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

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In this paper, we developed a method for covalent multi-biofunctionalization of titanium surface with mixtures of peptides at any desired ratios using click chemistry. We demonstrated that optimization of the types and ratios of the peptides on titanium surface resulted in excellent antimicrobial activity as well as good biocompatibility.

To improve the biological property of titanium surface, such as biocompatibility or antimicrobial activity, is important for clinical application of titanium based implantable materials, as the defects of these properties would lead to the failure of the surgery, patient disability and morbidity, and even death[1]. Peptides, which could exhibit excellent bioactivity with different amino sequences, are screened by researchers to solve this problem[2-6]. However, to modify the titanium surface with a single peptide is often limited by undesirable effects. For example, to improve the antimicrobial activity of titanium surface could lead to evident cytotoxicity[3, 7]. So how to multi-biofunctionalize the titanium surface with different peptides to achieve proper biological property is of great interest.

There have been many reports on attachment of peptides onto titanium surface. Physical adsorption was a simple method to reach this goal[3, 8]. However, it is closely dependent on the morphology and composition of the surfaces [3], and the burst release as well as the poor orientation of peptide on the surface would decrease the effect [3, 8, 9]. Conventional covalent immobilization, such as amidation reaction between amino and carboxylic acid groups[10-12], would affect the orientation and flexibility of the immobilized peptides and decrease their activities[9]. Also, these methods does not allow for control over the location and number of molecules binding to the substrate[9, 13]. Some other methods, such as integrating peptide with an anchor, were also tried[4, 14, 15]. For example, Mas-Moruno et al tried to use this method to integrate PHSRN and RGD peptides onto titanium surface at the same time. However, these two peptides they used have similar effects to improve the biocompatibility, and it could not change the ratios of the two peptides at desired ratios. Until now, it is still interesting to find new methods which could integrate several kinds of peptides with distinct functions at the same time at desired ratios.

In this paper, we developed a method to multi-biofunctionalize the titanium surface with peptides by immobilizing them with silane coupling agent. Using silane coupling agent is a useful method to integrate peptide onto implant surface[2]. However, conventional method has multistep, which should make it difficult to multi-biofunctionalize the titanium surface[2]. In this research, we first chose two kinds of peptides, HHC36 and RGD, as our model peptides. HHC36 (KRWWKWWRR, abbreviated as AMP) is a kind of short antimicrobial peptide[16]. Compared to other antimicrobial agents, antimicrobial peptides have several unique advantages, including a broad spectrum of antimicrobial activity, a low susceptibility of developing bacterial resistance and a short contact time to induce killing[9, 17, 18]. Besides these advantages, HHC36 peptide also had improved antimicrobial activity[3, 16]. Meanwhile, as the short amino acid sequences, it should have lower immunogenicity compared to traditional antimicrobial peptides and other cationic polymers[16, 19]. RGD peptide, which could specific combine with 11 species of integrin, is sometimes used to increase the biocompatibility of implants[2, 20, 21]. As HHC36 and RGD have different bioactivity, we chose these two peptides to multi-biofunctionalize the titanium surface with high antimicrobial activity as well as good biocompatibility. We integrated azido-PEG12-acid or azido-PEG24-acid onto the peptide's N' terminal by amide bond to introduce azido group in peptide, and prepared a kind of silane coupling agent with alkyl group (alkynyl-PEG-triethoxysilane, abbreviated as *APTS*). Then we integrated the peptides with *APTS* by Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC). As a kind of click chemistry, CuAAC could be processed in aqueous at room temperature at fast rate with high efficiency[22]. In addition, as reported, it could control the orientation of the peptide integrated on the implant surface[9, 13].

The scheme of the experiment was shown in Fig. 1 and the detail of the experiment was shown in supporting information. Briefly, after integrating the *APTS* with AMP or RGD peptides at different ratios by CuAAC, we directly rotary evaporated the click solution and re-dissolved the system into 95%ethanol/5%water (pH=4.6) to hydrolyze the *APTS* for 2 h. Then we dripped the hydrolyzed solution onto titanium surface to hydrolyze the *APTS* for another 2 h to form the film. For the control group, we use the peptide without azido group to replace the peptide-azide in

experimental groups, and other processes were the same. All the experiments were performed at room temperature. The abbreviations of different surfaces were shown in Table 1S.

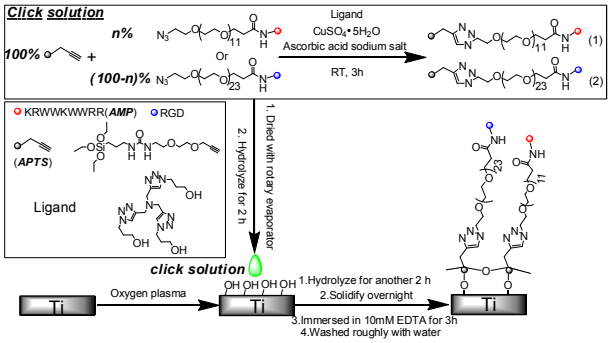

Figure. 1 The scheme of the process to multi-biofunctionalize the titanium surface with mixture of peptides.

Figure. 2 The fluorescent images of the surfaces stain with avidin-FITC: (a) *Ti*; (b) *Ti-biotin-control* and (c) *Ti-biotin*.

To demonstrate that this method could prepare bioactive molecular onto titanium surface, we first used this method to prepare biotin with azido group onto the substrate to replace peptides. Then the biotin in the film was stained with avidin-FITC, and observed with fluorescent microscopy. We used *Ti* and *Ti-biotin-control* (prepared in click solution without copper sulphate, and other processes were the same) as our control groups. The results shown in Fig. 2 showed that only *Ti-biotin* exhibited evident fluorescence, which demonstrated that a probe molecular could be integrated with *APTS* and prepared onto the titanium surface by this method.

We then characterized the morphology and composition of *Ti-100%AMP*. The AFM results shown in Fig. 3 (a) showed that *Ti* sample exhibited bulk morphology, which should be caused by the vapor deposition of titanium particles around 20 nm. After the *APTS* film formed, the morphology of *Ti-100%AMP* changed evidently and the bulk morphology disappeared. We also use ellipsometry to estimate the thickness of molecular (1) (as shown in Fig. 1) on *Ti-100%AMP*, which were about (9.9±0.2) nm. Note that the film thickness derived from ellipsometry is only a rough estimate, as fig. 3 (a) showed that the titanium surface obtained by evaporating titanium particle onto Si (100) was not very smooth, which would impact the results of ellipsometry. We then use the results of ellipsometry and the formula (1) [13, 23] in supporting information to estimate the density of molecular (1) on *Ti-100%AMP*, which was about $2.3\times10^{14}/cm^2$.

The C1s and N1s XPS spectrum of *Ti-100%AMP* shown in Fig. 3(b) could demonstrate the formation of the film furthermore. The C1s signal could be deconvoluted into the peaks of C-C, C-O&C-N and C=O in the results, in which the peak of C-O from OEG on the peptide or *APTS*, and the peak of C-N from triazole were overlapped[24]. The C1s spectrum of *Ti-AMP-control* shown in Fig. 1S showed that there should be only C-O peak from the OEG on APTS, which was much lower than that of *Ti-100%AMP*. The narrow N 1s scans of *Ti-100%AMP* in Fig. 3 (b) could be fitted into three peaks, which assigned to –CONH- and -N-N=N-, respectively. The ratio of the two peaks areas of -N-N=N- was about 1:2, which was consistent with the structure of the triazole.

The XPS results shown in Fig. 2S showed that the Ti2p signals of *Ti-100%AMP* and *Ti-AMP-control* became weaker compared to that of *Ti*, which demonstrated that there was *APTS* film on the titanium surface to shield the Ti signal from the substrate. Fig. 3S showed that the Si 2p signal of the *Ti-100%AMP* was lower than that of *Ti-AMP-control*, which should be caused by the peptide on the *APTS* to shield the Si signal.

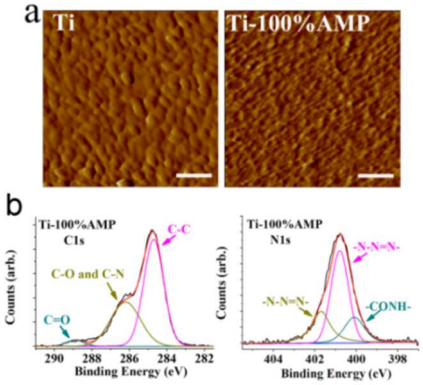

Figure. 3 (a) The AFM images of the indicated sample, the scale in the images denotes 100 nm, and the z scale for both images is 3 nm; (b) the C1s and N1s XPS spectrum of *Ti-100%AMP*.

The results above demonstrated that the bioactive molecular or peptide could be prepared onto pristine titanium surface by this method. In order to multi-biofunctionalize the titanium surface, we then prepared different surfaces by controlling the ratio of AMP and RGD. The contact angle results of these surfaces were shown in Table 2S. It showed that *Ti* had the lowest contact angle. As AMP has hydrophobic amino acid, such as tryptophan, *Ti-100%AMP* has larger contact angle than that of *Ti-100%RGD*. It also showed that *Ti-100%RGD*, *Ti-50%AMP-50%RGD*, *Ti-65%AMP-35%RGD* and *Ti-80%AMP-20%RGD* had similar contact angles, which were arranged from 58.83° to 63.03°. When the concentration of AMP was up to 90%, the contact angle of *Ti-90%AMP-10%RGD* had a sudden rise to 73.43°. It might be caused by the difference of OEG on the peptides. At N' terminal, the RGD had longer OEG (EG24) compared to that of AMP (EG12), which should make it upper on the AMP. It might affect the hydrophilic-hydrophobic property of the surface even at low concentration.

The antimicrobial activity and the biocompatibility of the surfaces with different concentrations of AMP and RGD were shown in Fig. 4. The results shown in Fig. 4(a) showed that without AMP, the *Ti*, *Ti-AMP-control* and *Ti-100%RGD* exhibited no antimicrobial activity. With the increase of AMP ratio on the surface, the antimicrobial activity of the sample increased. *Ti-50%AMP-50%RGD* could kill about 76.76% of *S. aureus* and 57.81% of *E. coli* on it. *Ti-80%AMP-20%RGD* could kill about 97.71% of *S. aureus* and 86.72% of *E. coli* on it. *Ti-90%AMP-10%RGD* and *Ti-100%AMP* could kill about 98.77% and 99.12% of *S. aureus*, as well as 92.03% and 94.24% of *E. coli*, respectively. In addition, the fluorescent images of live/dead assay in Fig. 4S also showed that the bacteria on *Ti-100%AMP* were died and those on *Ti-AMP-control* were alive. Interestingly, as shown in Fig. 5S, when there was no

RGD peptide, the antimicrobial activity of the surface with same concentration of AMP could increase. For example, the *Ti-50%AMP* could kill about 98.34% of *S. aureus* and 80.00% *E. coli* on it, which was much better than that of *Ti-50%AMP-50%RGD*.

The biocompatibility assay shown in Fig. 4(b) showed that the *Ti-100%AMP* exhibited cytotoxicity evidently, and the cells on it were about 80.03% compared to those on *Ti*. With the decrease of AMP and the increase of RGD, the biocompatibility of the samples increased. The cells on *Ti-90%AMP-10%RGD*, *Ti-80%AMP-20%RGD*, *Ti-65%AMP-35%RGD* and *Ti-50%AMP-50%RGD* were about 0.89, 1.02, 1.20 and 1.30 times compared to those on *Ti*, respectively. *Ti-100%RGD* had best biocompatibility, which was about 1.56 times compared to that of *Ti*, and about 1.95 times compared to that of *Ti-100%AMP*. Among these groups, the ideal group should be *Ti-80%AMP-20%RGD*, which had similar biocompatibility to *Ti* and exhibited excellent antimicrobial activity. The *Ti-RGD-control* showed similar biocompatibility to Ti, which illustrated that there should not be RGD on the surface and the *APTS* film would not affect the biocompatibility.

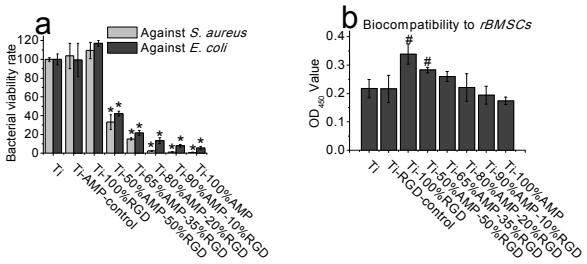

Figure 4. The antimicrobial activity (n=3) and biocompatibility (n=3) of the indicated samples. (# denotes $p<0.05$, and * denotes $p<0.001$ compared to *Ti* sample)

Conclusions

In this paper, we prepared different kinds of peptides onto titanium surface at desired ratios to multi-biofuntionalize the titanium surface. After modified with the peptides HHC36 and RGD at certain ratios, the surface could exhibit excellent antimicrobial activity as well as good biocompatibility.

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Notes and references

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1. M. Geetha, A. K. Singh, R. Asokamani and A. K. Gogia, *Progress in Materials Science*, 2009, **54**, 397-425.
2. S. J. Xiao, M. Textor, N. D. Spencer, M. Wieland, B. Keller and H. Sigrist, *Journal of Materials Science-Materials in Medicine*, 1997, **8**, 867-872.
3. M. Kazemzadeh-Narbat, J. Kindrachuk, K. Duan, H. Jenssen, R. E. W. Hancock and R. Wang, *Biomaterials*, 2010, **31**, 9519-9526.
4. C. Mas-Moruno, R. Fraioli, F. Albericio, J. M. Manero and F. J. Gil, *Acs Applied Materials & Interfaces*, 2014, **6**, 6525-6536.
5. A. W. G. Nijhuis, J. J. J. P. van den Beucken, O. C. Boerman, J. A. Jansen and S. C. G. Leeuwenburgh, *Tissue Engineering Part C-Methods*, 2013, **19**, 610-619.
6. L. Wang, M. Zhao, S. Li, U. J. Erasquin, H. Wang, L. Ren, C. Chen, Y. Wang and C. Cai, *Acs Applied Materials & Interfaces*, 2014, **6**, 8401-8406.
7. L. Wang, U. J. Erasquin, M. Zhao, L. Ren, M. Y. Zhang, G. J. Cheng, Y. Wang and C. Cai, *Acs Applied Materials & Interfaces*, 2011, **3**, 2885-2894.
8. M. Ma, M. Kazemzadeh-Narbat, Y. Hui, S. Lu, C. Ding, D. D. Y. Chen, R. E. W. Hancock and R. Wang, *Journal of Biomedical Materials Research Part A*, 2012, **100A**, 278-285.
9. F. Costa, I. F. Carvalho, R. C. Montelaro, P. Gomes and M. C. L. Martins, *Acta biomaterialia*, 2011, **7**, 1431-1440.
10. M. D. Steven and J. H. Hotchkiss, *Journal of Applied Polymer Science*, 2008, **110**, 2665-2670.
11. M. D. P. Willcox, E. B. H. Hume, Y. Aliwarga, N. Kumar and N. Cole, *Journal of Applied Microbiology*, 2008, **105**, 1817-1825.
12. M. S. Mannoor, S. Zhang, A. J. Link and M. C. McAlpine, *Proceedings of the National Academy of Sciences of the United States of America*, 2010, **107**, 19207-19212.
13. Y. Li, C. M. Santos, A. Kumar, M. Zhao, A. I. Lopez, G. Qin, A. M. McDermott and C. Cai, *Chemistry-a European Journal*, 2011, **17**, 2656-2665.
14. R. Beutner, J. Michael, B. Schwenzer and D. Scharnweber, *J. R. Soc. Interface*, 2010, **7**, S93-S105.
15. J. Michael, L. Schoenzart, I. Israel, R. Beutner, D. Scharnweber, H. Worch, U. Hempel and B. Schwenzer, *Bioconjugate Chemistry*, 2009, **20**, 710-718.
16. K. Hilpert, M. Elliott, H. Jenssen, J. Kindrachuk, C. D. Fjell, J. Koerner, D. F. H. Winkler, L. L. Weaver, P. Henklein, A. S. Ulrich, S. H. Y. Chiang, S. W. Farmer, N. Pante, R. Volkmer and R. E. W. Hancock, *Chemistry & Biology*, 2009, **16**, 58-69.
17. M. Chromek, Z. Slamova, P. Bergman, L. Kovacs, L. Podracka, I. Ehren, T. Hokfelt, G. H. Gudmundsson, R. L. Gallo, B. Agerberth and A. Brauner, *Nat. Med.*, 2006, **12**, 636-641.
18. F. H. Login, S. Balmand, A. Vallier, C. Vincent-Monegat, A. Vigneron, M. Weiss-Gayet, D. Rochat and A. Heddi, *Science*, 2011, **334**, 362-365.
19. M. Malmsten, *Soft Matter*, 2011, **7**, 8725-8736.
20. G. Vidal, T. Blanchi, A. J. Mieszawska, R. Calabrese, C. Rossi, P. Vigneron, J.-L. Duval, D. L. Kaplan and C. Egles, *Acta biomaterialia*, 2013, **9**, 4935-4943.
21. U. Hersel, C. Dahmen and H. Kessler, *Biomaterials*, 2003, **24**, 4385-4415.
22. V. V. Rostovtsev, L. G. Green, V. V. Fokin and K. B. Sharpless, *Angewandte Chemie-International Edition*, 2002, **41**, 2596-+.
23. S. J. Sofia, V. Premnath and E. W. Merrill, *Macromolecules*, 1998, **31**, 5059-5070.
24. Y. Li, M. Zhao, J. Wang, K. Liu and C. Cai, *Langmuir*, 2011, **27**, 4848-4856.