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Local co-delivery and release of antimicrobial peptide and RGD using the porous TiO_2

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We demonstrated for the first time to use two kinds of porous TiO_2 films to co-deliver peptide HHC36 (KRWWKWWRR) and RGD. The film co-delivering these two peptides exhibited excellent antimicrobial activity against *S. aureus* and *E. coli*, and low cytotoxicity to rat bone mesenchymal stem cells (*rBMSCs*).

1. INTRODUCTION

Porous TiO_2 prepared by anodic oxidation is one of potential biomaterials for hard tissue implants.[1, 2] As reported, it shows excellent biocompatibility and bioactivity in vitro and in vivo.[3] However, for hard tissue implants, especially the titanium-matrix implants, the peri-implant infections in early stage are common in clinic, which could lead to the failure of the surgery and patient disability, and even death.[4] Many methods have been used to improve the antimicrobial activity of the implants, such as UV treatment,[5] overlaying antimicrobial polymer on implant film,[6] incorporation with silver nanoparticles[7] or local delivery of various antibiotics.[8]

Among these methods, for titanium-matrix implants, antimicrobial peptides (AMPs), especially the short antimicrobial peptides,[9, 10] are screened by researchers. Although formed by less amino acids, the short antimicrobial peptides have improved antimicrobial activity, broad-spectrum activity, low susceptibility of developing bacterial resistance and short contact time to induce killing.[9] Moreover, after simple physical absorption, these peptides could be loaded and release from the porous TiO_2 film to show excellent antimicrobial activity.[11] However, in order to make sure the long actuation duration, excessive AMPs were often loaded on the porous TiO_2 film.[12] The large amount of AMPs could lead to cytotoxicity evidently, which still limits its application in clinic.[12, 13]

In this paper, in order to resolve the cytotoxicity of this control-release system, we use the porous TiO_2 film as the substrate to co-deliver HHC36 (KRWWKWWRR) and another kind of peptide, RGD, at the same time.[14] The RGD peptide mainly exists in extracellular matrix. It could specific combine with 11 species of integrin, and promote the conglutination between matrix and implants, which could apparently improve the biocompatibility of implants.[15, 16] We first prepared two kinds of porous TiO_2 on pure titanium in organic or inorganic solutions.[17, 18] Then we used the film to load these two kinds of peptides at different molar ratios, and characterized the co-release of the peptides from the film. The antimicrobial activity of the film against *S. aureus* and *E. coli* was tested in vitro, and the biocompatibility of the film was tested with rat bone mesenchymal stem cells (*rBMSCs*).

2. EXPERIMENTAL

2.1 Materials

Pure titanium foil ($10\times10\times0.3mm^3$, 99.8% purity) was purchased from Chenhui Metal Materials Ltd. (Baoji, China). The peptide HHC36 and RGD were purchased from GL Bio. (GL Biochem (Shanghai) Ltd.). The porous TiO_2-related reagents were purchased from Guangzhou Chemical Factory Co. Ltd. (Guangdong, China). The bacteria and cell-related reagents were purchased from Sigma-Aldrich (USA).

2.2 Anodic oxidation

The titanium foils were treated with 3vol% of HF and 5vol% of HNO_3. Then they were washed with acetone, ethanol and distilled water for 10 min, respectively, and were used as the anode, while the platinum foil was used as the cathode. This system was immersed into the electrolyte containing 0.27 M NH_4F in 75% glycerol (organic electrolyte) at 30 V for 6 h, or immersed into the electrolyte containing 1M of $(NH_4)_2SO_4$ and 0.5wt% NH_4F (inorganic electrolyte) at 30 V for 0.5 h. After anodization, the foils were washed with ethanol for 15 min by ultrasonic. Then the foils were annealed from room temperature to 500 °C at the rate of 5 °C/min, held for 3 h, and cooled down in the furnace. The foils treated in organic and inorganic electrolyte were noted as ***Org*** and ***Inorg***, respectively.

2.3 Loading peptides onto the porous TiO_2

The RGD peptide was added into a solution of AMP (HHC36, 1 mM in ethanol) at various molar concentrations (0, 1, 2 and 3 mM). Then 50 μl of the solution was added onto the porous TiO_2 film, and the film was dried under vacuum desiccator at room temperature for 30 min. This process was repeated for 5 times. After that, the film was washed with PBS for three times. The ***Org*** films treated with the solution containing AMP and RGD at different molar concentrations were abbreviated as ***Org-AMP***, ***Org-AMP-1RGD***, ***Org-AMP-2RGD*** and ***Org-AMP-3RGD***, respectively, while the ***Inorg*** films treated

with the solution containing AMP and RGD at different molar concentrations were abbreviated as ***Inorg-AMP***, ***Inorg -AMP-1RGD***, ***Inorg -AMP-2RGD*** and ***Inorg -AMP-3RGD***, respectively. The films treated with the solution only containing 1 mM RGD were abbreviated as ***Org-RGD*** and ***Inorg-RGD***, respectively.

2.4 The characterization of the film

We used an ELISA plate reader (Varioskan Flash 3001, Thermo, Finland) to quantify the release rate of AMP, and the BCA kit to calculate the release rate of RGD. The *Staphylococcus Aureus* (*S. aureus*, strain ATCC 29213) and *Escherichia Coli* (*E. coli*, strain ATCC 15224) were used to test the antimicrobial activity of the film, and the *rBMSCs* were used to test the biocompatibility of the film. The details of the experiments were shown in supporting information.

3. Results and discussion

The SEM images of the films shown in Fig. 1S showed that the average pore size of the ***Inorg*** film was about 125 nm, while that of ***Org*** film was about 70 nm. In addition, the porous structure on ***Inorg*** film was more regular. The XRD results in Fig. 2S showed that after annealing, the transformation of the phase from amorphous to anatase. These results demonstrated that as the high specific surface area and cell-favourite crystal, our films should be benefit to be used as hard tissue implants to load with peptides as other references.[2, 19]

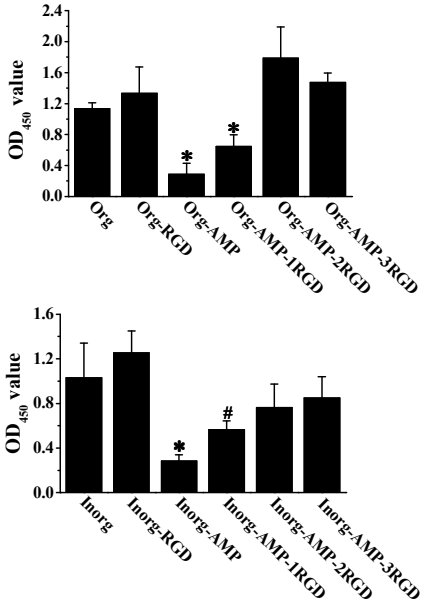

Fig. 1 Cell viability of *rBMSCs* on various films after cultured for 24 h (n=4). # denotes significant differences ($p< 0.01$) and *denotes significant differences ($p< 0.001$) compared with ***Org*** or ***Inorg***, respectively. The statistical significance was calculated with SPSS 17.0 statistical software and t-tests method.

We then used the CCK-8 assay to test the biocompatibility of different films to *rBMSCs*. The results shown in Fig. 1 showed that compared to the control (***Org*** and ***Inorg***), the films only loaded with AMP (***Org-AMP*** and ***Inorg-AMP***) exhibited obvious cytotoxicity, which could kill about 74.48% and 72.26% cells on them. Meanwhile, the RGD on the film could improve the biocompatibility. ***Org-RGD*** and ***Inorg-RGD*** films, which were the films only loading with RGD, exhibited better biocompatibility compared with ***Org*** or ***Inorg***, and the OD values increased about 17.39% and 21.50%, respectively. In addition, for the co-delivery system, the cells on ***Org-AMP-1RGD*** and ***Inorg-AMP-1RGD*** increased by about 1.24 and 0.98 times compared to ***Org-AMP*** and ***Inorg-AMP***, respectively. With the increase of RGD, the biocompatibility of the films improved. Compared to ***Org-AMP*** and ***Inorg-AMP***, respectively, the cells on ***Org-AMP-2RGD*** and ***Inorg-AMP-2RGD*** increased by 5.16 and 1.67 times, while the cells on ***Org-AMP-3RGD*** and ***Inorg-AMP-3RGD*** increased by 4.08 and 1.97 times.

As the film had excellent biocompatibility when the molar ratios of AMP and RGD were 1:2 or 1:3 (shown in Fig. 1), we chose ***Org-AMP-2RGD*** and ***Inorg-AMP-2RGD*** for the next experiment. In order to illustrated the effect of the peptides more clearly, we stained the cells on different films with FITC dye after cultured for 4 and 24 hours, and the fluorescent images were shown in Fig.3S and Fig. 4S. It showed that at 4 hours, ***Org-AMP-2RGD*** showed similar biocompatibility as ***Org***, which was better than ***Org-AMP*** but worse than ***Org-RGD***. It might be cause by that, although the AMP in both ***Org-AMP*** and ***Org-AMP-2RGD*** exhibited cytotoxicity, the RGD could improve the adhesion of cells at early time. After 24 h, ***Org-AMP-2RGD*** had similar biocompatibility as ***Org-RGD***, which was a little better than ***Org***. Meanwhile, the cells on ***Org-AMP*** were evident less than others. This result was corresponded to the CCK-8 results shown in Fig. 1, and it demonstrated that the RGD peptide in the co-delivery system could also improve the proliferation of the cells.

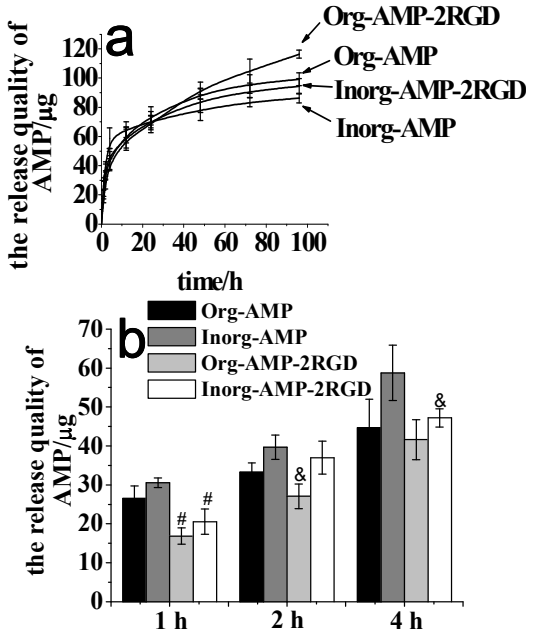

Fig. 2 (a) In vitro release of AMP from the indicated films; (b) the release amount of AMP from indicated films at first 4 h. (n=4). & denotes significant differences ($p< 0.05$) and #denotes significant differences ($p< 0.01$) (***Org-AMP-2RGD*** compared with ***Org-AMP***, and ***Inorg-AMP-2RGD*** compared with ***Inorg-AMP***, respectively.) The statistical significance was calculated with SPSS 17.0 statistical software and t-tests method.

We then detected the release of the peptides from the films. The release curve of AMP from the films (***Org-AMP***, ***Org-AMP-2RGD***, ***Inorg-AMP*** and ***Inorg-AMP-2RGD***) was detected by ELISA and the results were shown in Fig. 2 (a). The burst release of AMP from films was detected in the first 4 h in Fig. 2(a). About 45.03%, 35.77%, 68.12% and 49.98% of the AMP was released from ***Org***, ***Org-AMP-2RGD***, ***Inorg*** and ***Inorg-AMP-2RGD*** during this stage. However, this result was better compared to others,[9] which would release about 81.7% AMPs during the burst stage in the first 4 h. Fig. 2(b) showed the release of AMP from indicated films in the first 4 h.

Interestingly, it showed that the incorporation of RGD could improve the burst release of AMP at early stage (especially at 1 h). That could be figured by the possible interaction between the two peptides, which could alter the diffusion kinetics between them.[20] The release curves of RGD for different films detected by BCA kit was shown in Fig. 5S, which also showed the burst release in the first 4 h.

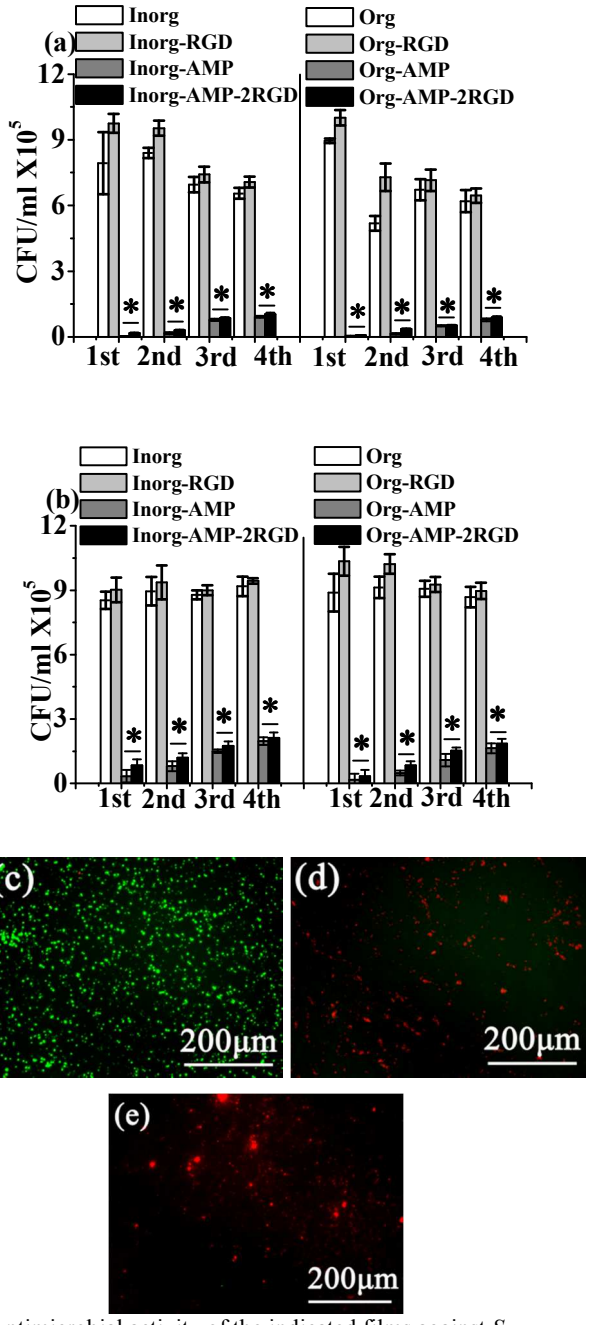

Fig.3 Antimicrobial activity of the indicated films against *S. aureus* (a) and *E.coli* (b) in 30 min for four cycles, and the live/dead assay of *E. coli* on ***Inorg*** (c), ***Inorg-AMP*** (d) and ***Inorg-AMP-2RGD*** films (the images were got under FITC and TRITC channels, and merged with the NIS software. The green bacteria were live, while the red bacteria were dead). * denotes significant differences ($p < 0.001$) compared with ***Inorg*** or ***Org***, respectively. The statistical significance was calculated with SPSS 17.0 statistical software and t-tests method.

In order to find the effect of the peptides released from the films on the biocompatibility, we tested the *rBMSCs*' viability in the medium containing the AMP or RGD at certain concentrations, and the results were shown in Fig. 6S and Fig. 7S. The release curves in Fig. 2 (a) showed that about 50-80 μg/mL of AMP released into the medium at the first 24 h. At these concentrations, the AMPs showed evident cytotoxicity to the *rBMSCs* (as shown in Fig. 6S), which illustrated that the cytotoxicity of the films containing AMP should be caused by the high concentrations of the peptide. Fig. 5S showed that about 80-140 μg/mL RGD released at the first 24 h. Interestingly, the *rBMSCs*' viability in the medium containing the RGD at these concentrations did not have evident difference (Fig. 7S). It illustrated that the better biocompatibility of the co-delivery film might be caused by the RGD left on the surface.

The antimicrobial activity of different films was tested with *S. aureus* and *E. coli*, and the results were shown in Fig. 3. The results demonstrated that the ***Org-AMP*** and ***Inorg-AMP*** films showed excellent antimicrobial activity against the *S. aureus* and *E. coli*. Meanwhile, after loaded with RGD, the antimicrobial activity of the films (***Org- AMP-2RGD*** and ***Inorg-AMP-2RGD***) did not decrease, which could also kill almost 100% of bacteria in 30 min. In addition, the ***Org-RGD*** and the ***Inorg-RGD*** films did not kill the bacteria evidently, which demonstrated that the RGD peptide had no antimicrobial activity. The consecutive killing assays, which means to reuse the samples after the last antimicrobial test, also demonstrated that the antimicrobial activity of the films could keep stability after four cycles. And after the fourth round, compared to the control (***Org*** and ***Inorg***), the ***Org-AMP***, ***Inorg-AMP***, ***Org-AMP-2RGD*** and ***Inorg-AMP-2RGD*** could also kill about 87.39%, 86.08%, 85.75% and 84.30% of the *S. aureus*, and 81.19%, 78.58%, 78.69% and 76.95% of the *E. coli*. The Live/Dead assay images shown in Fig. 3 (c), (d), (e) and Fig. 8S also showed that these antimicrobial films exhibited excellent antimicrobial activity, while the live bacteria exhibited green fluorescence and the dead bacteria exhibited red fluorescence.

Conclusions

We have successfully used the RGD to improve the biocompatibility of two kinds of porous TiO_2 films loaded with AMP. When the molar ratio of AMP and RGD is 1:2, the biocompatibility of the film has a tremendous increase. Meanwhile, these films show excellent antimicrobial activity against *S. aureus* and *E. coli* even after four cycles. This co-delivery system could be a potential solution for early stage peri-implant infection.

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Notes and references

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† Electronic Supplementary Information (ESI) available: [details of antimicrobial assay and biocompatibility assay, as well as the SEM image, XRD pattern of the porous TiO_2, the release curve of RGD, the fluorescent image of bacterial live/dead assay] See DOI: 10.1039/c000000x/

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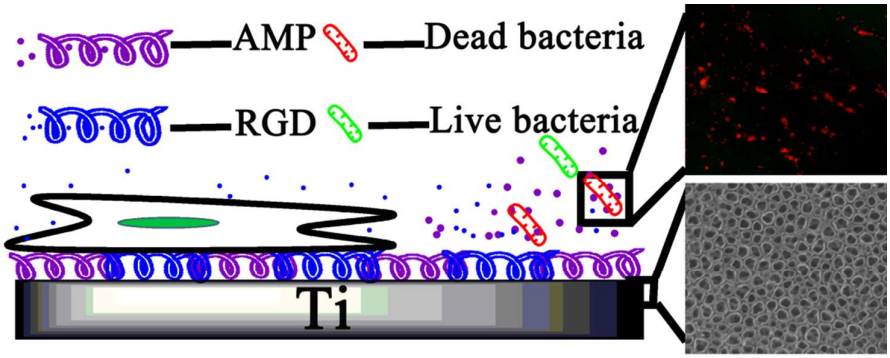
Graphical Abstract



The co-delivery system with AMP and RGD on porous titanium showed excellent biocompatibility and antimicrobial activity.