博士論文の内容の要旨

論文題目

Study on Antibacterial Effects of Phospholipid Copolymer Film with Cross-Linked Structure

（架橋構造をもつリン脂質ポリマー膜への抗菌性の研究）

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本文

1 Introduction

Bacterial adhesion and biofilm formation lead to serious problems in many areas, including sanitary items, food industry, marine constructions and medical devices. Biofilm formation is a multi-step process, numerous biomolecules (mainly consisting of protein and polysaccharide) usually predominate the surface prior to bacterial adhesion and form a so-called “conditioning film”. Planktonic bacteria then transport onto a solid surface and perform a reversible attachment through gravitational force, van der Waals force, electrostatic attraction, and hydrophobic interaction. Subsequently, molecular and/or cellular interaction at the proximity of bacterial cell and the solid surface turn the bacterial adhesion into the irreversible stage. Bacterial surface proteins play significant roles in this process. Surfaces that preventing protein adsorption turn out to be an ideal solution for the biofilm associated problems.

Phospholipid polymers emerged to be a useful material in antifouling modification. Among them, 2-methacryloyloxyethyl phosphorylcholine (MPC) copolymers are known to have outstanding performance,[1] and the bacterial anti-adhesiveness of the MPC copolymer modified surface has been demonstrated recently. Even though the MPC copolymer has been used for years, most of the successful example in both laboratorial studies and industrial applications were achieved through empirical method rather than full understanding of the fundamental principle and chemistry of this materials.

This research looked into the antibacterial property of phospholipid copolymer consisted of MPC, 3-methacryloxypropyl trimethoxysilane (MPTMSi) and 3-(methacryloyloxy) propyl-tris(trimethylsilyloxy) silane (MPTSSi), this copolymer was denoted as PMMMSi in the following. Mechanical property was thought to affect the bacterial adhesion.[2] Consequently, mechanical property of PMMMSi thin-films and their influences on bacterial adhesion were investigated as the first part of this research. Bacterial adhesion behavior (strength) on the PMMMSi thin-films was studied using a customer-designed microfluidic chip. Effects of protein condition film on bacteria adhesion was simulated by fibrinogen (Fg) adsorption layer. Finally, in order to improve the antibacterial properties, antimicrobial peptide was introduced onto the PMMMSi thin-film which enabled the surface to kill the approaching bacteria and release their corpse.

2 Influence of Mechanical Properties of Crosslinked Phospholipid Copolymer Thin-Films on Bacterial Anti-Adhesiveness

Chapter 2 describes fabrication of thickness and stiffness tunable PMMMSi thin-films.

PMMMSi was synthesized according to previous study.[3] Briefly. Initiator (2.5 mM 2,2'-Azobis(2-methylpropionitrile)) and monomers (0.3 M MPC, 0.1 M MPTSSi and 0.1 M MPTMSi) was dissolved in methanol and reacted at 65°C with stirring for 6 hours in Ar atmosphere. Product was collected from precipitation in acetone/ethanol (20/1, v/v) mixture solution. In order to coat the PMMMSi onto O_2 plasma treated glass or SiO_2 substrate, the polymer solution (0.01~0.5 wt% in methanol, coating samples corresponding to each concentration was denoted as C-0.01, C-0.05, C-0.10, C-0.20, and C-0.50, respectively) was mixed with 0.1M acetic acid aqueous solution (10/1, v/v) that used as a catalyst. a 1-h dip coating followed by vacuum drying and heating were performed to facilitate the silane coupling reaction. Thickness of PMMMSi coating film was measured by ellipsometry in both air and water environment. Surface hydrophilicity was examined by air bubble contact angle in water. Surface chemical composition was obtained using X-ray photoelectron spectrum (XPS). Surface topography and stiffness were obtained through AFM scanning in both air and aqueous environment.

In the bacterial adhesion assay, coating samples (Ø13 mm cover slide) were stuck to the bottom of a 9 cm Petri dish using PDMS. 20 mL bacterial medium (10^7 cells/mL in TSB or 10^8 cells/mL in M9 medium) was added into the dish. After a certain incubation time, the bacterial medium was discarded and the samples were rinsed with PBS. For optical microscope observation, the sample was then fixed with 0.2 % PFA/PBS solution for 1 hour before methylene blue (MB) according to Loeffler.

The contents of the obtained PMMMSi determined to be MPC:MPTSSi:MPTMSi = 68%:17%:15% by ^{1}H NMR. The number-averaged molecular weight (M_n) and the polydispersity index (M_w/M_n, where Mw is the weight-averaged molecular weight) estimated by GPC were 1.7×105 and 1.74, respectively. The atomic ratios of phosphorous versus carbon (P/C) and nitrogen versus carbon (N/C) did not display significant deviation among samples. Thus, changing the PMMMSi concentration in the coating solution did not affect the chemical status of the resulted coating films. The contact angle of bubble of each surface was as large as ca. 160° which indicates the super-hydrophilic feature of all the PMMMSi thin-films.

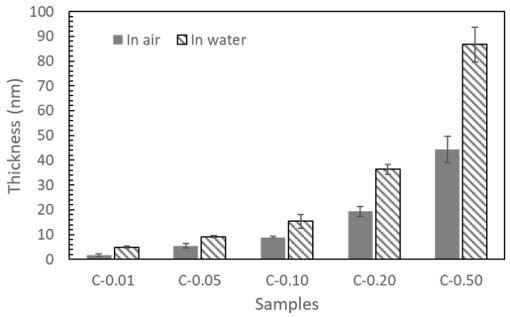

Figure 1 PMMMSi thin-films thickness measurement in air and water by ellipsometry measurement.

Thickness of the obtained PMMMSi thin-film was controlled as a function of the PMMMSi concentration in the coating solution, that is, higher PMMMSi concentration resulted in a thicker coating film.

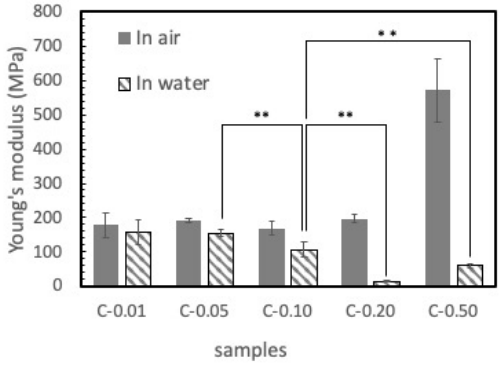

Figure 2 Young's modulus of PMMMSi thin-films in air and water calculated with classical Hertzian model. ** indicate $p < 0.01$ (n=3

Figure 2 shows the sample's modulus of C-0.01, C-0.05, C-0.10, and C-0.20 which did not vary significantly, while C-0.50 displayed an obvious higher modulus. When the samples were soaked in PBS, sample's modulus decreased in all the samples, which probably due to the swelling of the PMMMSi thin-films in aqueous media, as shown in **Figure 2.3**. In the combination of **Figure 2.3** and **Figure 2.5**, it can be learned that with the increase in the coating thickness, sample's modulus decreased from C-0.01 to C-0.20 in PBS. The sample's modulus value of C-0.50 decreased to a value between those of C-0.10 and C-0.20 because sample's modulus of C-0.50 in the air was higher than the other thin-film samples.

Bacterial adhesion behavior was investigated on the PMMMSi coating films It is found that the thicker and softer PMMMSi thin-films performed better than the stiffer ones in terms of inhibiting *S. aureus* adhesion during

EPS-production in the TSB medium. Under non-EPS-producing conditions (M9 medium), the PMMMSi thin-films showed a satisfactory anti-adhesion property that lasted over a period of 24 h. The PMMMSi thin-films prevented the Gram-negative *P. aeruginosa* adhesion; however, the influence of mechanical property was not clear. These results suggest that not only protein-repellence but also mechanical property plays an important role in bacterial anti-adhesiveness.

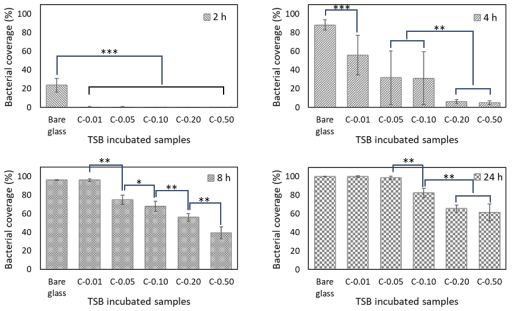

Figure 3 Statistical analysis of *S. aureus* coverage development, * indicate $p < 0.05$, ** indicate $p < 0.01$ (n=5)

On the basis of these findings, the PMMMSi thin-films with controllable thickness and mechanical property would be of instructive significance to the investigation of bacterial adhesion behaviors and the development of bacterial anti-adhesive surface, like the design of the indwelling medical devices.

3 Microfluidic Shear Device for Quantitative Investigation of Bacterial Anti-Adhesiveness of Phospholipid Copolymer Thin-films

Hydrodynamic shear assay is useful in approaching the cell-surface interactions.[4] Chapter 3 describes an original designed microfluidic chip which was used to study the bacterial adhesion strength (**Figure 4**). The microfluidic chip was built via the 3D printing mold and rapid prototyping technique with polydimethylsiloxane (PDMS). After O_2 plasma treatment, the molded PDMS can be bound onto transparent glass. The whole fluidic device was constructed through connecting the chip with a syringe pump system

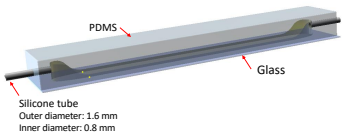

Figure 4 Illustration of the fabricated microfluidic chip.

With this device, *Staphylococcus aureus* initial adhesion strength on PMMMSi thin-films of different thickness (20 nm and 40 nm, denoted as C-20 and C-40) were investigated. The method described in **Figure 5**. Bacterial adhesion strength was analyzed via the detachment effect of fluidic flow of known shear stress.

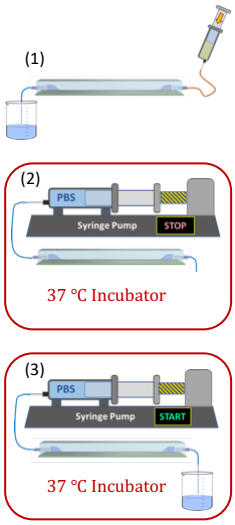

Figure 5 Bacterial adhesion (1) bacterial medium was then manually ejected into the chip; (2) the chip was moved into a incubator in where bacteria was incubated at 37 °C for 2 h under static condition; (3) PBS was pumped through the chip at a setting speed.

The deviation of the bacterial adhesion strength among these coating films with different thickness were revealed by the controllable shear stress. Phospholipid copolymer reduced the adhesion of *S. aureus* and the thicker film had better bacterial anti-adhesiveness.

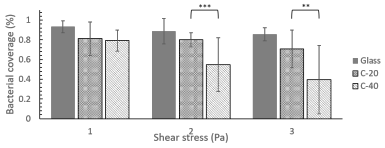

Figure 6 Statistical analysis of the fraction of adhered bacterial under shear rinse. Fraction of adhered bacterial represents the ratio of bacterial coverage on the surface after and before shear rinse** *indicates $p<0.01$ and *** indicates $p<0.001$*

4 Antimicrobial Peptide Immobilization Improved Antibacterial Property of Crosslinking Phospholipid Copolymer Film

The immobilization of antimicrobial peptide on the anti-adhesive surface can enhance its antibacterial property.[5] Chapter 4 aims to

introduce AMP onto PMMMSi thin-film. A two-steps method was presented. The PMMMSi was firstly coated on the substrate as described in the earlier chapters and aminated by 3-Aminopropyl)triethoxy silane (ATPSi), consequently, AMP was able graft onto the surface through a glutaraldehyde bridge. The bacterial anti-adhesion and contact killing functions was verified using *S. aureus*.

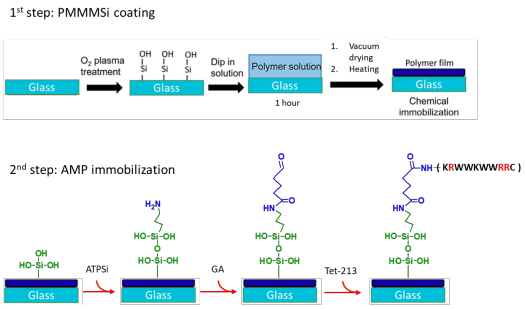

Figure 7 microscopic images of bacteria adhered on the surfaces of glass (a-c), C-20 (d-f) and C-40 (g-i) after fluidic PBS rinse with shear stress of 1 Pa (a, d and g), 2 Pa (b, e and h) and 3 Pa (c, f and i), respectively; bottom graph shows

The successful immobilization of AMP on the surface could be confirmed from the XPS spectra as the additional peak area represented the immobilized AMP on the PMMMSi thin-film surface (**Figure 8**).

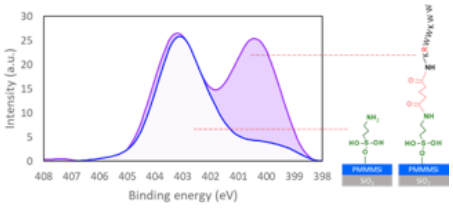

Figure 8 High-resolution of XPS spectra of the N 1s of ATPSi grafted PMMMSi and AMP immobilized PMMMSi surfaces

the contact killing effect of the AMP immobilized surface was tested with S. aureus. (Figure 9)The test sample was incubated in S. aureus/PBS suspension (107 cell/mL) for 24 h and LIVE/DEAD staining was applied to the bacteria that adhered on the sample surface. S. aureus is a strong biofilm formation species. After 24 h incubation, vast S. aureus cells were found to adhered on the bare glass. Almost all the adhered cells are alive (green dots in Figure 9). The adhesion was stable even rinse was applied, S. aureus was rarely detached from glass surface (Figure 9b). On the other hand, PMMMSi reduced the S. aureus adhesion strength as the rinse process detached numerous adhered bacteria (Figure 9c, d). Figure 9e, f show the contact killing effect of AMP immobilized PMMMSi thin-film where dead S. aureus cells (red dots) were observed. Moreover, the killed bacteira can be removed by rinse. the functionalized surface display both bacterial anti-adhesive and bactericidal properties.

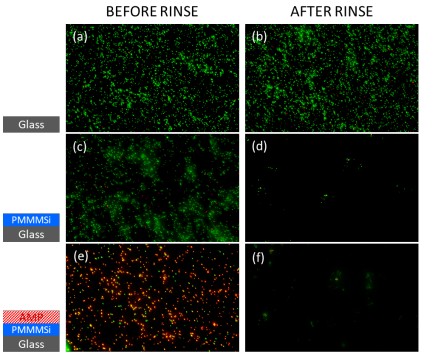

Figure 9 LIVE/DEAD staining applied to the surfaces of Glass, PMMMSi, AMP/PMMMSi, incubated with *S. aureus* for 24 h

5 Conclusion

The antibacterial property of crosslinked phospholipid copolymer thin-film was firstly investigated from the standpoint of physical properties. Results demonstrated that the thicker and softer PMMMSi thin-films have better bacterial anti-adhesiveness. Bacterial adhesion strength was quantitatively studied via a customer-designed microfluidic platform. This platform interpreted the anti-adhesiveness of PMMMSi thin-films into numerical value. Finally, AMP immobilization turned the PMMMSi thin-film into bifunctional surface that possessed both anti-adhesive and bactericidal properties. These findings would be of instructive significance to the investigation of bacterial adhesion behaviors and the development of antibacterial surface, like the design of the indwelling medical devices.

Reference

(1) Y Xu, *et al.* **Ann Biomed Eng**, 2010 38(6): p. 1938-53.224

(2) K.W. Kolewe, *et al.* **ACS Appl Mater Interfaces,** 2018. 10(3): p. 2275-2281.

(3) Nagahashi, K., *et al.* **Colloids and Surfaces B-Biointerfaces,** 2015 134 p. 384-391

(4) H. Lu *et al.* **Analytical chemistry** 2004 76: p. 5257-5264

(5) J Chen *et al.* **ACS Biomate Sci & Eng**. 2018, 5(2): p. 1034-1044