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*Supporting Information*

# Multi-biofunctionalization of titanium surface with mixture of peptides to achieve excellent antimicrobial activity and biocompatibility

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## Experimental

### Materials

Pristine titanium substrate (10×10×1mm$^3$, 99.8% purity) was purchased from Chenhui Metal Materials Ltd. (Baoji, China). The titanium substrate (5×5×1mm$^3$, 99.8% purity), which was obtained by evaporating titanium particle (around 20 nm) onto Si (100), was purchased from Tsinghua-Foxconn nano technology research center for AFM assay and ellipsometry assay. The alkynyl-PEG-triethoxysilane (*APTS*) was purchased from Xing Jia Feng Science and Technology Development Co. Ltd. (Shenzhen, China). The azido-dPEG®12-acid and azido-dPEG®24-acid were purchased from Quanta Biodesign (Ohio, USA). HHC36 with/without tethering azido-dPEG®12-acid and the RGD peptide with/without tethering azido-dPEG®24-acid at the N-terminus were got from ChinaPeptides Co., Ltd. (Shanghai, China). Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (ligand), azide-PEG3-biotin conjugate (biotin-azide), avidin-FITC and all bacteria/cell-related reagents were purchased from Sigma-Aldrich (USA). The other reagents used in this experiment were purchased from Guangzhou Chemical Factory Co. Ltd. (Guangdong, China).

### Preparation of titanium substrate

The titanium substrate (10×10×1mm$^3$) was polished by abrasive papers (graded roughness from 800 to 5000). After that, the two kinds of the titanium substrates (10×10×1mm$^3$ and 5×5×1mm$^3$) were washed with acetone and ethanol for 15 min by ultrasonic, respectively, and dried in 70 °C oven. Before application, all the substrates were treated with oxygen plasma for 5 min to activate the hydroxyl on the surfaces.

### Multi-biofunctionalization of the titanium surface

The click solution contained 100 μM $CuSO_4$, 200 μM ligand, 5mM ascorbic acid sodium salt, 100 μM *APTS* and different concentrations of peptides (0-100 μM AMP and 0-100 μM RGD). After mixing the reagents, the reaction system was shaken for 3 h in dark at room temperature under nitrogen environment in the glove box. Then the click solution was directly rotary evaporated immediately, and resolved with hydrolytic solution (95% ethanol and 5% distilled water, adjust pH to 4.6 with acetic acid), whose volume was 1/40 compared to original click solution, to hydrolyte the

silane coupling agent in the system in dark at room temperature. After hydrolyzed for 2 h, 40 μL of the hydrolytic solution was dripped onto titanium surface ($10\times10\times1mm^3$), or 10μL of the hydrolytic solution was dripped onto the titanium surface ($5\times5\times1mm^3$) for AFM assay, and hydrolyzed for another 2 h under nitrogen environment in dark at room temperature to form different surfaces. Then the samples were immersed into 1 mL of 10 mM EDTA2Na solution for 3 h to remove the copper and washed roughly with distilled water, and dried under nitrogen atmosphere in the glove box for 12 h.

The titanium substrate only treated with oxygen plasma and immersed into 10 mM EDTA2Na solution for 3 h was abbreviated as ***Ti***. The samples treated with the click solution containing 100 μM AMP, 90 μM AMP/10μM RGD, 80μM AMP/20μM RGD, 65μM AMP/35μM RGD, 50μM AMP/50μM RGD and 100μM RGD were abbreviated as *Ti-100%AMP*, *Ti-90%AMP-10%RGD*, *Ti-80%AMP-20%RGD*, *Ti-65%AMP-35%RGD*, *Ti-50%AMP-50%* and *RGD Ti-100%RGD*, respectively. The samples treated with the click solution containing 80μM, 50μM, 40μM, 30μM and 20μM of AMP were abbreviated as *Ti-80%AMP*, *Ti-50%AMP*, *Ti-40%AMP*, *Ti-30%AMP* and *Ti-20%AMP*, respectively. The samples treated with the click solution containing 100 μM peptides (AMP or RGD) without azido group were abbreviated as *Ti-AMP-control* and *Ti-RGD-control*, respectively.

**Fluorescent detection of the bioactive molecular**

To demonstrate this method could prepare bioactive molecular onto titanium surface, the surface was treated with click solution in which 100 μM of biotin-azide was used to replace peptides. All other processes were the same, and the sample was abbreviated as *Ti-biotin*. For the control group, the surface was treated with click solution as *Ti-biotin* but without $CuSO_4$, and all other processes were the same. This control group was abbreviated as *Ti-biotin-control*. In addition, *Ti* sample was used as another control group. Then all the samples were placed into 24-well plate and stained with 1 mL of avidin-FITC (0.1 mg/mL in PBS) for 20 min at 4 ºC in the dark, and washed again with PBS for 3 times. After that, the samples were observed with a Nikon eclipse 80i fluorescence microscope at FITC channel using a 10 X objective. A

CoolSnap HQ2 camera (Photometrics, Tuscon, AZ) and NIS Elements software (Version 3.0, Nikon Instruments, Melville, NY) were used for image acquisition and analysis.

**The characterization of the sample**

X-ray Photoelectron Spectroscopy (XPS) was performed with a PHI 5700 X-ray photoelectron spectrometer equipped with a monochromatic Al Kα X-ray source (1486.7eV) at a takeoff angle (TOA) of 45º from the surface. Atomic Force Microscopy (AFM) image of the surface was performed using a MultiMode Nanoscope IIIa AFM (Digital Instruments Inc., Santa Barbara, CA). Images were acquired in tapping mode using a silicon nitride cantilever (MikroMasch, San Jose, CA) with a resonance frequency of 132.9 kHz and a nominal force constant of 1.75 N/m.

The ellipsometer (VASE, J.A.Woollam Co., Inc, NE, USA) was employed for thickness measurement. It was operated with a 400nm-1000nm Xe laser with a 5nm step at an incident angle of 70°. The thickness was fitting by the software Ellipsometry Solutions matched with the ellipsometer. The surface density of molecular (1) could be estimated by the formula (1)[1, 2]:

$$D = \rho d_{ML} N_A / M_w \qquad (1)$$

where D is the number of molecules per unit area; ρ is the density of molecular (1), which is assumed to be 1 g/cm$^3$;[1, 2] $d_{ML}$ = 9.9 nm is the thickness of the layer measured by ellipsometry; $N_A$ is Avogadro's number ($6.02 \times 10^{23}$) and Mw is the molecular weight of molecular (1) shown in Fig. 1 (2502.9 g/mol).

The contact angle of the surface (n=3) was measured by the liquid drop method on a contace angle goniometer (OCA15, Dataphysics, Germany) with distilled water.

**Antimicrobial assay**

*Bacterial culture*

*Staphylococcus Aureus* (*S. Aureus*, strain ATCC 29213) and *Escherichia Coli* (*E. coli*, strain ATCC 15224) were purchased from VWR International, LLC. Single colony of the bacteria was inoculated in 5 ml of LB media overnight at 37 ºC with shaking (150 rpm). After that, 1 ml of the bacterial suspension was inoculated in 50

ml of fresh LB media, respectively, which was incubated for 5 h with shaking (250 rpm) at 37 ºC to achieve mid-log phase growth.

*Bacterial culture on the surfaces*

The bacteria were re-suspended to PBS with the concentration of $1.0\times10^7$ cfu/ml. Prior to seeding, the samples were placed into a 24-well culture plate. Then 40 μl of the bacterial suspension ($1.0\times10^7$ cfu/ml in PBS) was added onto each sample to fully cover the substrate.

*Antimicrobial activity of the surfaces*

For agar plate assay, after cultured the bacterial on different surfaces for 2.5 h at 37 ºC, 1960 μL of PBS was added into each well to dilute the bacterial solution. After mixed with the pipettor roughly, each sample as well as the bacterial suspension were transferred to a new tube, ultrasonic for 5 min and votex for 1 min to detach the adhered bacteria. Then the bacterial solution was diluted to $10^0$, $10^1$ and $10^2$ times, and 10 μL of the bacterial suspension was taken to evaluate the viability of bacteria by using agar plates.

For live/dead assay, after cultured the bacteria on different surfaces for 2.5 h at 37 ºC, the samples were rinsed gently with PBS for three times. Then 20 μl of 0.5% propidium iodide solution and 40 μl of 0.05% Fluorescein diacetate were added onto the sample and the system was incubated for 5 min at room temperature. After that, the films were washed with PBS for three times and observed with Eclipsc Ti-U (Nikon, Japan) immediately.

**Cell assay**

The biocompatibility of the functionalized surface was tested with the rat bone mesenchymal stem cells (*rBMSCs*) as follows.

*Cell culture and seeding*

The *rBMSCs* were cultured in High glucose Dulbecco's modified Eagle's medium (H-DMEM) (Hyclone, Logan, Utah) containing 10% fetal bovine serum (FBS) and in a 5% $CO_2$ atmosphere at 37 ºC. Medium was replaced every third day. The adherent cells were allowed to reach about 80% confluence. Cells were passaged in culture and passage 5-10 (P5-P10) cells were used for the experiments.

All the samples used for cell test were sterilized with 75% ethanol for 2 h in the 24-well plates before treatment. Then the samples were washed with PBS for three times. The cells were added directly into each well (30000 cells in 1 ml media suspension per well) and were cultured for 24 h before the test.

*CCK-8 assay*

After being cultured for 24 h, the biocompatibility of the material was evaluated with CCK-8 assay. Briefly, at indicated time point, the films were transferred to a new 24-well plate and washed three times with PBS. Then 350 μL of complete medium containing 35 μL of CCK-8 solution was added into each well. After 2 h at 37 ºC in dark, 100 μL of the incubated solution was transferred to a new 96-well plate and the optical density (OD) value of the solution was measured with an ELISA plate reader (Varioskan Flash 3001, Thermo, Finland) at 450 nm wavelength

**Statistics**

The antimicrobial assay and cell assay were repeated at least three times and the results were expressed as means ± standard deviations. Statistical significance was calculated using the SPSS 17.0 statistical software. Statistical significance was defined as $p<0.05$.

Table 1S The abbreviation of the samples.

| **Sample Abbreviation** | | **Treatment method** |
|---|---|---|
| *Ti* | | Ti substrate |
| *Ti-biotin-control* | | Ti + click solution with 100μM biotin-azide and 0μM copper |
| *Ti-biotin* | | Ti + click solution with 100μM biotin-azide |
| *Ti-AMP-control* | | Ti + click solution with 100μM AMP without azide group |
| *Ti-RGD-control* | | Ti + click solution with 100μM RGD without azide group |
| *Ti-100%AMP* | | Ti + click solution with 100μM AMP-azide |
| Ti-AMP | *Ti-20%AMP* | Ti + click solution with 20μM AMP-azide |
| | *Ti-30%AMP* | Ti + click solution with 30μM AMP-azide |
| | *Ti-40%AMP* | Ti + click solution with 40μM AMP-azide |
| | *Ti-50%AMP* | Ti + click solution with 50μM AMP-azide |
| | *Ti-80%AMP* | Ti + click solution with 80μM AMP-azide |
| Ti-AMP-RGD | *Ti-90%AMP-10%RGD* | Ti + click solution with 90μM AMP-azide and 10μM RGD-azide |
| | *Ti-80%AMP-20%RGD* | Ti + click solution with 80μM AMP-azide and 20μM RGD-azide |
| | *Ti-65%AMP-35%RGD* | Ti + click solution with 65μM AMP-azide and 35μM RGD-azide |
| | *Ti-50%AMP-50%RGD* | Ti + click solution with 50μM AMP-azide and 50μM RGD-azide |
| | *Ti-100%RGD* | Ti + click solution with 100μM RGD-azide |

Table 2S The contact angles of the indicated samples. (n=3, # denotes p<0.05, & denotes p<0.01 and * denotes p<0.001 compared to ***Ti***)

| Sample | Contact angle (°) |
|---|---|
| *Ti* | 45.23±4.12 |
| *Ti-RGD-control* | 70.63±1.75 * |
| *Ti-100%RGD* | 61±2.17 & |
| *Ti-50%AMP-50%RGD* | 58.83±3.13 # |
| *Ti-65%AMP-35%RGD* | 63.03±1.11 & |
| *Ti-80%AMP-20%RGD* | 62.30±2.55 & |
| *Ti-90%AMP-10%RGD* | 73.43±1.95 * |
| *Ti-100%AMP* | 79.3±4.07 * |

Fig. 1S The C1s XPS spectrum of *Ti-AMP-control*.

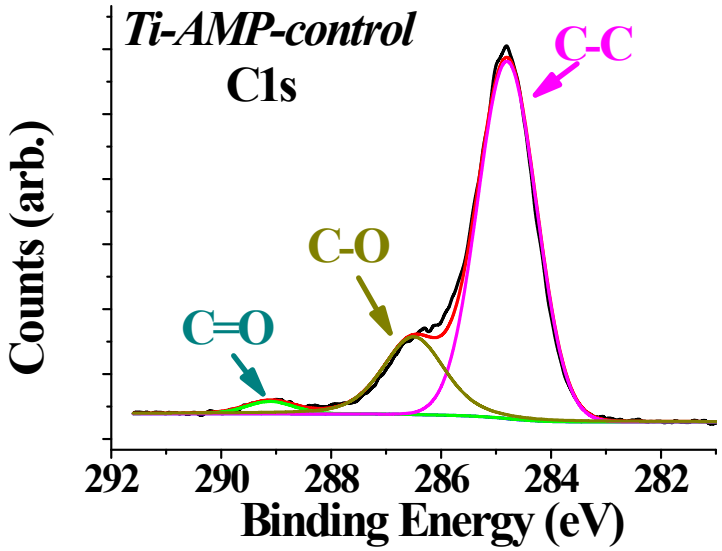


Fig. 2S The Ti2p XPS spectrum of indicated samples.

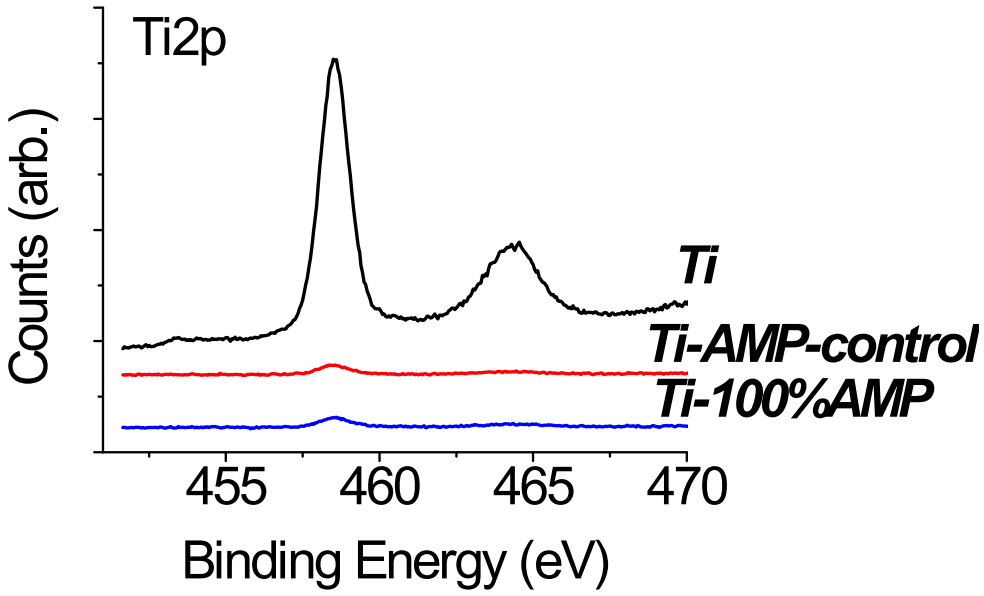


Fig. 3S The Si2p XPS spectrum of the indicated samples.

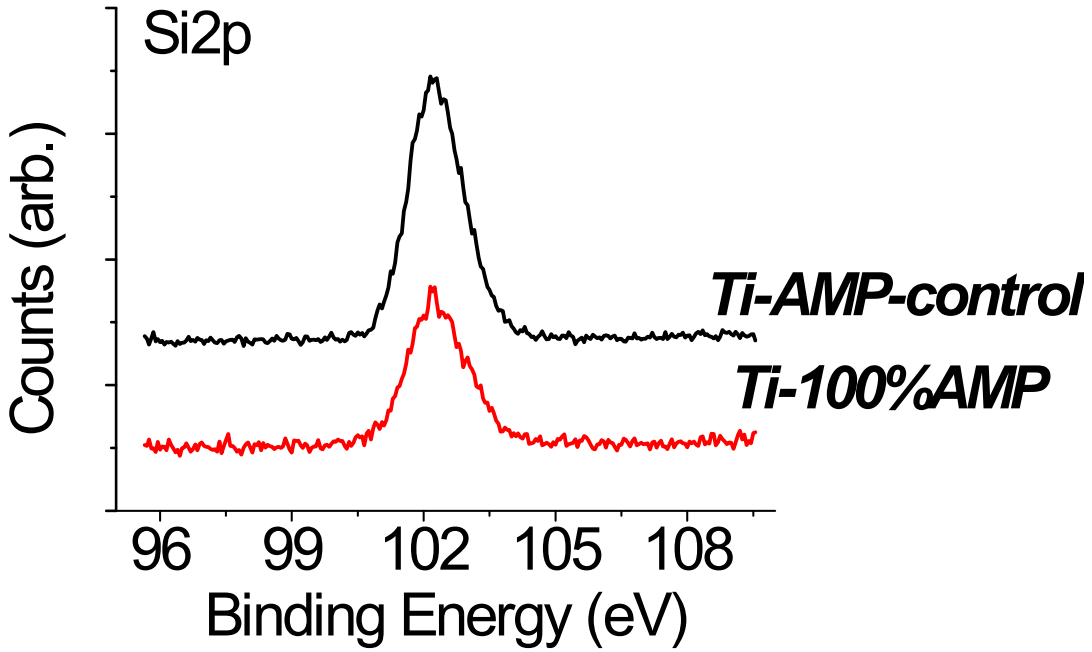


Fig. 4S The live/dead assay of *E. coli* on the indicated samples (the images were got under FITC and TRITC channels, and merged with the NIS software. The green bacteria were live, while the red bacteria were dead).

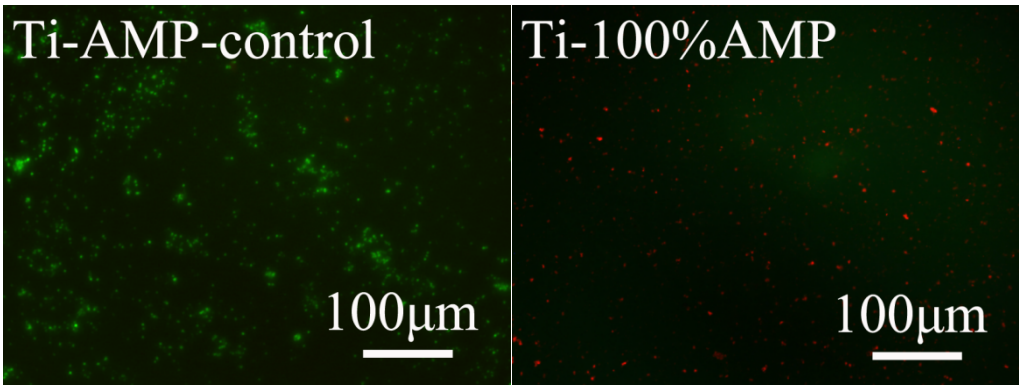
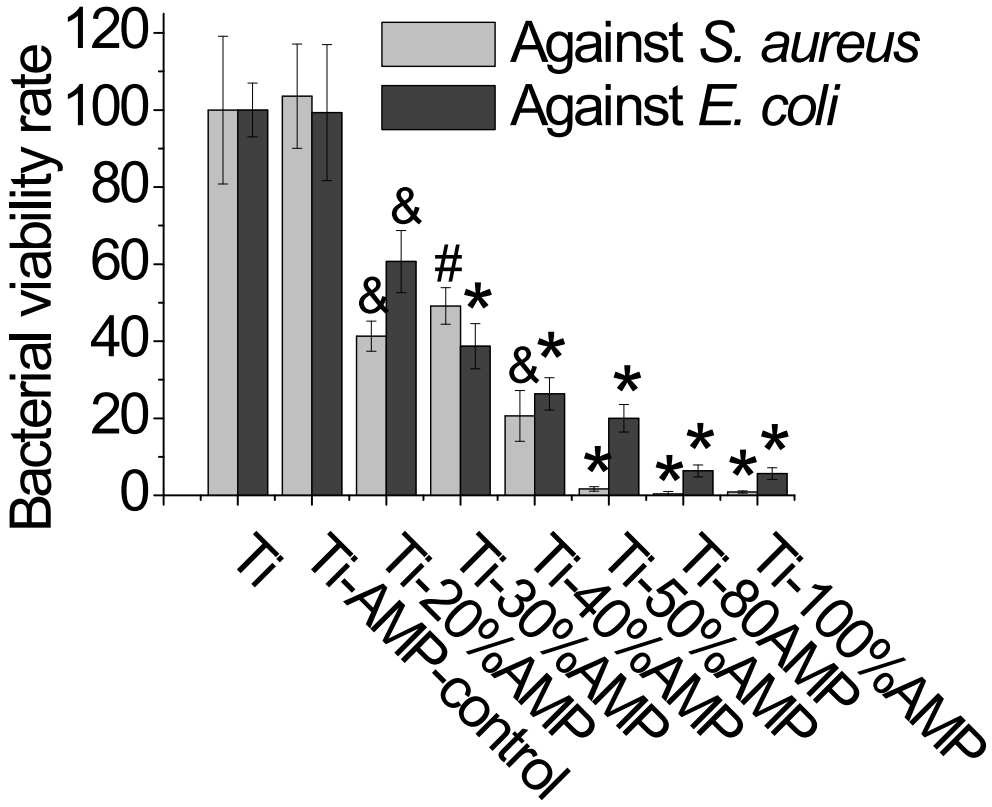


Fig. 5S The antimicrobial activity of the indicated samples against *S. aureus* and *E. coli*. (n=3, # denotes p<0.05, & denotes p<0.01 and * denotes p<0.001 compared to ***Ti***)





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2. S. J. Sofia, V. Premnath and E. W. Merrill, *Macromolecules*, 1998, **31**, 5059-5070.