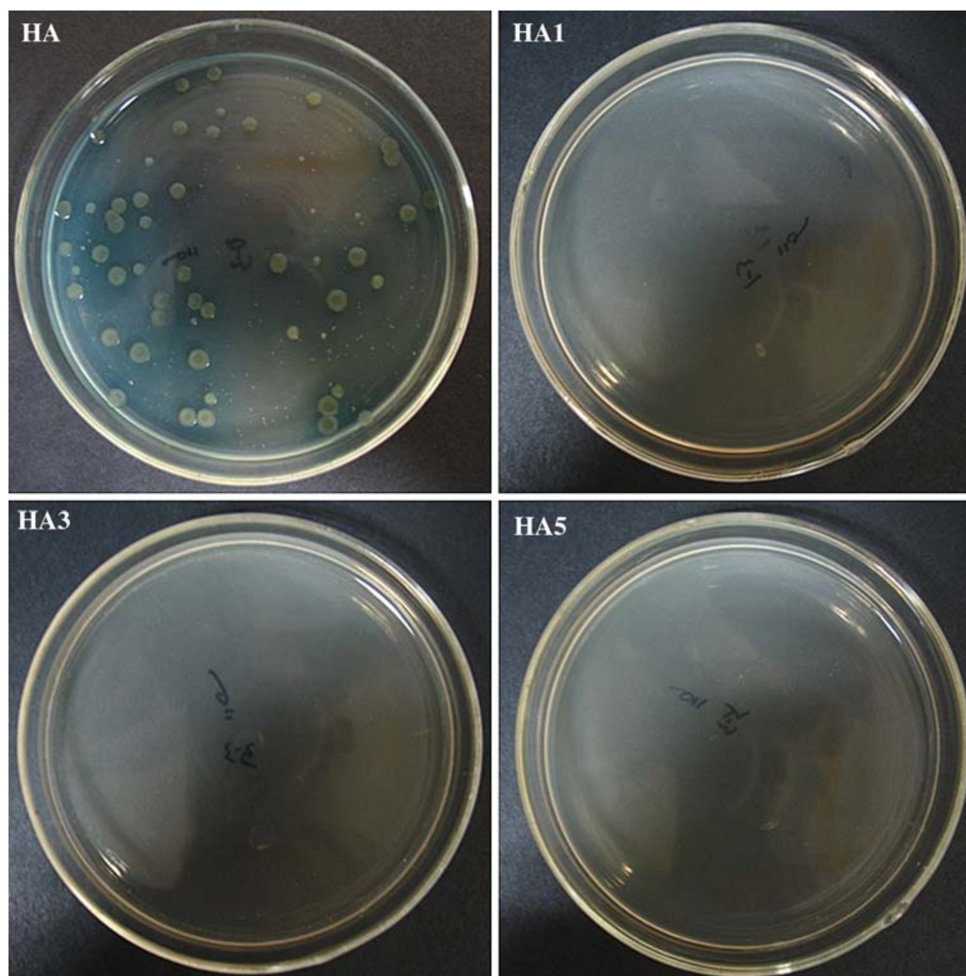

Fig. 3 Anti-microbial effect of the silver-containing HA coatings against *P. aeruginosa*

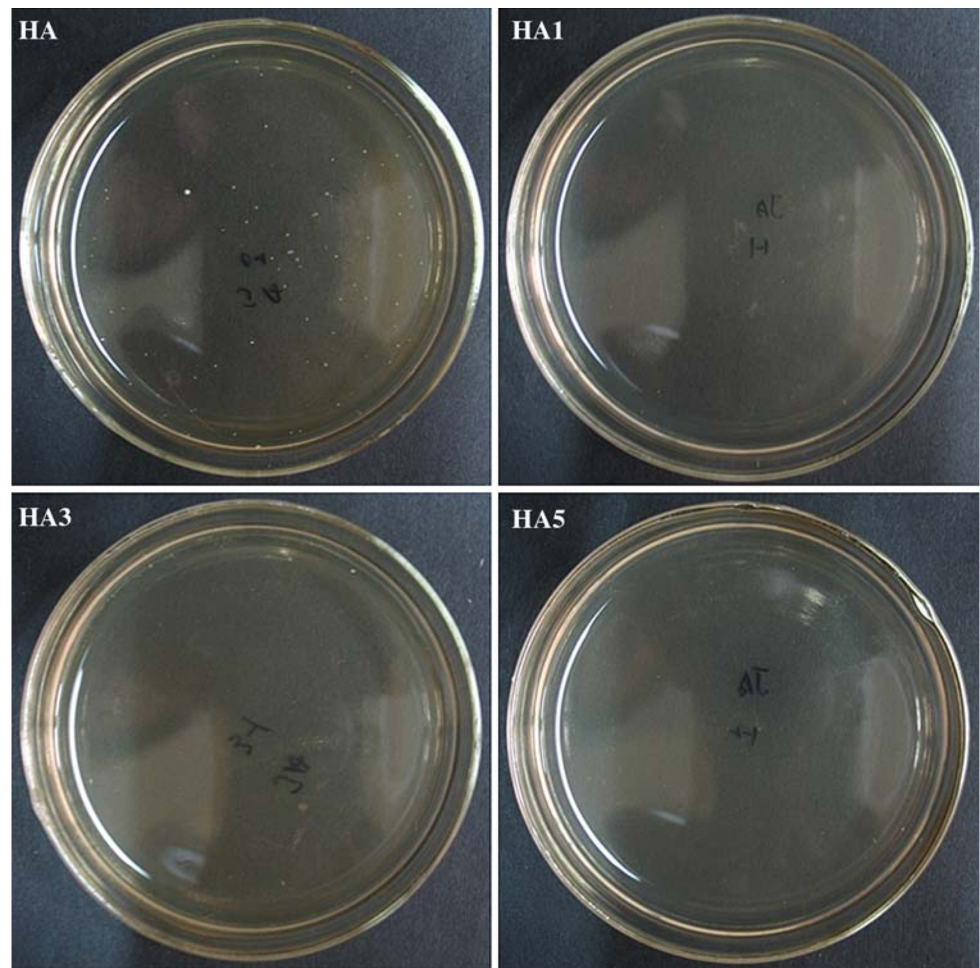

Fig. 4 Anti-microbial effect of the silver-containing HA coatings against *S. aureus*

Table 3 The anti-bacterial ratio of the samples

	Anti-bacterial ratio (%)			
	HA	HA1	HA3	HA5
E. coli	0	96.94	99.13	100
P. aeruginosa	0	97.55	99.75	100
S. aureus	0	97.48	98.74	100

and then the release speed slowed down after 14 days. The largest concentration of silver ion in SBF at the end of 49 days is 2.27 ppm for the HA5 coating.

A bioactivity delivery system, which can release anti-bacterial ions, is expected to an antibiotic-delivering system at the bone-implant interface. It is reported that the parenteral administration of antibiotics may not be effective for the infection since the formation of a biofilm on the surface of the implant increases the resistance of the bacteria to antibiotics, so a high concentration of antibiotics at the bone-implant interface would be essential to prevent these bacterial infections [23]. From Fig. 5, it can be observed that the plasma sprayed silver-

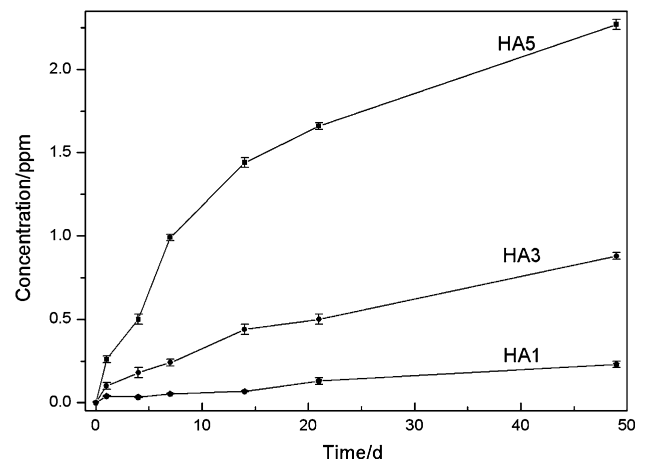

Fig. 5 Changes in concentration of the silver ions in SBF after the immersion of silver-containing HA coatings for different times

containing HA coatings are an effective system for delivering silver ions, which can inhibit the proliferation of bacteria effectively.

Biocompatibility should be seriously taken into account while excellent anti-bacterial ability is achieved for the HA

Table 4 The average absorbance, cell relative rate, and cytotoxicity rank of the samples for 7 days

	Average absorbance	Cell relative rate	Cytotoxicity rank
HA	0.091	101.11	0
HA1	0.093	103.33	0
HA3	0.108	120.00	0
HA5	0.106	117.78	0
Positive	0.061	67.78	2
Negative	0.090	–	–

coatings by the addition of silver. The cell relative rate and cytotoxicity rank of the HA and silver-containing HA coatings are shown in Table 4, which reveals that the cytotoxicity ranks after 7 days for the silver-containing HA coatings are all 0. This means that the silver-containing coatings with <5% silver have no cytotoxicity. Figure 6 shows the cell morphologies at the end of 7 days. There are no obvious differences in the cell morphology between the samples and negative controls, but most of the cells cultured in the extracts of positive controls are dead. The results of cell morphology are accordance with the cytotoxicity ranks.

The hemolysis ratios of the samples are showed in Table 5. From Table 5, it can be seen that the hemolysis ratio increases gradually with increasing in silver content. According to ASTM F 756-00, samples with hemolysis ratio <2 can be considered nonhemolytic. In this experiment, all of the hemolysis ratio of the coatings are <0.4 and therefore, it can be concluded that all of the four coatings are nonhemolytic.

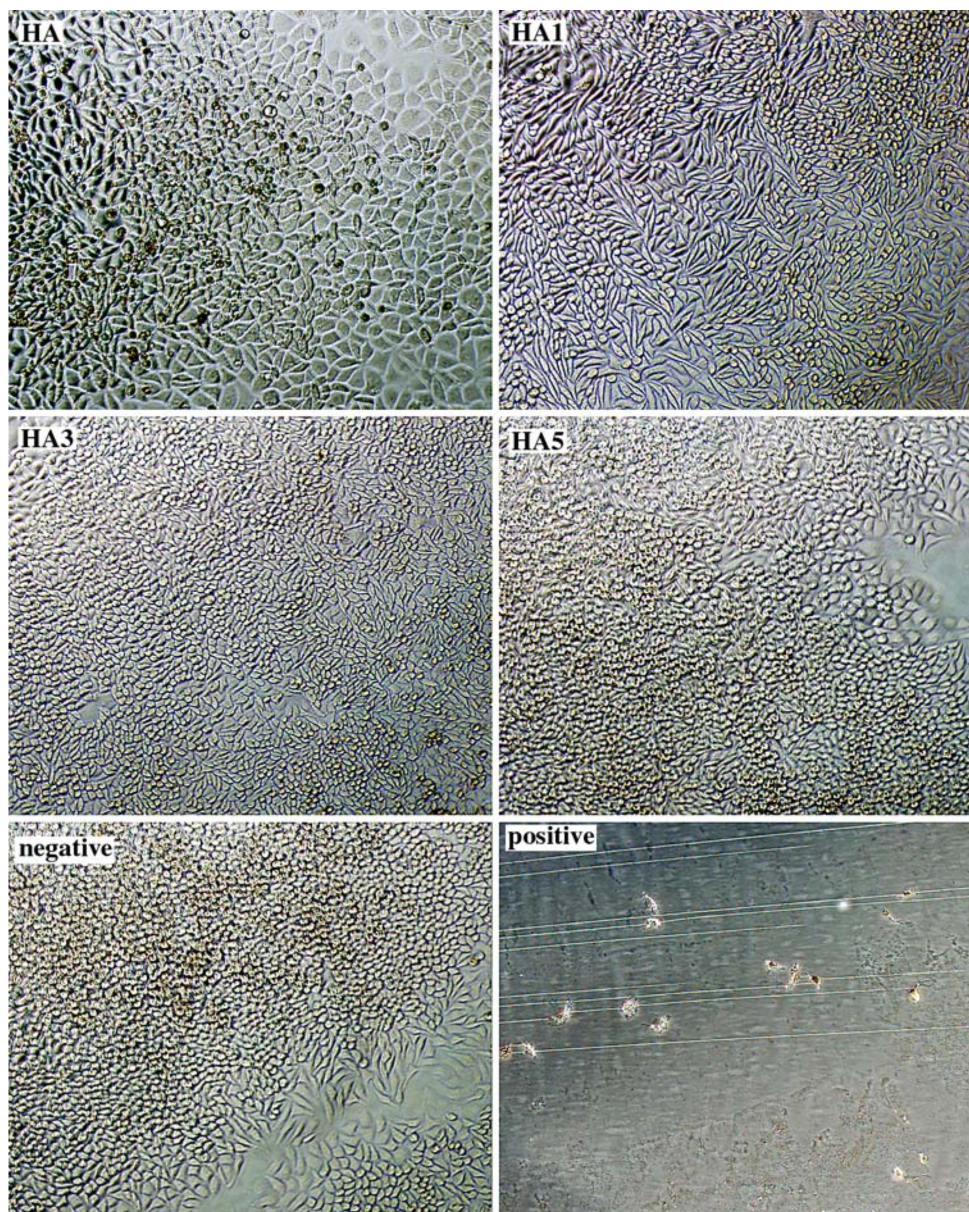

Fig. 6 Cell morphology after the extracts and phenol were removed from the 96-well plates at the end of 7 days

Table 5 Hemolysis ratio of the samples

	HA	HA1	HA3	HA5	Negative	Positive
Absorbance	0.011	0.016	0.013	0.015	0.012	1.185
	0.011	0.010	0.013	0.016	0.011	0.978
	0.015	0.013	0.015	0.016	0.012	0.993
Average	0.012	0.013	0.014	0.016	0.012	1.052
Hemolysis ratio (%)	0	0.10	0.19	0.38	0	–

From the results of cytotoxicity and hemolysis tests, it can be known that the silver-containing HA coatings with <5% silver have no cytotoxicity and hemolysis. It is reported in some literatures [24, 25] that the maximum value of released Ag^+ (19 μg/mm^2) is well below the critical level of cytotoxicity. In this study the highest calculated value for the silver ions release in SBF is 0.38 μg/mm^2.

4 Conclusions

Plasma sprayed silver-containing HA coatings have been fabricated and exhibited a marked anti-bacterial effect against *E. coli*, *P. aeruginosa*, and *S. aureus*. The silver and its opportune release in physiological environment played an important role on inhibiting proliferation of bacteria. No significant cytotoxicity and hemolysis were observed for the silver-containing coatings. It was concluded that the silver-containing coatings had good anti-bacterial and biological properties.

References

1. W.R. Lacefield, in *Hydroxyapatite Coating*, ed. by L.L. Hench, J. Wilson. An Introduction to Bioceramics, 1st edn. (World Scientific, Singapore, 1993), p. 223
2. R. Capanna, H.G. Morris, D. Campanacci, M. Del Ben, M. Campanacci, J. Bone Jt. Surg. Br. **76B**, 178 (1994)
3. F. Zhang, G.K. Wolf, X.H. Wang, X.H. Liu, Surf. Coat Technol. **148**, 65 (2001). doi:10.1016/S0257-8972(01)01305-6
4. A. Oyane, Y. Yokoyama, M. Uchida, A. Ito, Biomaterials **27**, 3295 (2006). doi:10.1016/j.biomaterials.2006.01.029
5. Z.L. Shi, K.G. Neoh, E.T. Kang, Biomaterials **26**, 501 (2005). doi:10.1016/j.biomaterials.2004.02.060
6. Q. Zhao, Y. Liu, C. Wang, S. Wang, Appl. Surf. Sci. **253**, 7254 (2007). doi:10.1016/j.apsusc.2007.03.011
7. Q.L. Feng, T.N. Kim, J. Wu, E.S. Park, J.O. Kim, D.Y. Lim, F.Z. Cui, Thin Solid Films **335**, 214 (1998). doi:10.1016/S0040-6090(98)00956-0
8. T.N. Kim, Q.L. Feng, J.O. Kim, J. Wu, H. Wang, G.C. Chen et al., J. Mater. Sci. Mater. Med. **9**, 129 (1998). doi:10.1023/A:1008811501734
9. Q.L. Feng, J. Wu, G.Q. Chen, F.Z. Cui, T.N. Kim, J.O. Kim, J. Biomed. Mater. Res. **52**, 662 (2000). doi:10.1002/1097-4636(20001215)52:4<662::AID-JBM10>3.0.CO;2-3
10. I. Sondi, B. Salopek-Sondi, J. Colloid Interface Sci. **275**, 177 (2004). doi:10.1016/j.jcis.2004.02.012
11. M. Bellantone, H.D. Williams, L.L. Hench, Antimicrob. Agents Chemother. **46**, 1940 (2002). doi:10.1128/AAC.46.6.1940-1945.2002
12. H.J. Klasen, Burns **26**, 117 (2000). doi:10.1016/S0305-4179(99)00108-4
13. G. Gosheger, J. Hardes, H. Ahrens, A. Streitburger, H. Buerger et al., Biomaterials **25**, 5547 (2004). doi:10.1016/j.biomaterials.2004.01.008
14. M. Kawashita, S. Tsuneyama, F. Miyaji, T. Kokubo, H. Kozuba, K. Yamamoto, Biomaterials **21**, 393 (2000). doi:10.1016/S0142-9612(99)00201-X
15. H.J. Jeon, S.C. Yi, S.G. Oh, Biomaterials **24**, 4921 (2003). doi:10.1016/S0142-9612(03)00415-0
16. K. Yamamoto, S. Ohashi, M. Aono, T. Kokubo, I. Yamada, J. Yamauchi, Dent. Mater. **12**, 227 (1996). doi:10.1016/S0109-5641(96)80027-3
17. S. Di Nunzio, C. Vitale Brovarone, S. Spriano, D. Milanese, J. Eur. Ceram. Soc. **24**, 2935 (2004). doi:10.1016/j.jeurceramsoc.2003.11.010
18. W. Chen, Y. Liu, H.S. Courtney, M. Bettenga, C.M. Agrawal, J.D. Bumgardner, J.L. Ong, Biomaterials **27**, 5512 (2006). doi:10.1016/j.biomaterials.2006.07.003
19. A.J. Betts, D.P. Dowling, M.L. Mcconnell, C. Pope, Mater Des **26**, 21718 (2005). doi:10.1016/j.matdes.2004.02.006
20. M. Shirkhanzadeh, M. Azadegan, G.Q. Liu, Mater. Lett. **24**, 7 (1995). doi:10.1016/0167-577X(95)00059-3
21. T. Kokubo, H. Kushitani, S. Sakka, T. Kitsugi, T. Yamamuro, J. Biomed. Mater. Res. **24**, 721 (1990). doi:10.1002/jbm.820240607
22. S.P. Chen, G.Z. Wu, H.Y. Zeng, Carbohyr. Polym. **60**, 33 (2005). doi:10.1016/j.carbpol.2004.11.020
23. Z. Ruszczak, W. Friess, Adv. Drug Deliv. Rev. **55**, 1679 (2003). doi:10.1016/j.addr.2003.08.007
24. D.P. Dowling, K. Donnelly, M.L. Mcconnell, R. Eloy, M.N. Arnaud, Thin Solid Films **398–399**, 602 (2001). doi:10.1016/S0040-6090(01)01326-8
25. J.C. Wataha, P.F. Lockwood, A. Schedle, J. Biomed. Mater. Res. **52**, 360 (2000). doi:10.1002/1097-4636(200011)52:2<360::AID-JBM16>3.0.CO;2-B