CHEMISTRY
A EUROPEAN JOURNAL

DOI: 10.1002/chem.201100084

Facile Method for Development of Ligand-Patterned Substrates Induced by a Chemical Reaction

Hyunjung Seo,[a] Inseong Choi,[a] Jeongwook Lee,[a] Sohyun Kim,[a] Dong-Eun Kim,[a] Sang Kyung Kim,[b] and Woon-Seok Yeo*[a]

Patterned substrates have been widely used in various applications, including arrays of biomolecules and cells, high-throughput assays, and direct target sensing.[1] In practice, those demands have been achieved by either of or a combination of two strategies: 1) direct incorporation of biomolecules or functional-group-containing molecules into desired patterns and 2) generation of functional-group-presenting patterns by way of chemical conversions on the surface. The former encompasses microcontact printing (μCP),[2] dip-pen nanolithography (DPN),[3] polymer-pen lithography (PPL),[4] microfluidic networks (μFNs),[5] and microarrays.[6] The latter utilizes the "turning-on" strategy, in which inactive substrates are switched to an active state to reveal organic functional groups, in most cases by electrochemical or photochemical conversions.[7] Patterned functional groups in both strategies are further used as chemical handles for immobilization of biomolecules, such as cell-adhesion ligands, enzyme substrates, proteins, oligosaccharides, and oligonucleotides, to afford patterned substrates. As a typical recent example, Rozkiewicz et al. reported on modified μCP for the preparation of oligonucleotide micropatterns.[8] In their report, oxidized PDMS stamps were first coated with positively charged dendrimers followed by negatively charged oligonucleotides in a layer-by-layer arrangement, and were transferred to a solid support for the generation of microarrays. Smith and co-workers introduced a photo-labile protecting group to a thiol functionality.[9] Various patterns of small molecules and proteins were prepared by using a photolithographic method in combination with thiol-specific conjugation chemistry. Yousaf et al. showed that ligand density and composition influence the rate of stem-cell differentiation by using hydroquinone-based electroactive substrates, which were patterned with a variety of ligands by using microarray technology.[10] Although these two strategies are reliable, well established, and, therefore, widely used, each of the strategies offers limitations on practical use as a general platform for ligand-patterned substrates. For instance, direct contact printing methods, such as μCP, cannot control ligand density on the surface, which can provide important quantitative information for use in experimental design. A concern with regard to the turning-on strategy is that in some cases activated functional groups require specified conjugation chemistry and, therefore, necessitate preparatory steps (tagging steps) to make the ligands compatible with the conjugation reaction.

Herein, we describe a simple, efficient, and straightforward method for ligand patterning on a surface, induced by a non-invasive organic chemical reaction—which we have termed a chemical-reaction-induced patterning (CRIP)—and equipped with the capability for control of ligand density. In addition, our method is compatible with common patterning tools and conjugation chemistry. Herein, we demonstrate our strategy by using two popular patterning tools, μCP and microarray, and verify the fidelity of the preparation of ligand-patterned substrates by patterning cell-adhesion ligands and aptamers.

Our strategy for preparation of ligand-patterned substrates relies on conversion of a substrate from a dormant (inactive) state to an active state by way of a chemical reaction grafted with a patterning method (Figure 1). The resulting patterned area presents a chemically reactive functional group, that is, a primary amine, which can be harnessed for the immobilization of biomolecules of interest. Our approach utilizes self-assembled monolayers (SAMs) on gold terminated with masked functional groups. Figure 1B shows the structure of the monolayer and the chemical reactions taking place on the surface for the preparation of the amine-functionalized substrate (for the synthesis of the quinone-terminated alkanethiol, see the Supporting information). The quinone-presenting monolayer[11] was treated with a reducing agent by using patterning tools. Upon reduction of the quinone to the corresponding hydroquinone, a cyclization reaction ensues to afford an amine group. The resulting amine can react with linkers or ligands through amine-specific bioconjugation chemistry. The surrounding tri(ethylene glycol) groups provide inertness towards the nonspecific adsorption of proteins and cells, which is the most demanding feature of substrates in biological/biochemical studies at interfaces.[12] Herein, we demonstrate this CRIP strategy by preparing various patterns of RGD (Arg-Gly-Asp)

[a] H. Seo, I. Choi, J. Lee, Dr. S. Kim, Prof. D.-E. Kim, Prof. W.-S. Yeo
Department of Bioscience and Biotechnology
Konkuk University, Seoul, 143-701 (Korea)
Fax: (+82) 2-2030-7890
E-mail: wsyeo@konkuk.ac.kr

[b] S. K. Kim
Nano-Bio Research Center
Korea Institute of Science and Technology, Seoul, 136-791 (Korea)

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/chem.201100084.

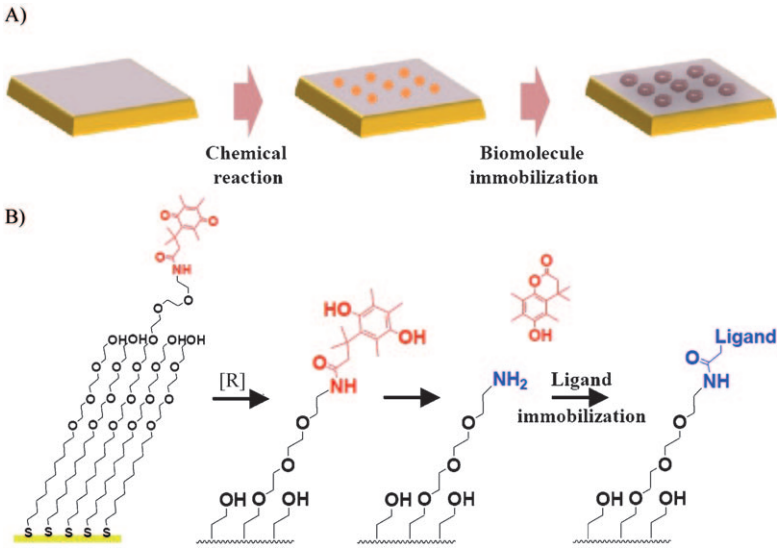

Figure 1. A) Strategy of the chemical reaction induced patterning method. B) Structure of the monolayer-presenting quinone functional group used in this study for preparation of amine-functionalized surfaces. Using patterning tools, the quinone-presenting monolayer is treated with a reducing agent. Upon reduction of the quinone to the corresponding hydroquinone, a cyclization reaction takes place to afford an amine group that can be harnessed for immobilization of biomolecules through amine-specific bioconjugation chemistry.

tripeptide, a cell-adhesion ligand.[13] The ratio of the quinone moiety to the tri(ethylene glycol) group was maintained at <0.01 (1%) to ensure that cell adhesion to the patterned monolayer was mediated by specific interactions between receptors of cells and ligands on the patterns. As another demonstration, the peptide aptamer DUP-1 (FRPNRAQDYNTN), specific to the PC3 cell (one of the prostate cancer cell lines), was patterned on the monolayer and captured PC3 cells specifically.[14]

SAMs of alkanethiolates on gold are well suited for analysis with matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MS), termed SAMDI MS.[15] SAMDI analysis affords the molecular weight of constituents of monolayers (alkanethiolates) and thus provides not only structural information but also quantitative information with regard to the composition of monolayers. In addition, because molecular weight is an intrinsic property of a molecule, it allows us to discriminate between signals from analytes and deleterious background noise. Therefore, SAMDI is one of the most reliable techniques for the investigation of chemical/biochemical transformation of SAMs on gold.[16] We used SAMDI analysis to establish chemical conversions on the monolayer, as depicted in Figure 1B. First, we prepared a quinone presenting monolayer at a density of 20%. MS analysis of the monolayer gave a major peak at m/z 1057 $[M+Na]^+$, corresponding to the quinone-containing disulfide (Figure 2A). This quinone monolayer was treated with $NaBH_4$ (10 μL, 50 mM in deionized water) for 30 min, and then analyzed by MS. New peaks corresponding to the amine product were observed at m/z 803 $[M+H]^+$ and m/z 825 $[M+Na]^+$ (Figure 2B). To verify that the resulting amine could serve to immobilize ligands, the amine-presenting monolayer was treated with a bifunctional crosslinker, maleimide-NHS (10 μL, 50 mM in DMSO). MS analysis showed that the peaks at m/z 803 and 825 were absent and showed a new peak at m/z 976 $[M+Na]^+$, which was assigned to the maleimide functionality on the monolayer (Figure 2C). Treatment of the maleimide-presenting monolayer with CGRGDS peptide (10 μL, 1 mM in phosphate buffer solution (PBS), pH 7.4) gave peaks at m/z 1212 $[M+H]^+$ and 1546 $[M+H]^+$, corresponding to peptide-conjugated products in a free thiol form and a disulfide form, respectively (Figure 2D). Note that the peak at m/z 693 $[M+Na]^+$ corresponds to a tri(ethylene glycol)-terminated disulfide and the peak at m/z 1178 in Figure 2D corresponds to the molecular ion $[M+H-SH_2]^+$,

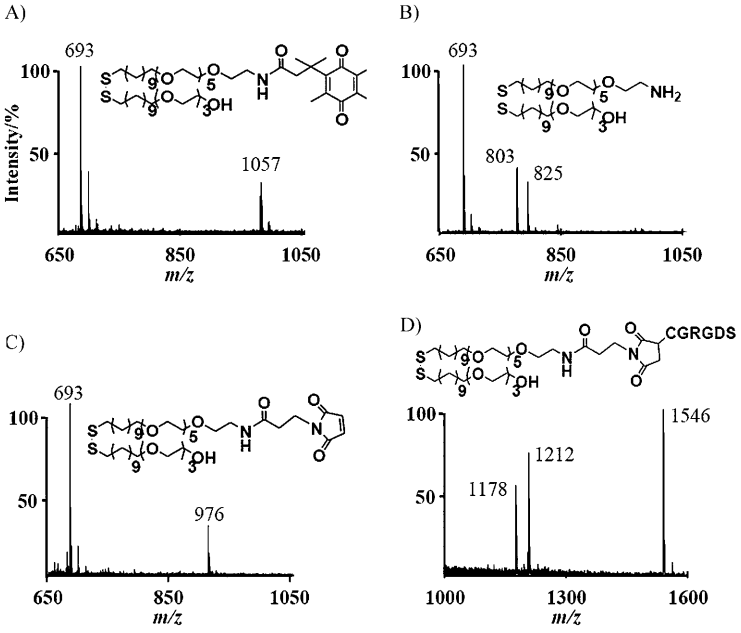

Figure 2. Verification of surface chemistry by MALDI-TOF MS: quinone-presenting monolayer (A), after reduction (B), after treatment with maleimide-NHS bifunctional cross-linker (C), and after CGRGDS conjugation (D). For details, see the main text.

which is often observed in the MALDI-TOF analysis of SAMs on gold and results from the abstraction of SH_2. Taken together, these results establish the fact that the chemical conversions proceeded as proposed in high yield. Furthermore, SAMDI analyses ensured that we would exclude undesired effects, which can be attributed to unreacted or unidentified species on the surface, from our experimental results.

The fidelity of our strategy was verified by fluorescence by using two common patterning methods, μCP and microarray, on glass substrates. PDMS stamps with various features, such as circles, squares, stripes, and mixtures (100 μm size), were inked with a solution of $NaBH_4$ in THF (100 mM) and applied to the quinone-presenting glass substrate for 30 min. Next, we patterned the quinone-presenting glass substrate into circular regions by using a microarrayer with an array pin (inner diameter of 100 μm) and $NaBH_4$ solution (50 mM, 10% DMSO in water). After thorough washing, the resulting substrates were treated with FITC, an amine-specific fluorescent dye (0.25 μM in PBS, pH 7.4). We examined the immobilization of FITC on the patterned surfaces by using a microarray scanner. Figure 3 shows fluorescence images from the substrates patterned by μCP (Figure 3A, B, C, D) and microarray (Figure 3E), which displayed clear green fluorescence exclusively from the patterns. As a control, the quinone-presenting glass substrate was treated by using the same process, except that the substrate was incubated with acetic anhydride (100 mM in THF) before FITC. As expected, no distinctive fluorescence was observed (data not shown). This result verifies that amine patterns were generated as desired and that FITC was selectively conjugated to this patterned amine.

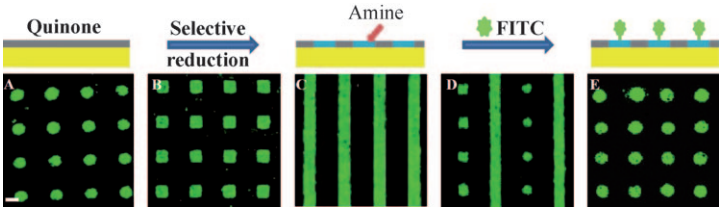

Figure 3. Verification of CRIP by using fluorescence. Using μCP and microarray, the quinone-presenting glass substrate was patterned into various features, which were proved by fluorescence. Scale bar: 100 μm.

To demonstrate biologically relevant applications of the CRIP strategy, Arg-Gly-Asp (RGD) peptide was conjugated for the preparation of cell patterns. The RGD peptide is a ligand that mediates cell adhesion through a specific interaction with integrin receptors on the cell surface. As described above, we prepared quinone-presenting monolayers and patterned them by using μCP and microarray. The resulting amine-patterned monolayer was reacted with the maleimide-NHS bifunctional linker (50 mM in DMSO) to afford maleimide groups, and was subsequently treated with the CGRGDS peptide (1 mM in PBS, pH 7.4). The RGD was anchored to the monolayer by a Michael addition reaction between the thiol in cysteine and the maleimide presented on the surface.[17] This RGD-presenting monolayer was treated with HeLa cells and incubated for 2 h. After brief washing with sterilized PBS, the monolayer was transferred to fresh media in a 12-well plate, incubated further for 10 h, and photographed. Optical micrographs in Figure 4 display cells that were patterned in accordance with the features of the stamps and microarray, indicating that the RGD peptide promoted selective cell adhesion to the chemically induced

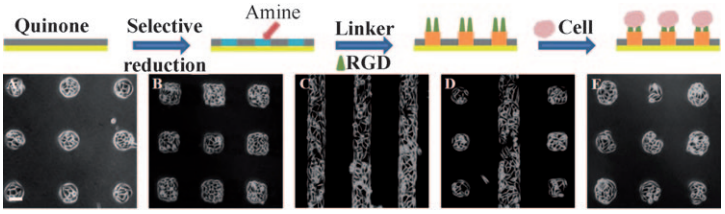

Figure 4. Application of the CRIP to cell patterning. Using μCP and microarray, the quinone-presenting monolayer underwent a series of chemical conversions to afford RGD patterns. After HeLa cell seeding and incubation for 12 h, optical micrography clearly showed cell patterns as designed. Scale bar: 100 μm.

regions. As a control, we repeated this experiment with a tri(ethylene glycol)-terminated monolayer. Cells did not attach to the monolayer, which provided additional evidence that cell adhesion to the substrate is biospecific and that the patterning methods used in the study did not compromise the inertness of the substrate. As another demonstration of biologically relevant applications of the strategy, we tested aptamer-based cell capture on the patterned substrate. The peptide aptamer DUP-1, which is specific to the PC3 cell, was patterned by the same method described above by using a microarrayer. As shown in Figure 5, PC3 cells were captured on the aptamer-patterned substrate and was further treated with the rhodamine-labeled aptamer (Rho-DUP-1) to give the fluorescent image in accordance with the cell pattern. Control experiments with a different prostate cancer cell line (LNCaP cell) and with an RGD-patterned substrate did not afford distinctive cell patterns, which indicates that the PC3 cells were captured on the substrate through specific interactions to DUP-1 aptamers on the monolayer (data not shown).

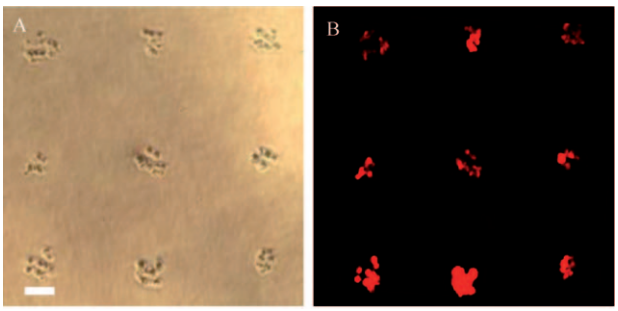

Figure 5. The DUP-1 peptide aptamer-patterned substrate was seeded with PC3 cells (A) and further treated with Rho-DUP-1 to give a fluorescent image in accordance with the cell pattern. Scale bar: 100 μm.

Important aspects of the CRIP method described herein include compatibility with many existing patterning methods and versatility of the resulting reaction product, that is, amines. Because our approach relies on chemical reduction of terminal groups on the surface, any patterning tools that deliver reducing agents to the surface can be harnessed. In fact, Mirkin et al. reported on a nanopatterning method by delivery of the oxidant to a hydroquinone-presenting surface

by using DPN.[18] Nucleophilic addition of amine-containing molecules to the localized benzoquinone resulted in nanopatterns. However, conjugation chemistry to the resulting benzoquinone is not as versatile as popular organic functional groups, such as amines, carboxylic acids, aldehydes, and thiols, which are the most commonly used functional groups not only in chemistry but also in biology. Moreover, the stability of hydroquinone against oxidation under ambient conditions sometimes causes unexpected results. Amine functionalities, unlike other common functional groups, such as carboxylic acids, aldehydes, and thiols, are quite stable under physiological conditions, are tolerable to reduce or oxidize, and maintain chemical reactivity under aqueous conditions over a wide pH range. Furthermore, along with development and commercialization of various amine-specific crosslinkers, almost every biomolecule can be immobilized on the amine-presenting surface. Another significant aspect of this work is the use of SAMs of alkanethiolates on gold. For interrogation of specific interactions between ligands on the surface and biomolecules of interest in a quantitative manner, the ligand density on the surface should be controllable and the surface should be inert to nonspecific adsorption. In this context, mixed SAMs prepared from a solution of ligand-terminated thiol and oligo(ethylene glycol)-terminated thiol in various ratios can perfectly supply those demands.

In conclusion, this report describes a simple ligand-patterning strategy induced by a chemical reaction that generates a pattern of amine functional groups. We proved the fidelity of our strategy by demonstrating the RGD patterning and subsequent cell patterns and the capture of prostate cancer cells to a specific aptamer-patterned substrate. Due to the chemical flexibility of the resulting amine functional group and the compatibility of this strategy with many existing patterning methods, we believe that this method will be a useful platform technology for the preparation of ligand-patterned substrates. For example, we are currently carrying out nano-patterning of multiple ligands by using grafting dip-pen nanolithography for investigation of cellular behavior on multi-ligand-presenting substrates.

Acknowledgements

This research was supported by a Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (grant 2009-0064280).

Keywords: aptamers · cell adhesion · mass spectrometry · monolayers · patterning

[1] a) E.-H. Song, N. L. B. Pohl, *Curr. Opin. Chem. Biol.* **2009**, *13*, 626; b) S. Ray, G. Mehta, S. Srivastava, *Proteomics* **2010**, *10*, 731; c) B. J. Hong, V. Sunkara, J. W. Park, *Nucleic Acids Res.* **2005**, *33*, e106; d) J. Hahm, C. M. Lieber, *Nano Lett.* **2004**, *4*, 51; e) W. U. Wang, C. Chen, K. Lin, Y. Fang, C. M. Lieber, *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 3208; f) Y. L. Bunimovich, Y. S. Shin, W. S. Yeo, M. Amori, G. Kwong, J. R. Heath, *J. Am. Chem. Soc.* **2006**, *128*, 16323.

[2] a) S. A. Lange, V. Benes, D. P. Kern, J. K. H. Hörber, A. Bernard, *Anal. Chem.* **2004**, *76*, 1641; b) C. S. Chen, M. Mrksich, S. Huang, G. M. Whitesides, D. E. Ingber, *Science* **1997**, *276*, 1425; c) M. Mrksich, C. S. Chen, Y. Xia, L. E. Dike, D. E. Ingber, G. M. Whitesides, *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 10775; d) Y. Xia, G. M. Whitesides, *Angew. Chem.* **1998**, *110*, 568; *Angew. Chem. Int. Ed.* **1998**, *37*, 550.

[3] a) D. S. Ginger, H. Zhang, C. A. Mirkin, *Angew. Chem.* **2004**, *116*, 30; *Angew. Chem. Int. Ed.* **2004**, *43*, 30; b) K. Salaita, Y. Wang, C. A. Mirkin, *Nat. Nanotechnol.* **2007**, *2*, 145; c) R. D. Piner, J. Zhu, F. Xu, S. Hong, C. A. Mirkin, *Science* **1999**, *283*, 661.

[4] a) F. Huo, Z. Zheng, G. Zheng, L. R. Giam, H. Zhang, C. A. Mirkin, *Science* **2008**, *321*, 1658; b) A. B. Braunschweig, F. Huo, C. A. Mirkin, *Nat. Chem.* **2009**, *1*, 353.

[5] a) B. M. Murphy, X. He, D. Dandy, C. S. Henry, *Anal. Chem.* **2008**, *80*, 444; b) R. T. Petty, H.-W. Li, J. H. Maduram, R. Ismagilov, M. Mrksich, *J. Am. Chem. Soc.* **2007**, *129*, 8966.

[6] a) B. J. Hong, S. J. Oh, T. O. Youn, S. H. Kwon, J. W. Park, *Langmuir* **2005**, *21*, 4257; b) V. Sunkara, B. J. Hong, J. W. Park, *Biosens. Bioelectron.* **2007**, *22*, 1532.

[7] a) H. Kaji, M. Kanada, D. Oyamatsu, T. Matsue, M. Nishizawa, *Langmuir* **2004**, *20*, 16; b) K. Kim, M. Jang, H. Yang, E. Kim, Y. T. Kim, J. Kwak, *Langmuir* **2004**, *20*, 3821; c) X. Jiang, R. Ferrigno, M. Mrksich, G. M. Whitesides, *J. Am. Chem. Soc.* **2003**, *125*, 2366; d) M. N. Yousaf, B. T. Houseman, M. Mrksich, *Angew. Chem.* **2001**, *113*, 1127; *Angew. Chem. Int. Ed.* **2001**, *40*, 1093; f) W. S. Dillmore, M. N. Yousaf, M. Mrksich, *Langmuir* **2004**, *20*, 7223; g) J. Nakanishi, Y. Kikuchi, T. Takarada, H. Nakayama, K. Yamaguchi, M. Maeda, *J. Am. Chem. Soc.* **2004**, *126*, 16314; h) Y. Kikuchi, J. Nakanishi, T. Shimizu, H. Nakayama, S. Inoue, K. Yamaguchi, H. Iwai, Y. Yoshida, Y. Horiike, T. Takarada, M. Maeda, *Langmuir* **2008**, *24*, 13084.

[8] D. I. Rozkiewicz, W. Brugman, R. M. Kerkhoven, B. J. Ravoo, D. N. Reinhoudt, *J. Am. Chem. Soc.* **2007**, *129*, 11593.

[9] S. Chen, L. M. Smith, *Langmuir* **2009**, *25*, 12275.

[10] W. Luo, E. W. L. Chan, M. N. Yousaf, *J. Am. Chem. Soc.* **2010**, *132*, 2614.

[11] a) C. D. Hodneland, M. Mrksich, *J. Am. Chem. Soc.* **2000**, *122*, 4235; b) W. –S. Yeo, M. Mrksich, *Langmuir* **2006**, *22*, 10816.

[12] M. Mrksich, G. M. Whitesides, *ACS Symp. Ser.* **1997**, *680*, 361.

[13] E. Ruoslahti, M. D. Pierschbacher, *Science* **1987**, *238*, 491.

[14] a) S. Zitzmann, W. Mier, A. Schad, R. Kinscherf, V. Askozylakis, S. Kramer, A. Altmann, M. Eisenhut, U. Haberkorn, *Clin. Cancer Res.* **2005**, *11*, 139; b) K. Min, K.-M. Song, M. Cho, Y.-S. Chun, Y.-B. Shim, J. K. Ku, C. Ban, *Chem. Commun.* **2010**, *46*, 5566.

[15] a) J. R. Lee, J. Lee, S. K. Kim, K. P. Kim, H. S. Park, W.-S. Yeo, *Angew. Chem.* **2008**, *120*, 9660; *Angew. Chem. Int. Ed.* **2008**, *47*, 9518; b) V. L. Marin, T. H. Bayburt, S. G. Sligar, M. Mrksich, *Angew. Chem.* **2007**, *119*, 8952; *Angew. Chem. Int. Ed.* **2007**, *46*, 8796; c) W.-S. Yeo, D.-H. Min, R. W. Hsieh, G. L. Greene, M. Mrksich, *Angew. Chem.* **2005**, *117*, 5616; *Angew. Chem. Int. Ed.* **2005**, *44*, 5480; d) M. Mrksich, *ACS Nano* **2008**, *2*, 7.

[16] a) S. Kim, J. I. Kim, H. R. Park, M. K. Kim, Y. Chong, W.-S. Yeo, *Bull. Korean Chem. Soc.* **2009**, *30*, 2574; b) D. H. Min, W.-S. Yeo, M. Mrksich, *Anal. Chem.* **2004**, *76*, 3923; c) W.-S. Yeo, M. Mrksich, *Adv. Mater.* **2004**, *16*, 1352; d) W.-S. Yeo, M. Mrksich, *Angew. Chem.* **2003**, *115*, 3229; *Angew. Chem. Int. Ed.* **2003**, *42*, 3121.

[17] B. T. Houseman, E. S. Gawalt, M. Mrksich, *Langmuir* **2003**, *19*, 1522.

[18] A. B. Braunschweig, A. J. Senesi, C. A. Mirkin, *J. Am. Chem. Soc.* **2009**, *131*, 922.

Received: January 10, 2011
Published online: April 18, 2011

