博士論文（要約）

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）

リク　シュウ

陸　　洲

1 Introduction

Bacterial adhesion and subsequent biofilm formation lead to serious problems in many areas, including sanitary items[1], food industry[2], marine constructions[3,4], and medical devices[5,6]. In particular, biofilm-induced infections in indwelling medical devices have been responsible for high mortality around the world, which warrants further investigation[7,8]. The 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[9,10]. Subsequently, molecular and/or cellular interaction at the proximity of bacterial cell and the solid surface turn the bacterial adhesion into the irreversible stage[11,12]. In this process, bacterial surface proteins play significant roles[13-15]. With time extension, bacterial cells accumulate on the primary adhered cells, and a multicellular biofilm was consequently formed. Bacterial cell-cell connections occur in the biofilm, which was established mainly through protein-based binding[16] as well as the secretion of extracellular polysaccharide (EPS)[17-19]. It is reported that the ability to construct and maintain a structural multicellular biofilm depends critically on the production of EPS[19], but the adhesion strength on the material surface is determined by the primary-adhered bacteria[20].

To effectively solve the biofilm-associated problems, it is necessary to prevent the protein-surface interaction during the primary adhesion of bacteria. For reducing protein adsorption on materials surfaces, various materials which have an ability of anti-protein adsorption, are applied. Among them, 2-methacryloyloxyethyl phosphorylcholine (MPC) copolymers are known to have outstanding performance[21], and the bacterial anti-adhesiveness of the modified MPC copolymer surface has been demonstrated[22-26]. Moreover, it was reported that not only the surface properties but also mechanical properties control the prokaryotic and eukaryotic cell behaviors[27-30]. Kolewe et al. reported that stiffer polyethylene glycol (PEG) hydrogel was more likely to attract bacterial adhesion[31,32]. However, a sub mm-sized hydrogel is not a suitable material for surface

modification on a medical device, like the inner wall of a thin catheter. Considering the applications in medical devices, we focused on the effects of the mechanical properties of the nm-sized polymer coating thin-film on the bacterial adhesion.

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. Physical properties of PMMMSi thin-films and their influences on bacterial adhesion was 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

PMMMSi was synthesized through free radical polymerization using α,α′-Azobisisobutyronitrile (AIBN) as initiator. Bacterial anti-adhesiveness of PMMMSi thin-film was investigated. The MPC unit is the main antibiofouling content, the MPTMSi unit with silane coupling sites covalently bound the polymer chains onto the hydroxylic substrate as well as crosslinks the adjacent polymer chains into a network structure, and the MPTSSi unit functions to maintain the homogeneity of the copolymer thin-films by hydrophobic interactions[33]. The contents of the obtained PMMMSi was determined to be MPC:MPTSSi:MPTMSi = 68:17:15 (mol%) by ^{1}H NMR, which was close to the feeding monomer ratio. This copolymer has a sufficient MPC component that can retain the antibiofouling feature as well as includes enough silane coupling sites for stable coating. PMMMSi can be easily modified onto the hydroxylic

substrate such as glass, the thickness and stiffness were modulated by changing the PMMMSi concentration in the coating solution and the crosslinking of each polymer chain[33].

Table 2.1 Characteristics of PMMMSi thin-film

Sample	Thickness in air/in water (nm)[a]	[P]/[C][b]	[N]/[C][b]	Static air contact angle (°)	RMS roughness in air (nm)[c]	RMS roughness in PBS (nm)[c]
C-0.01	$1.6 \pm 0.5/4.9 \pm 0.4$	0.104	0.089	164.9 ± 6.3	0.3 ± 0.0	0.5 ± 0.3
C-0.05	$5.5 \pm 1.0/9.6 \pm 0.3$	0.155	0.107	163.8 ± 3.8	0.8 ± 0.1	0.8 ± 0.1
C-0.10	$8.9 \pm 0.5/15.4 \pm 2.8$	0.120	0.109	165.5 ± 5.6	0.5 ± 0.0	0.6 ± 0.0
C-0.20	$19.3 \pm 2.0/36.4 \pm 2.0$	0.133	0.103	166.2 ± 5.9	0.5 ± 0.0	1.2 ± 0.7
C-0.50	$44.5 \pm 5.3/86.7 \pm 7.0$	0.113	0.106	163.7 ± 4.2	1.5 ± 1.0	2.3 ± 0.8

[a] Measured by ellipsometry

[b] Measured under vacuum condition

[c] Calculated on the basis of the topography measured by AFM

Table 2.1 summarizes the characteristics of the PMMMSi thin-films prepared by changing the polymer concentrations (0.01 wt%, 0.05 wt%, 0.10 wt%, 0.20 wt%, and 0.50 wt%; 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). The elemental composition of the surface was examined by XPS. Nitrogen and phosphorous are the characteristic elements of the phosphoryl choline moiety of the MPC unit. The elemental ratios of phosphorous versus carbon (P/C) and nitrogen versus carbon (N/C) were calculated based on the integration of the peaks. Changing the PMMMSi concentration in the coating solution did not affect the chemical status of the resulting thin-films. Surface wettability by air bubble contact angle was subsequently measured in water. The contact angle of the air bubble on each surface was as large as ca.160°, which indicates the hydrophilic feature of the PMMMSi thin-film, and no significant deviation was observed among the samples. Root mean square (RMS) roughness of the samples of C-0.01 to C-0.20 in air and PBS, measured by AFM were small, but that of C-0.50 was a little larger.

The thickness of the PMMMSi thin-film in air and distilled water were obtained by the spectroscopic ellipsometry measurement (**Table 2.1** and **Figure 2.1**). The thickness of the PMMMSi thin-film increased as the PMMMSi concentration in the coating solution increased.

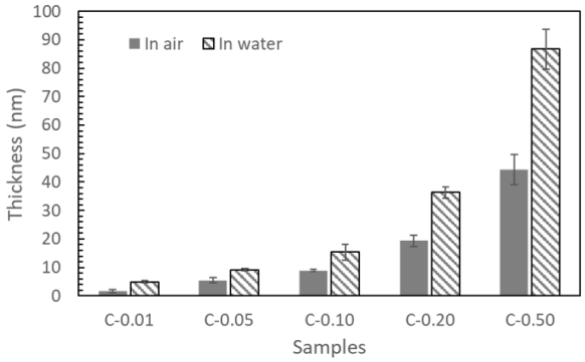

Figure 2.1 PMMMSi thin-films thickness measurement in air and water by ellipsometry measurement.

Young's modulus measurements were also carried out in dry and wet states. **Figure 2.2** 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. 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.

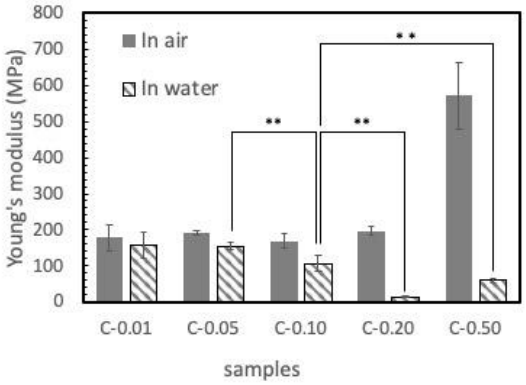

Figure 2.2 Young's modulus of PMMMSi thin-films in air and water calculated with classical Hertzian model. ** indicate $p < 0.01$ (n=3)

Bacterial adhesion behaviors on the PMMMSi thin-films was subsequently investigated. The PMMMSi-coated glass slides were incubated with the *Staphylococcus aureus* in nutrition rich

TSB medium. The samples were washed with PBS before the observation.

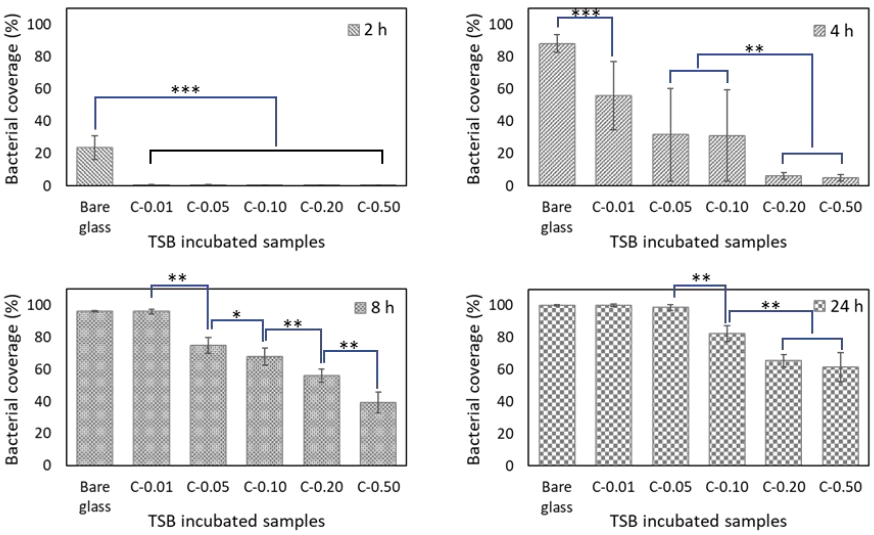

Figure 2.3 Statistical analysis of *S. aureus* coverage development, * indicate $p < 0.05$, ** indicate $p < 0.01$ (n=5)

Figure 2.3 shows that *S. aureus* rapidly colonized the bare glass surface in the first 2 h, while few bacteria were found to adhere to the PMMMSi thin-film surfaces. With the extension of the incubation time, the *S. aureus* biofilm developed quickly on the bare glass surface and dominated the entire area within 4 h. At this time point, the surface of C-0.01 was also colonized by *S. aureus*, but the thicker and softer PMMMSi thin-films of C-0.20 and C-0.50 had less *S. aureus* adhesion. After 8 h, *S. aureus* biofilm completely covered the surface of C-0.01, while fewer *S. aureus* adhered to C-0.20 and C-0.50, even after 24 h incubation. *S. aureus* is an extracellular-polysaccharide (EPS) produced species, EPS determines the characteristics of the top surface of the bacterial cells that control the bacterial initial adhesion. Bacteria probably use different mechanical to finish the adhesion under the environment with or without nutrient supply[34]. EPS coat not only helps *S. aureus* stick to the surface but are also involved in the construction of EPS[17,18]. EPS connected the adjustment *S. aureus* cells together when a number of bacterial cells accumulated on the surface due to gravitational force.

From these investigations, the bacteria adhesion behavior on protein non-adsorbed

PMMMSi thin-films of a series of thicknesses and stiffness are summarized as follows: thicker and softer PMMMSi thin-films exhibit better anti-adhesiveness toward *S. aureus*. It is still unclear how bacteria recognize the mechanical properties of a surface; however, the bacterial cell surface appendages, like the flagella[35], probably served as a mechanical sensor. We hope that these findings would be helpful in eradicating the bacteria-associated issues.

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

In this section, a hydrodynamic shear assay based on a customer-designed microfluidic chip to study the bacterial adhesion behaviors was proposed. Microfluidic devices have emerged as a mutual technology and it is shown that microfluidic shear stress also offered unique advantages such as improving throughput, resolution, and fidelity in antibacterial surface evaluation[36-39]. 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 that enable the use of real-time observation (**Figure 3.1**). This strategy imparts benefits by allowing a fast turnaround time for device design and fabrication[40]. Particularly, microfluidic chip in this study provided a large dimension (4.2 mm in width (w), 0.5 mm in height (h) and 42 mm in length (L)) that ensured the broad observation area while guaranteed laminar flow even at very high linear fluid velocities. This is important because: 1) the susceptibility of bacterial adhesion is a result of statistical analysis which needed substantial data that collected from a wide area of the surface in question; and 2) the bacteria usually perform strong adhesion on material surface which needs strong shear to detach. 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.

As a rectangular channel whose width was much larger than its height, the effects of the side walls on the fluidic pattern is negligible, and the fluent thus can be considered to run in between

a pair of parallel plate (the top and bottom walls). the shear stress corresponding to a given flow rate can be calculated from *Eq. 3.1*. (the water molecules at the proximity of the channel walls was considered not moved along the main fluent body (non-slip condition)).

$$\tau = \frac{6\mu Q}{h^2 w} \qquad Eq.\,3.1$$

where μ is the viscosity of the fluid, Q is the flow rate and h and w are the height and width of the channel.

Microfluidic chip device was able to modulate the retraction strength (shear stress) upon the adhered bacterial cell, and the bacterial adhesion strength was quantitatively analyzed under this condition. Bacteria was firstly introduced into the fluidic channel and allowed to attach to the bottom under static condition. Subsequently, shear stress was applied to the adhered bacteria using a syringe pump. When shear stress was 1 Pa, the bacteria still adhered on all the surfaces. The bacterial anti-adhesiveness in C-20 and C-40 surfaces were not shown as well as the non-coated glass surface. Increase the shear stress lead to the removal of the attached bacteria. Under 2 Pa shear stress, a number of attached bacteria were detached from C-40 surface while relative smaller amount of bacteria were moved away from C-20 surface despite the modification of phospholipid copolymer thin-film. The equal shear stress resulted in variated retraction effects among C-20 and C-40 surfaces demonstrates the variated bacterial anti-adhesiveness of the phospholipid copolymer films of different thickness.

Further increased the shear stress as large as 3 Pa for the rinse caused greater detachment of the adhered bacteria from the phospholipid copolymer films. However, the deviation between C-20 and C-40 was weaken ($p < 0.01$). It is rational to speculate that approximately 100% of adhered bacteria would be detached from both the C-20 and C-40 surfaces under a much greater shear stress.

These results demonstrate that phospholipid copolymer reduced the adhesion of *S. aureus* and the thicker film had better bacterial anti-adhesiveness. The microfluidic device provides an easy and reliable way for the estimation of bacterial adhesion susceptibility of a variety surfaces under multiple conditions which was supposed to serve as a useful tool in the investigation of

bacterial adhesion behaviors and antibacterial surface development.

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

An anti-adhesive surface reduces bacterial adhesion rather than completely blocks the bacterial attachment., It is necessary to incorporate the bactericidal agents into the anti-adhesive modification. Here, a two-steps technique of fabricating a bacterial anti-adhesive and bactericidal bifunctional surface is described. In the first step, PMMMSi was coated on glass. the polymer film was aminated by 3-Aminopropyl)triethoxy silane (ATPSi) which enable AMP to bind to the surface through a glutaraldehyde (GA) bridge[41]. Immobilization of AMP (Tet-213, KRWWKWWRRC) enhance the antibacterial property and enable the surface to against a wide variety of bacteria, they are nontoxic to humans, and bacteria hardly acquire resistance[42,43].

Subsequently, the contact killing effect of the AMP immobilized surface was tested with *S. aureus*. The test sample was incubated in *S. aureus*/PBS suspension (10_7 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. When PMMMSi was coated onto the surface, the bacterial adhesion strength was weakened. Before rinse, many bacteria can still attach to the PMMMSi surface, but the rinse removed most of the attached *S. aureus* cells from the PMMMSi surface. On the AMP immobilized PMMMSi thin-film, *S. aureus* was killed on the surface which demonstrated the contact killing effect. Moreover, the dead bacteria can be released from the surface

5 Conclusion

In 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. ÖZcan S, ÇAliŞ AÇIkbaŞ N, AÇIkbaŞ G. Formation of Antibacterial Effect on Ceramic Tile Surfaces. *Anadolu Univ J of Sci and Technology A– Appl Sci and Eng* 2017; **18**(1): 122-.
2. Liu Y, Jiang Y, Zhu J, Huang J, Zhang H. Inhibition of bacterial adhesion and biofilm formation of sulfonated chitosan against *Pseudomonas aeruginosa*. *Carbohydrate Polymers* 2019; **206**: 412-9.
3. Chen SQ, Wang P, Zhang D. Corrosion behavior of copper under biofilm of sulfate-reducing bacteria. *Corrosion Sci* 2014; **87**: 407-15.
4. Wang P, Lu Z, Zhang D. Slippery liquid-infused porous surfaces fabricated on aluminum as a barrier to corrosion induced by sulfate reducing bacteria. *Corrosion Sci* 2015; **93**: 159-66.
5. Adlhart C, Verran J, Azevedo NF, et al. Surface modifications for antimicrobial effects in the healthcare setting: a critical overview. *J Hosp Infect* 2018: 1-11.
6. Lebeaux D, Ghigo JM, Beloin C. Biofilm-related infections: bridging the gap between clinical management and fundamental aspects of recalcitrance toward antibiotics. *Microbiology and molecular biology reviews : MMBR* 2014; **78**(3): 510-43.
7. Vertes A, Hitchins V, Phillips KS. Analytical challenges of microbial biofilms on medical devices *Anal Chem* 2012; **84**(9): 3858-66.
8. Klevens RM, Jonathan RE, Chesley L. Richards, Jr., et al. Estimating Health Care-Associated Infections and Deaths in U.S. Hospitals, 2002. *Public Health Reports* 2007; **122**(2): 160-6.
9. Hori K, Matsumoto S. Bacterial adhesion: From mechanism to control. *Biochemical Engineering Journal* 2010; **48**(3): 424-34.
10. Kim SE, Zhang C, Advincula AA, Baer E, Pokorski JK. Protein and bacterial antifouling behavior of melt-coextruded nanofiber mats. *ACS Appl Mater Interfaces* 2016; **8**(14): 8928-38.
11. Hermansson M. The DLVO Theory in Microbial Adhesion. *Colloids Surf B Biointerfaces* 1999; **14**(1): 105-19.
12. Katsikogianni M, Missirlis YF. Concise Review of Mechanisms of Bacterial Adhesion to Biomaterials and of Techniques Used in Estimating Bacteria-Material Interactions. *European cells and materials* 2004; **8**: 37-57.
13. Cucarella C, Solano C, Valle J, Amorena B, Lasa I, Penades JR. Bap, a *Staphylococcus aureus*

Surface Protein Involved in Biofilm Formation. *J Bacteriol* 2001; **183**(9): 2888-96.
14. Lasa I, Penades JR. Bap: a Family of Surface Proteins Involved in Biofilm Formation. *Research in Microbiology* 2006; **157**(2): 99-107.
15. Chagnot C, Zorgani MA, Astruc T, Desvaux M. Proteinaceous determinants of surface colonization in bacteria: bacterial adhesion and biofilm formation from a protein secretion perspective. *Frontiers in microbiology* 2013; **4**: 303.
16. Speziale P, Pietrocola G, Foster TJ, Geoghegan JA. Protein-based biofilm matrices in Staphylococci. *Frontiers in Cellular and Infection Microbiology* 2014; **4**: 1-10.
17. Formosa-Dague C, Feuillie C, Beaussart A, et al. Sticky matrix: Adhesion mechanism of the staphylococcal polysaccharide intercellular adhesin. *ACS Nano* 2016; **10**(3): 3443-52.
18. Limoli DH, Jones CJ, Wozniak DJ. Bacterial extracellular polysaccharides in biofilm formation and function. *Microbiology Spectrum* 2015; **3**(3): 1-30.
19. Chew SC, Yam JKH, Matysik A, et al. Matrix polysaccharides and SiaD diguanylate cyclase alter community structure and competitiveness of *Pseudomonas aeruginosa* during dual-species biofilm development with *Staphylococcus aureus*. *American Society for Microbiology* 2018; **9**(6): e00585-18.
20. Busscher HJ, Bos R, Mei HCvd. Initial Microbial Adhesion Is a Determinant for the Strength of Biofilm Adhesion. *FEMS Microbiology Letters* 1995; **128**: 229-34.
21. Xu Y, Takai M, Ishihara K. Phospholipid Polymer Biointerfaces for Lab-on-a-Chip Devices. *Annals of Biomedical Engineering* 2010; **38**(6): 1938-53.
22. Wang BL, Jin TW, Han YM, et al. Bio-inspired terpolymers containing dopamine, cations and MPC: a versatile platform to construct a recycle antibacterial and antifouling surface. *J Mat Chem B* 2015; **3**(27): 5501-10.
23. Zhang N, Zhang K, Melo MAS, et al. Effects of long-term water-aging on novel anti-biofilm and protein-repellent dental composite. *International Journal of Molecular Sciences* 2017; **18**(1): 1-15.
24. Wang BL, Ye Z, Tang YH, et al. Fabrication of nonfouling, bactericidal, and bacteria corpse release multifunctional surface through surface-initiated RAFT polymerization. *Int J Nanomed* 2017; **12**: 111-25.
25. Yuan B, Chen Q, Ding WQ, et al. Copolymer coatings consisting of 2-methacryloyloxyethyl phosphorylcholine and 3-methacryloxypropyl trimethoxysilane via ATRP to improve cellulose biocompatibility. *ACS Appl Mater Interfaces* 2012; **4**(8): 4031-9.
26. Fujii K, Matsumoto HN, Koyama Y, Iwasaki Y, Ishihara K, Takakuda K. Prevention of biofilm formation with a coating of 2-methacryloyloxyethyl phosphorylcholine polymer. *J Vet Med Sci* 2008; **70**(2): 167-73.
27. Azuma T, Teramura Y, Takai M. Cellular response to non-contacting nanoscale sublayer: cells sense several nanometer mechanical property. *ACS Appl Mater Interfaces* 2016; **8**(17): 10710-6.
28. Guegan C, Garderes J, Le Pennec G, et al. Alteration of bacterial adhesion induced by the substrate stiffness. *Colloids Surf B Biointerfaces* 2014; **114**: 193-200.
29. Wang Y, Guan A, Isayeva I, et al. Interactions of *Staphylococcus aureus* with ultrasoft hydrogel biomaterials. *Biomaterials* 2016; **95**: 74-85.
30. Guégan C, Garderes J, Le Pennec G, et al. Alteration of bacterial adhesion induced by the

substrate stiffness. *Colloids Surf B Biointerfaces* 2014; **114**: 193-200.
31. Kolewe KW, Dobosz KM, Emrick T, Nonnenmann SS, Schiffman JD. Fouling-resistant hydrogels prepared by the swelling-assisted infusion and polymerization of dopamine. *ACS Applied Biomaterials* 2018; **1**(1): 33-41.
32. Kolewe KW, Zhu J, Mako NR, Nonnenmann SS, Schiffman JD. Bacterial adhesion is affected by the thickness and stiffness of poly(ethylene glycol) hydrogels *ACS Appl Mater Interfaces* 2018; **10**(3): 2275-81.
33. Nagahashi K, Teramura Y, Takai M. Stable surface coating of silicone elastomer with phosphorylcholine and organosilane copolymer with cross-linking for repelling proteins. *Colloids Surf B Biointerfaces* 2015; **134**: 384-91.
34. Moryl M, Kaleta A, Strzelecki K, Różalska S, Różalski A. Effect of nutrient and stress factors on polysaccharides synthesis in Proteus mirabilis biofilm. *Acta Biochimica Polonica* 2014; **61**(1).
35. Song F, Brasch ME, Wang H, Henderson JH, Sauer K, Ren D. How Bacteria Respond to Material Stiffness during Attachment: A Role of Escherichia coli Flagellar Motility. *ACS Appl Mater Interfaces* 2017; **9**(27): 22176-84.
36. Sun H, Chan CW, Wang Y, et al. Reliable and reusable whole polypropylene plastic microfluidic devices for a rapid, low-cost antimicrobial susceptibility test. *Lab on a chip* 2019; **19**(17): 2915-24.
37. Siddiqui S, Chandrasekaran A, Lin N, Tufenkji N, Moraes C. Microfluidic Shear Assay to Distinguish between Bacterial Adhesion and Attachment Strength on Stiffness-Tunable Silicone Substrates. *Langmuir* 2019; **35**(26): 8840-9.
38. Lee WB, Chien CC, You HL, Kuo FC, Lee MS, Lee GB. An integrated microfluidic system for antimicrobial susceptibility testing with antibiotic combination. *Lab on a chip* 2019; **19**(16): 2699-708.
39. Zarabadi MP, Paquet-Mercier F, Charette SJ, Greener J. Hydrodynamic Effects on Biofilms at the Biointerface Using a Microfluidic Electrochemical Cell: Case Study of Pseudomonas sp. *Langmuir* 2017; **33**(8): 2041-9.
40. Lu H, Koo LY, Wang WM, Lauffenburger DA, Griffith LG, Jensen KF. Microfluidic shear devices for quantitative analysis of cell adhesion. *Anal Chem* 2004; **76**: 5257-64.
41. Alagem-Shafir M, Kivovich E, Tzchori I, et al. The formation of an anti-restenotic/anti-thrombotic surface by immobilization of nitric oxide synthase on a metallic carrier. *Acta biomaterialia* 2014; **10**(5): 2304-12.
42. Gao G, Lange D, Hilpert K, et al. The biocompatibility and biofilm resistance of implant coatings based on hydrophilic polymer brushes conjugated with antimicrobial peptides. *Biomaterials* 2011; **32**(16): 3899-909.
43. Zhao G, Zhong H, Zhang M, Hong Y. Effects of antimicrobial peptides on Staphylococcus aureus growth and biofilm formation in vitro following isolation from implant-associated infections. *Int J Clin Exp Med* 2015; **6**(1): 1546-51.