Photoreactive immobilization of 11-(2,4-dinitro-5-fluorobenzene)-undecenamide on a hydrogenated silicon (100) surface for protein immobilizations†

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Several nucleophiles such as proteins or poly(ethyleneimine) could be easily conjugated with a 11-(2,4-dinitro-5-fluorobenzene)undecenamide (DFUA) monolayer photochemically prepared on a silicon (100) surface.

Patterned protein arrays on a solid surface have attracted much attention as a platform technology for biosensor and biochip applications.[1] In order to immobilize proteins at a specific location on a solid sensor surface, a variety of techniques have been employed such as a simple spotting method,[2] a conventional photolithographic patterning,[3] or a soft lithography.[4] The proteins of interest can be immobilized on a surface simply through electrostatic adsorption onto a charged surface[5] or through a covalent linkage of the abundant amine groups with a aldehyde or carboxylated surface.[6] For more oriented surfaces, a specialized tag can be recombinantly prepared on either a C- or N-terminus of a probe protein along with the development of a specific counterpart on the solid surface.[2,7]

Meanwhile, the preparation of a well-ordered organic monolayer on a silicon surface has been an important issue to fabricate a functional surface, which is applicable to biosensors or biochips based on modern electronics.[8,9] In contrast to many alkanethiols formed on a gold surface, which can be quite easily removed either oxidatively or thermally, organosilane compounds on an oxidized or hydrogenated silicon surface have demonstrated higher thermal stability with moderate surface densities.[10–13] In this regard, photoimmobilization of compounds bearing an alkene group on a hydrogenated silicon surface has been continually attempted to circumvent inhomogeneities of monolayer formation that are often observed in surface hydrolysis reactions on an oxidized silicon surface.[14,15] Presumably, this reaction scheme appears to be advantageous in terms of relatively diverse ω-functionalities[8] as well as the high thermal and chemical stability of the Si–C bond;[10–12] the photo-immobilization of an alkene compound appears to occur through a surface chain reaction along with the formation of silicon radicals.[14]

Herein, we report a novel photochemical protocol for a ligand layer, 11-(2,4-dinitro-5-fluorophenyl)undecenamide (DFUA) monolayer, as a reactive intermediate to tether proteins of interest. The reactive benzene ring at one end of this compound is deliberately chosen to facilitate the protein uptake (or other nucleophiles) after the photoimmobilization, which is contrasted with methylene or carboxyl termini dominantly found in previous investigations.[14,15] The protein capture capability of the DFUA layer was compared with those of similarly immobilized undecanoic acid (UA) or stearic acid (SA) monolayers as control surfaces. In addition, the DFUA layer has been extended for an oriented immobilization of glutathione S-transferase (GST) tagged proteins.

Fig. 1 shows a schematic illustration describing the photochemical immobilization of DFUA and a subsequent protein immobilization. Two distinct protocols were employed in order to tether the alkene compound on a silicon surface, one being a micro-contact printing (μCP) method and the other a conventional

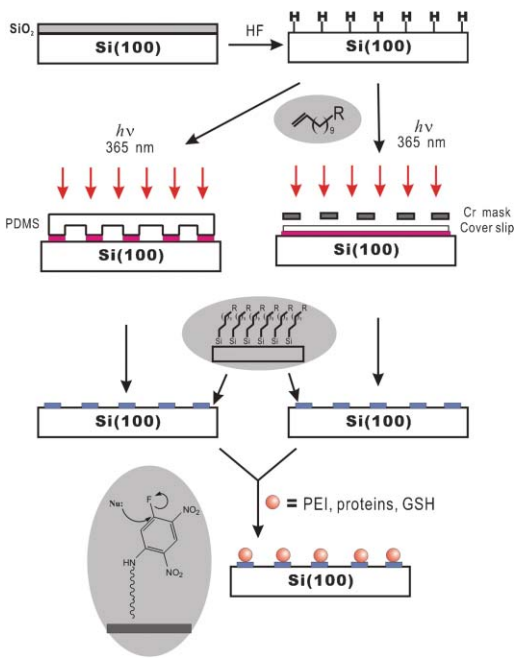

Fig. 1 Schematic illustration showing two distinct methods (*i.e.*, μCP and a conventional photolithography set-up) for the photochemical immobilization of DFUA on a silicon (100) surface.

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photolithography using a pattern mask. PDMS stamps were molded from a master pattern made of a photoresister for the μCP, while a stainless steel mask pattern with arrays of holes was employed for the photolithography protocol; the diameter of a single hole was ~1 mm. On these patterned DFUA layers, the immobilization of mainly three different target molecules such as proteins, cationic poly(ethyleneimine), and glutathione were investigated.

Fig. 2(a) shows an AFM image of the patterned DFUA layer fabricated *via* the μCP method. The average height of the pattern was 2.4 ± 0.5 nm, which is consistent with the theoretical value (~2.1 nm) estimated from the DFUA structure. In the enlarged image (Fig. 2(b)), a nanoporous structure was clearly seen with a dimension of several tens of nanometers, and these nanostructures are largely attributed to the stacking of benzene rings among the self-assembled DFUA layer;[16] this stacking appears to degrade more or less the sensitivity to the nucleophiles of interest. As will be demonstrated below, the patterned DFUA layer was highly susceptible to all of the potential nucleophiles (*e.g.*, amine groups of a protein that are exposed to the solution phase or polyamines).

Indeed, it was clearly seen that a substantial amount of BSA was conjugated with the patterned DFUA layer as shown in the AFM image (Fig. 2(c) and (d)). The height difference was estimated to be 3.4 ± 0.8 nm, which is quite close to the dimension of BSA (66 kDa, 8.4 × 7.8 × 3.2 nm^3).[17] The patterned BSA layer appears not to be close-packed due largely to a steric effect during the immobilization or the reduced activity caused by a π-stacked benzene groups (see Fig. 2(b)); the protein capture ability of the DFUA layer was totally conserved even after a week (in a dark refrigerator at 4 °C). The liability of DFUA layer to the attack of a nucleophiles was more evident in the interaction with poly(ethyleneimine) (PEI, MW 150 000) as a polymeric source of amino-nucleophiles. A PEI layer was successfully immobilized on the DFUA layer as shown in Fig. 3(a). The average height of the patterned PEI layer was estimated to be 600 ± 100 nm and this

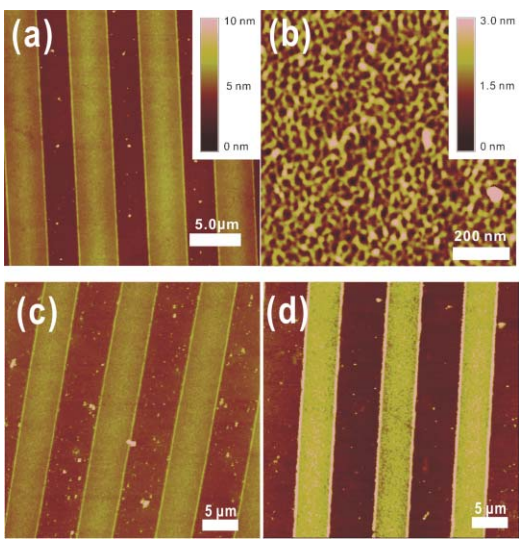

Fig. 2 (a) An AFM image of a line-patterned DFUA layer and (b) an enlarged version showing details of surface morphology in the DFUA layer. AFM images of the line-pattern sequentially acquired (c) before and (d) after soaking the patterned silicon chip with a BSA solution (0.1 mg ml^{-1}).

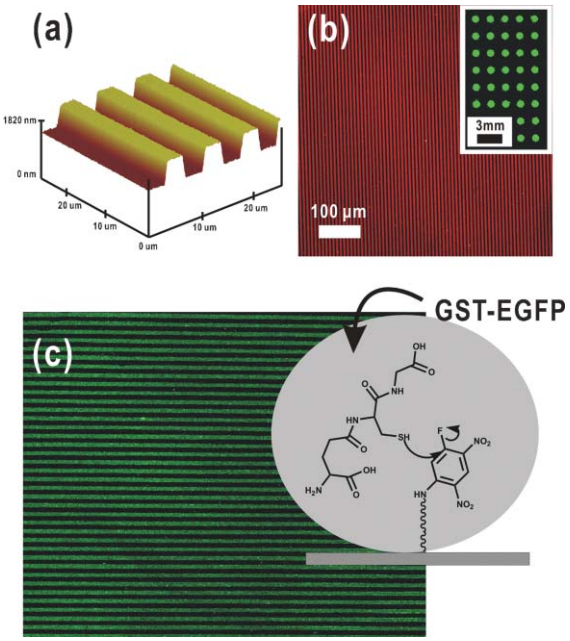

Fig. 3 (a) An AFM image of PEI tethered on a line-patterned DFUA layer and (b) a fluorescence image recorded after soaking the DFUA line pattern with Cy-5 labeled streptavidin (0.1 mg ml^{-1}) *inset*: FITC-labeled BSA captured on DFUA spots fabricated *via* photolithographic method. (c) A fluorescence image of captured GST-EGFP on a line-patterned glutathione-DFUA layer.

enhanced load of PEI can be ascribed to its polymeric nature; a few specific interactions are sufficient to tether a whole polymeric chain.

In addition, the effective capture of proteins was more clearly demonstrated by acquiring the fluorescent image of the patterned DFUA layer that was prepared by soaking it with a Cy5-labeled streptavidin solution after a μ-CP (see images in Fig. 3(b)). Compared to the AFM analysis, an even fluorescent signal over a large area was clearly observable. On the other hand, the inset shows the fluorescent pattern of FITC-labeled BSA captured on a DFUA layer that was prepared by the photolithographic method. These observations indicate that a variety of proteins can be immobilized onto the DFUA layers fabricated *via* these two immobilization protocols, simply by means of soaking them in a protein solution; the DFUA layer might represent a potential active region for biosensor or biochip applications.

Another useful system considered for protein immobilization is a simple UA layer that is photo-immobilized on the same silicon surface and with the same tethering protocol; for this preparation, the immobilization is assumed to occur through the same photoactivation of the alkene moiety. As shown in Fig. S1(a) of ESI,† apparently very similar fluorescent signals were observed both in the DFUA and in the UA layers upon soaking with FITC-BSA solution (0.1 mg ml^{-1}); this surface was highly hydrophilic compared to a DFUA or SA layer (see below). In spite of some advantages (*i.e.*, the simple chemical structure), it seems inferior to the present DFUA ligand owing to the electrostatic nature of the interaction. In other words, the electrostatic (or nonspecific) protein adsorption on the UA surface was substantially alleviated when employing a buffer solution with a high ionic strength (1 M NaCl), as demonstrated in Fig. S1(b) of ESI.†

Moreover, according to a previous result on the photoreactive immobilization of an alkenoic acid on a silicon surface, a possibility of carbonyl immobilization was raised in the work of Asanuma *et al.*, instead of photoreaction at the alkene moiety;[15] at this stage, a possibility of DFUA immobilization through a carbonyl group can be plausibly excluded by the presence of a bulky benzene ring. In an effort to examine the extent of the carbonyl tethering in an immobilization of alkenoic acid, SA was spin-coated and photo-immobilized on a silicon (100) surface according to the same protocol as in the DFUA layer. However, as shown in Fig. S1(c) of ESI,† this surface demonstrated substantially low fluorescent intensity even at a ten times larger concentration of SA (*i.e.*, 100 mM), indicating that the carbonyl-immobilization in a fatty acid is negligible; no noticeable fluorescent signal was observed in the photo-immobilized SA below 20 mM. The fluorescent signal is ascribed to a hydrophobic and nonspecific interaction of the protein on the SA layer; the surface was hydrophobic compared to a bare silicon surface.

In addition to a UA system, *N*-hydroxysuccinimide ester of UA (NHS-UA) was further investigated as an alternative ligand for the preparation of an active protein layer. In this surface, the dependence on ionic strength was not so significant as on the UA surface; the protein immobilization appears to be accomplished *via* a covalent linkage with the NHS ester. However, an NHS-UA layer pre-soaked in a buffer solution for 2 h, before the protein immobilization, appears to degenerate, as in the case of the UA surface (Fig. S1(d) of ESI).† The NHS ester group was totally hydrolyzed in the pre-soaking and proteins were negligibly adsorbed on the carboxylated surface at a high ionic strength (PBS plus 1 M NaCl). Obviously, the advantage of the present DFUA can be concluded as due to its resistance to water attack and its independence upon the ionic strength of the buffer used.

Compared to the protein immobilization performed hitherto, one more additional step was applied to the current scheme (before the protein immobilization), in order to achieve a more oriented immobilization of proteins using the DFUA as a protein capture layer. In this scheme, reduced glutathione molecules were first immobilized on a DFUA layer to capture GST-tagged proteins, instead of a direct and random immobilization. Recalling that the dinitrofluorophenyl (DNF) group of DFUA, known as "Sanger's reagent", has been used for peptide sequencing processes, the phenyl group as a nucleophilic center appears to be more susceptible to a thiol group at a lower solution pH (down to ~6.5) compared to other amino-nucleophiles; in the conventional peptide sequencing, an alkaline buffer is preferred to guarantee the N-terminus binding with a DNF group. The glutathione immobilization followed by a subsequent soaking with GST-EGFP appears to indeed work successfully for the capture of the GST-tagged proteins (see Fig. 3(c)). On the other hand, a negligible amount of EGFP without the GST tag could be immobilized on the glutathione modified surface as shown in Fig. S2(b) of ESI,† indicating a specific interaction of GST-EGFP with the glutathione modified DFUA layer. After one week preservation at 4 °C in a buffer solution, it was clearly seen that ~90% of the initial fluorescence survived, implicating that a surface induced denaturing of EGFP was negligible on the DFUA-glutathione surface.

Overall, these observations implicate that the DFUA layer on a hydrogenated silicon surface can be widely applicable both to a random protein immobilization and to an oriented tethering of GST-tagged proteins; the DFUA ligand efficiently tethers a reduced glutathione as an affinity counterpart for the GST tag. Moreover, the surface exhibited a relative robustness to side reactions compared to a simple carboxylated surface.

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Notes and references

1 R. S. Kane, S. Takayama, E. Ostuni, D. E. Ingber and G. M. Whitesides, *Biomaterials*, 1999, **20**, 2363.
2 S. O. Jung, H.-S. Ro, B. Ho. Kho, Y.-B. Shin, M. G. Kim and B. H. Chung, *Proteomics*, 2005, **5**, 4427.
3 K. L. Christman, M. V. Requa, V. D. Enriquez-Rios, S. C. Ward, K. A. Bradley, K. L. Turner and H. D. Maynard, *Langmuir*, 2006, **22**, 7444.
4 D. Falconnet, G. Csucs, H. M. Grandin and M. Textor, *Biomaterials*, 2006, **27**, 3044; G. M. Whitesides, E. Ostuni, S. Takayama, X. Jiang and D. E. Ingber, *Annu. Rev. Biomed. Eng.*, 2001, **3**, 335.
5 S. Chen, L. Liu, J. Zhou and S. Jiang, *Langmuir*, 2003, **19**, 2859; T. H. Ha, J. Y. Jeong and B. H. Chung, *Chem. Commun.*, 2005, 3959.
6 J. Herrwerth, T. Rosendahl, C. Feng, J. Fick, W. Eck, M. Himmelhaus, R. Dahint and M. Grunze, *Langmuir*, 2003, **19**, 1880; M. Veiseh, M. H. Zareie and M. Zhang, *Langmuir*, 2002, **18**, 6671; D. A. Nivens and D. W. Conrad, *Langmuir*, 2002, **18**, 499.
7 J. M. Abad, S. F. L. Mertene, M. Pita, V. M. Fernandez and D. J. Schiffrin, *J. Am. Chem. Soc.*, 2005, **127**, 5689.
8 L. C. P. M. de Smet, G. A. Stork, G. H. F. Hurenkamp, Q.-Y. Sun, H. Topal, P. J. E. Vronen, A. B. Sieval, A. Wright, G. M. Vissar, H. Zuilhof and E. J. R. Sudholter, *J. Am. Chem. Soc.*, 2003, **125**, 13916; H. B. Yin, T. Brown, R. Greef, J. S. Wilkinson and t. Melvin, *Microelectron. Eng.*, 2004, **73–74**, 830.
9 M. C. Howland, A. R. Sapuri-Butti, S. S. Dixit, A. M. Dattelbaum, A. P. Shreve and A. N. Parikh, *J. Am. Chem. Soc.*, 2005, **127**, 6752.
10 M. M. Sung, G. J. Kluth, O. W. Yauw and R. Maboudian, *Langmuir*, 1997, **13**, 6164.
11 A. B. Sieval, A. L. Demirel, J. W. M. Nissink, M. R. Linford, J. H. van der Maas, W. H. de Jeu, H. Zuilhof and E. J. R. Sudhölter, *Langmuir*, 1998, **14**, 1759; G. F. Cerofolini, C. Galati, S. Reina and L. Renna, *Mater. Sci. Eng., C*, 2003, **23**, 253.
12 R. L. Cicero, M. R. Linford and C. E. D. Chidsey, *Langmuir*, 2000, **16**, 5688.
13 M. Calistri-Yeh, E. J. Kramer, R. Sharma, W. Zhao, M. H. Rafailovich, J. Sokolov and J. D. Brock, *Langmuir*, 1996, **12**, 2747; R. Wang and S. L. Wunder, *J. Phys. Chem. B*, 2001, **105**, 173; N. Rozlosnik, M. C. Gerstenberg and N. B. Larsen, *Langmuir*, 2003, **19**, 1182.
14 B. J. Eves, Q.-Y. Sun, G. P. Lopinski and H. Zuihof, *J. Am. Chem. Soc.*, 2004, **126**, 14318; R. L. Cicero and C. E. D. Chidsey, *Langmuir*, 2002, **18**, 305.
15 H. Asanuma, G. P. Lopinski and H. Yu, *Langmuir*, 2005, **21**, 5013; F. Effenberger, G. Götz, B. Bidlingmaier and M. Wezstein, *Angew. Chem., Int. Ed.*, 1998, **37**, 2462.
16 N. Sanyal and P. M. Lahti, *Cryst. Growth Des.*, 2006, **6**, 1253.
17 M. L. Ferrer, R. Duchowicz, B. Carrasco, J. G. de la Torre and A. U. Acuña, *Biophys. J.*, 2001, **80**, 2422.