Tunable Coverage of Immobilized Biomolecules for Biofunctional Interface Design

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Tunable Coverage of Immobilized Biomolecules for Biofunctional Interface Design

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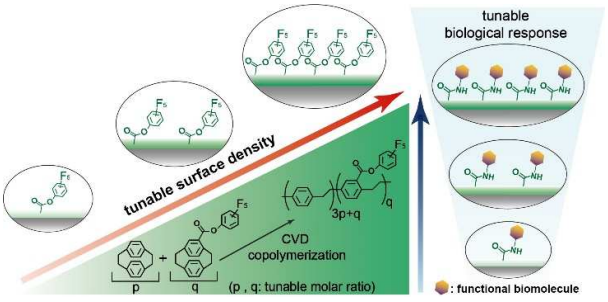
The controlled coverage of immobilized biomolecules is introduced, illustrating a concept for designing biomaterial surfaces such that the extent of manipulation employed to elicit biological responses is controlled according to density changes in the underlying chemical motifs and the density of immobilized biomolecules.

The surface density of conjugated biomolecules has become an essential issue in advanced biomaterial design, in which the immobilization of functional biomolecules plays an important role in determining the effectiveness of the current biomaterials used.[1] Although the immobilization of functional biomolecules provides the advantage of a significantly low dosage of use, the exhibited biological effectiveness may not be equally extrapolated when used in solution[2] compared with being immobilized on surfaces; and the association of the immobilized biomolecules concentrations to the effectiveness of the induced biological responses yet has only been sporadically discussed[3] or only in a case-by-case manner. Correlations regarding the immobilization efficiency of biomolecules and surface chemical motifs, the relationship between the coverage of attached biomolecules and the resulting biological efficacy, and the mechanisms explaining the activities exhibited by these immobilized biomolecules may require substantial research and may need to be incorporated as important parameters for designing new biomaterials. From a practical point of view, the immobilization of biomolecules onto material surfaces also requires a precisely controlled configuration of biomolecules such that tailored coverage may be realized; such control could allow for the manipulation of effective biological responses or the generation of gradient guidance.[4]

The objective of this study was to illustrate the important concept of controlling the coverage of the immobilized biomolecules and the resulting tunable biological activities for advanced biomaterials designs. The tunable mechanism was demonstrated by precisely adjusting the density of underlying anchoring sites, which was achieved by introducing a series of density-varied poly-*p*-xylylenes formed during the modification process of chemical vapor deposition (CVD) copolymerization. Pentafluorophenol (PFP) ester, a chemical motif that provides a bioorthogonal linkage for amine-rich molecules, and forms an amide bond through the amine-PFP ester coupling reaction,[5] was chosen to realize the idea and was used for the immobilizations of biomolecules including polyethylene glycols, aptamers, proteins of bovine serum albumin and fibronectin. The binding efficacy of these molecules and the tunable biological responses with respect to cell adhesion and proliferation as well as protein adsorption were verified.

The density-varied PFP ester coating was prepared via CVD copolymerization on a self-designed multi-sourced CVD copolymerization system.[6] During the CVD process, the density of PFP ester groups was adjusted by copolymerizing a controlled molar ratio of PFP ester-[2.2]paracyclophane and nonsubstituted-[2.2]paracyclophane (1:1, 1:20, 1:80,



Scheme 1. Schematic illustration of preparation of density-varied PFP ester coatings by copolymerizing a controlled molar ratio (1:0, 1:1, 1:20, 1:80, 0:1) of PFP ester-[2.2]paracyclophane and nonsubstituted-[2.2]paracyclophane during the CVD copolymerization process. The ability to tailor the coated surfaces to elicit biological responses was controlled by varying the density of the underlying PFP ester groups and the biomolecules immobilized.

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Electronic Supplementary Information (ESI) available: XPS and IRRAS characterizations of the density-varied PFP ester coatings; confirmation of protein immobilization on the coating surfaces; standard calibration curves of fluorescence probe intensity; images of cell nuclei staining. See DOI: 10.1039/x0xx00000x

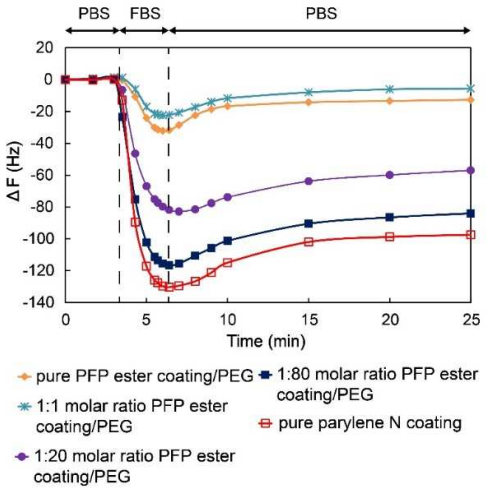

Figure 1. Adsorption of 10% fetal bovine serum (FBS) on the modified surfaces with varied immobilization densities of PEGs. Dynamic QCM analysis was performed to monitor the adsorption of FBS molecules on these surfaces. FBS solution was injected into the system at 3 min, and after an interval of 3 min, PBS buffer solution was introduced into the system at 6 min.

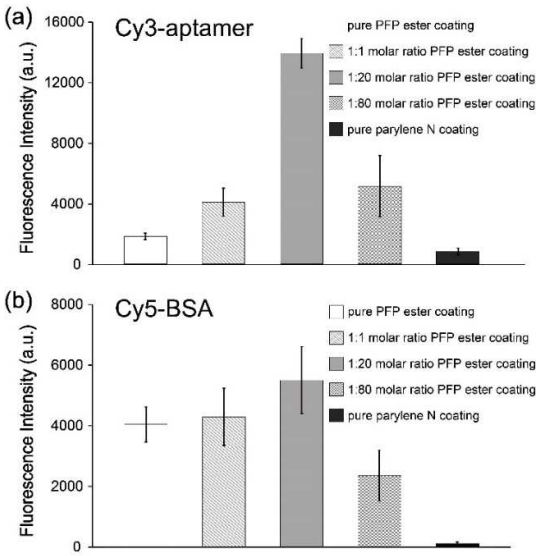

Figure 2. Fluorescence intensities were characterized at various immobilization densities of (a) Cy3-aptamer and (b) Cy5-BSA. Immobilizations were performed using density-varied PFP ester coatings on glass substrates. Each experiment was performed in triplicate.

respectively), and the resulted in a series of density-varied PFP ester-functionalized poly-*p*-xylylenes, as illustrated in **Scheme 1**. Characterizations using a combination of XPS and IRRAS to verify the resulting density-varied PFP ester coatings are presented in the ESI. The surface densities of the resulting modified surfaces were calculated by using molecular modeling simulation[7] and also by chemical analysis approaches from ultraviolet–visible spectroscopy and quartz crystal microbalance (QCM). The densities were well correlated to the molar ratios 1:0, 1:1, 1:20, 1:80, 0:1, and are summarized in Table S1 in the ESI. Consistent results were also verified by the characterizations from both XPS and IRRAS analysis, and are revealed in the ESI. Several types of biomolecules, including amine-PEG, aptamer (GGTTGGTGTGGTTGG), and proteins of BSA and fibronectin, were used as model molecules to examine the conjugation efficiency with respect to the varied density of PFP ester anchoring sites to which these molecules were tethered covalently through reaction with the highly reactive PFP ester group to form an amide bond with the terminal primary amine. In the experiments, amine-PEG was first immobilized on QCM crystal surfaces that were modified with varied densities of PFP ester groups (contain the controlled molar ratios of 1:0, 1:1, 1:20, 1:80, and 0:1 described above). The antifouling PEG surfaces exhibited an anticipated suppression of protein molecules, as previously observed by QCM analysis by us[8] and others.[9] The results are presented in **Figure 1** and reveal the anticipated resistance toward the selected protein system containing 10% FBS; in addition, high resistance is observed for the groups in which more PEG was tethered to the surfaces, including pure PFP ester and the 1:1 molar ratio PFP-ester-coated surfaces. Although a higher density of anchoring sites was postulated for the former surface compared with the later, the two surfaces exhibited relatively equal results; approximately 6.5×10^{-4} mol·cm^{-2} of adsorbed FBS was detected due to the steric effect observed similarly for other PEG systems.[10] A saturated immobilization density may have been reached for the PEG molecules for the 1:1 molar ratio PFP-ester-coated surfaces. However, a decreased resistance dependence was observed for surfaces on which fewer PEGs were immobilized, and the highest fouling of FBS ($1.1 \pm 0.14 \times 10^{-2}$ mol·cm^{-2}) was observed on the surface on which no PEG was immobilized (pure parylene N coating). The immobilizations were further implemented by utilizing two fluorescence-labeled molecules, including an aptamer with a sequence of 15 nucleic acids (GGTTGGTGTGGTTGG) and BSA, and tethering these molecules to the density-varied PFP ester coating surfaces. The resulting fluorescence intensities of the immobilized Cy3-aptamer and Cy5-BSA were analyzed using a fluorescence scanner, and the densities of the immobilized molecules were extrapolated by comparison with standard calibration curves (details of the calibration curves are included in the ESI). As demonstrated in **Figure 2**, increased fluorescence intensities were observed on surfaces with more concentrated PFP ester groups, indicating an increased attachment of aptamers or BSA. Notably, the maximum intensity was detected for the 1:20 group for both cases; thus, the hypothesis that steric effect and electrostatic repulsion resulted from crowded proteins or aptamers may be formulated in the present study, similarly to other systems.[11] Finally, amine-PEG and fibronectin protein were also immobilized at varied densities. The resulting cellular responses were examined by culturing mouse embryonic fibroblast (3T3-L1) cells on such surfaces. After 24 hours of cell culture, the attachment of 3T3-L1 on these surfaces was analyzed (**Figure 3 (a)**), and an opposite trend from a confluent cell growing pattern to a suppressed growth was observed accordingly with a decrease in the ratio of immobilized PEGs; thus, the cell-repelling activity of PEG was confirmed and, more practically,

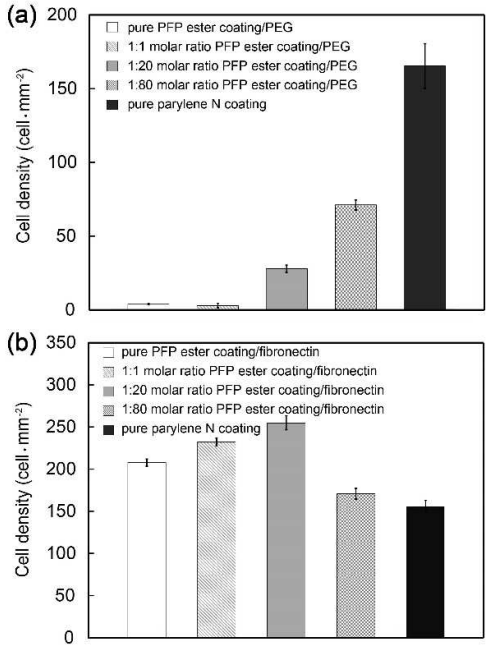

Figure 3. Quantitative analysis of 3T3-L1 cell attachment on surfaces with (a) varied ratios of PEG immobilizations and (b) varied ratios of immobilized fibronectin proteins. Each experiment was performed in quintuplicate.

was addressed in a tunable manner. The attached cells were also counted (by counting the stained nuclei as shown in the ESI) to analyze the surfaces, and 3.8 ± 0.5 cell·mm^{-2} of cells were observed on the surface on which the most PEG groups were immobilized compared with the 157.3 ± 14.3 cell·mm^{-2} cells attached to the surface without PEG modification. The statistical results also showed increasing resistance to cell attachment with the increase in the density of attached PEGs, which is consistent with aforementioned results obtained for resistance to protein repulsion. The immobilization of fibronectins of varied density was examined in a separate experiment for 3T3-L1 adhesion, in which the fibronectin protein was shown to be an important factor affecting cell attachment and proliferation.[12] In contrast to PEGs, the results obtained by observing the cell growth pattern and quantitatively counting the cells (**Figure 3 (b)**) indicated that cell proliferation increased with fibronectin density. The maximum cell density was also detected for the 1:20 group, which is consistent with the finding observed previously for BSA proteins and unambiguously confirmed the above-mentioned hypothesis concerning the steric effect and electrostatic repulsion, which was also observed to hold for fibronectin proteins during the immobilization process. Taken together, the biological activities, i.e., cell attachment and protein adsorption, can effectively respond to the changes in the density of the immobilized functional molecules. Although the details of the dependencies and mechanism may still require further investigation, the results demonstrate that the biological activities of biomaterial surfaces are tunable and can be tailored by controlling the densities of the underlying chemical motifs or the immobilized biomolecules.

Immobilizing biofunctional molecules onto the surfaces of biomaterials is an important step toward the design of new materials. Searching for an optimal approach to immobilize these molecules can barely satisfy our current needs when used in more sophisticated biological microenvironments. A precise control over the density of the attached molecules to enable an a tailored and optimized biological response and effectiveness is encouraged for new biomaterials design, and a better understanding of the mechanisms governing the activity of the tethered compounds would be helpful but requires a detailed investigation. The proposed immobilization technique is facile and versatile and may help advance studies and applications that rely on the immobilization of biomolecules on material surfaces, and we foresee the extension of exploiting other functional poly-*p*-xylylenes for tuning the biointerface with more biomolecules, and/or enabling synergistic and gradient control over multiple functions, as well as the development of industrial products.

Acknowledgements

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1 T. Sun, G. Qing, B. Su and L. Jiang, *Chemical Society Reviews*, 2011, **40**, 2909; D. G. Castner and B. D. Ratner, *Surface Science*, 2002, **500**, 28.
2 Y. Luo and M. S. Shoichet, *Biomacromolecules*, 2004, **5**, 2315.
3 D. Alves and M. Olívia Pereira, *Biofouling*, 2014, **30**, 483.
4 Y. M. Elkasabi, J. Lahann and P. H. Krebsbach, *Biomaterials*, 2011, **32**, 1809.
5 G. T. Hermanson, *Bioconjugate techniques*, Academic Press, Boston, 2008.
6 M.-Y. Tsai, Y.-C. Chen, T.-J. Lin, Y.-C. Hsu, C.-Y. Lin, R.-H. Yuan, J. Yu, M.-S. Teng, M. Hirtz, M. H.-C. Chen, C.-H. Chang and H.-Y. Chen, *Advanced Functional Materials*, 2014, **24**, 2281; H.-Y. Chen, T.-J. Lin, M.-Y. Tsai, C.-T. Su, R.-H. Yuan, C.-C. Hsieh, Y.-J. Yang, C.-C. Hsu, H.-M. Hsiao and Y.-C. Hsu, *Chemical Communications*, 2013, **49**, 4531.
7 S. M. Kerwin, *Journal of the American Chemical Society*, 2010, **132**, 2466.
8 C.-T. Su, R.-H. Yuan, Y.-C. Chen, T.-J. Lin, H.-W. Chien, C.-C. Hsieh, W.-B. Tsai, C.-H. Chang and H.-Y. Chen, *Colloids and Surfaces B: Biointerfaces*, 2014, **116**, 727-733.
9 R. Yang and K. K. Gleason, *Langmuir*, 2012, **28**, 12266; G. B. Rusu, M. Asandulesa, I. Topala, V. Pohoata, N. Dumitrascu and M. Barboiu, *Biosensors & Bioelectronics*, 2014, **53**, 154.
10 K. Yoshioka, E. Sakai, M. Daimon and A. Kitahara, *Journal of the American Ceramic Society*, 1997, **80**, 2667.
11 X. Zhao, F. Pan, B. Cowsill, J. R. Lu, L. Garcia-Gancedo, A. J. Flewitt, G. M. Ashley and J. Luo, *Langmuir*, 2011, **27**, 7654; D. Lee, M. Yoo, H. Seo, Y. Tak, W.-G. Kim, K. Yong, S.-W. Rhee and S. Jeon, *Sensors and Actuators B: Chemical*, 2009, **135**, 444.
12 U. Hersel, C. Dahmen and H. Kessler, *Biomaterials*, 2003, **24**, 4385.

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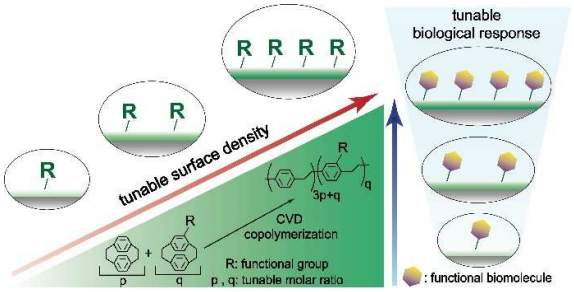

Tunable biological responses can be controlled by the modifications of the density of the underlying chemical motifs and the density of the biomolecules immobilized.