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Asymmetrically Functionalized, Four-Armed, Poly(ethylene glycol) Compounds for Construction of Chemically Functionalizable Non-Biofouling Surfaces

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Abstract: Asymmetrically functionalized, four-armed, Tween 20 derivatives that formed stable monomolecular films on solid substrates were designed and synthesized. Thiol-modified Tween 20 was used for forming self-assembled monolayers (SAMs) on gold, and maleimide-modified Tween 20 was introduced onto SiO_2 surfaces with SAMs of (3-mercaptopropyl)trimethoxysilane through Michael addition. These structurally modified Tween 20 compounds gave the original characteristics of Tween 20, non-biofouling (from ethylene glycol groups) and functionalizable (from OH groups) properties, to each substrate. The non-biofouling properties of the Tween 20-coated gold and SiO_2 surfaces were investigated by surface plasmon resonance spectroscopy and ellipsometry, and these surfaces showed strong resistance against nonspecific adsorption of proteins. In addition, the biospecific binding of streptavidin was achieved after coupling of (+)-biotinyl-3,6,9-trioxaundecanediamine onto the non-biofouling surfaces through amide-bond formation.

Keywords: non-biofouling surfaces · proteins · self-assembly · surface chemistry · surface plasmon resonance

Introduction

One of the most critical issues in the development of high-performance bioassay devices is the construction of a solid surface that enables biospecific recognition for target biomolecules at interfaces.[1–6] Biospecific recognition is achieved on device surfaces through the inherent, strong attraction between a surface-immobilized probe and a target, only when nonspecific interaction between device surfaces and biomolecules is eliminated. Therefore, the first step toward the development of the bioassay devices is to establish simple and mass-processable strategies for both constructing biologically "inert" surfaces, which eliminate nonspecific interaction/adsorption of biomolecules, and introducing probes of interest onto the inert surfaces. Various organic materials, such as polysaccharides,[7–9] zwitterionic compounds,[10–13] and poly(ethylene glycol) (PEG)[14–28] have been employed for constructing biologically inert surfaces (in other words, non-biofouling surfaces). In particular, ethylene glycol (EG) moieties have intensively been used to realize non-biofouling surfaces owing to their inertness to cell and protein adhesion, good biocompatibility, low toxicity, non-immunogenicity, and high water solubility.[29,30] Innumerable methods have been suggested for the introduction of EG moieties onto surfaces, exemplified by the formation of self-assembled monolayers (SAMs),[14–17] covalent grafting,[18–24] and physisorption.[25–28]

Commercially available Tween 20 and its series have recently attracted attention because of their structural and functional characteristics; the three divergent EG arms make the Tween 20 series non-biofouling; the long alkyl chain of the fourth arm can be utilized as a surface-anchoring portion through hydrophobic interactions; and the hydroxy (−OH) group of the EG arms is amenable to post-

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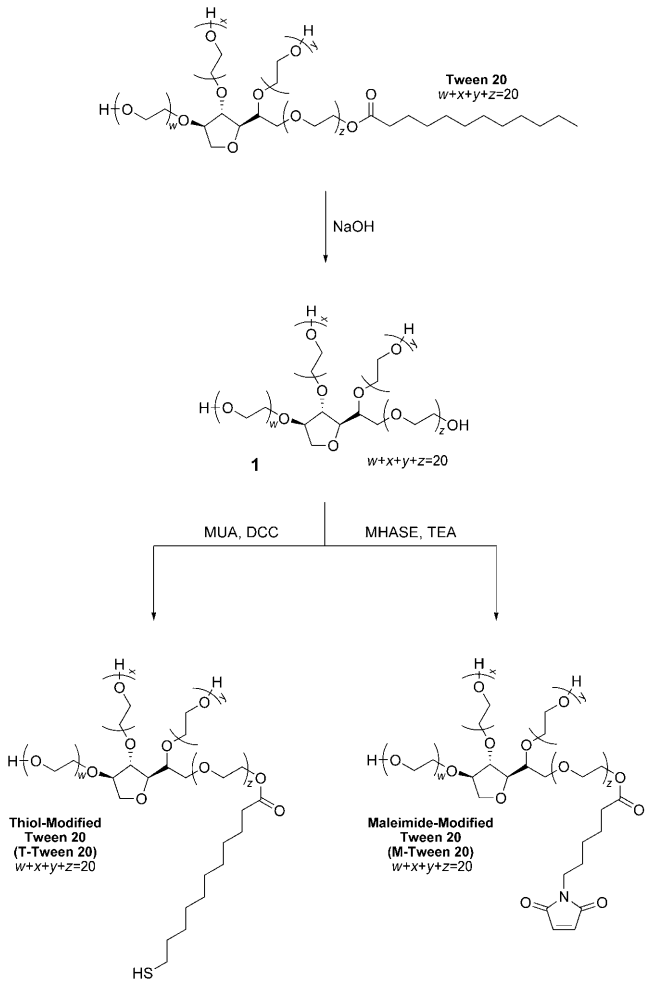
functionalization. Tween 20 has been used primarily as a non-biofouling coating material for carbon nanotubes in which hydrophobic interactions between Tween 20 and the sidewalls of carbon nanotubes led to the physical adsorption of Tween 20 onto carbon nanotubes.[27,31,32] In addition to carbon nanotubes, we demonstrated that the long alkyl chain of Tween 20 also could be utilized for immobilizing Tween 20 onto low-density SAMs.[28]

Several branched EG structures, such as star-shaped PEG molecules,[33,34] hyperbranched polyglycerols,[35] and poly-(oligo(ethylene glycol)methacrylate),[24] have been attempted as coating materials for constructing bioactive surfaces; these branched structures could increase both probe-coupling and target-binding capacity because of multiple functional groups.[24,35] In addition, such branched polymers sometimes showed superior non-biofouling properties when compared with their linear analogues. For example, hyperbranched polyglycerols were more resistant to protein adsorption than linear PEG of a similar molecular weight.[35] Nonspecific adsorption of large proteins decreased to zero on the star-shaped PEG-coated surface even at intermediate values of grafting density, whereas it was only prevented on the linear PEG-coated surface at the highest grafting density.[33] We also observed a similar phenomenon in the previous study:[28] nonspecific adsorption of proteins was prevented more effectively on the branched Tween 20-coated surface than on the linear Brij 35-coated surface even though Brij 35 had 23 EG groups per molecule, more than Tween 20 (20 EG groups per molecule). Therefore, the use of branched EG structures would potentially increase the sensitivity of bioassays owing to the decreased nonspecific adsorption and increased binding capacity. Although Tween 20 is one of the promising, commercially available materials for this purpose, it is not simply applicable to prevalent chip platforms, such as Au and SiO_2, because of the instability associated with Tween 20 immobilized on flat surfaces.[28,36] Low-density SAMs have been used to stably immobilize Tween 20,[28] however, the stability of the adsorbed Tween 20 was only maintained in aqueous solutions and delicate and cumbersome processes are required for the construction of low-density SAMs. The instability of Tween 20 in organic solvents precluded the direct introduction of probes onto the surface through chemical reactions. Herein, we describe the design and synthesis of two kinds of Tween 20 derivatives with the aim of immobilizing Tween 20 moieties simply and robustly onto gold and SiO_2 (Scheme 1): for gold, thiol-modified Tween 20 (T-Tween 20) that had a thiol end-group instead of the methyl-terminated dodecyl chain as is the case in Tween 20; for SiO_2, maleimide-modified Tween 20 (M-Tween 20) that had a maleimide end-group. The functionality incompatibility between hydroxy and trichlorosilyl (or trialkoxysilyl) groups led us to design M-Tween 20, which could be coupled with sulfhydryl groups at neutral pH values with high reactivity and chemoselectivity.[37–40] With these structural changes, we successfully formed stable SAMs of T-Tween 20 and M-Tween 20, which still possessed the original characteristics of Tween 20, non-biofouling (from EG groups) and functionalizable (from OH groups) properties.



Scheme 1. Chemical structures and synthetic schemes of thiol-modified Tween 20 (T-Tween 20) and maleimide-modified Tween 20 (M-Tween 20). DCC = *N*,*N*′-Dicyclohexylcarbodiimide, MHASE = 6-maleimido-1-hexanoic acid succinimidyl ester, MUA = 11-mercaptoundecanoic acid, TEA = triethylamine.

Results and Discussion

Preparation of SAMs of Thiol-Modified Tween 20 (T-Tween 20) On Gold

Gold surfaces have extensively been used as a platform for studying interactions between bio-entities, such as proteins and cells, and organic functionalities on surfaces, because of several characteristics of gold surfaces.[41] Of most importance, gold surfaces are coated easily with thiol-containing organic molecules in the form of SAMs, because gold binds to thiols with a high affinity. Gold thin films are suitable for a number of spectroscopic and analytical techniques, including surface plasmon resonance (SPR) spectroscopy, quartz crystal microbalance (QCM), polarized infrared external reflectance spectroscopy (PIERS), and ellipsometry. In addi-

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tion, gold thin films are easy to obtain and handle because gold is an inert metal. For these reasons, we chose a gold substrate as the first target substrate in the molecular design of Tween 20 derivatives and synthesized T-Tween 20 that contained a thiol end-group (see the Experimental Section).

We formed the SAMs of T-Tween 20 by immersing a gold substrate in an ethanol solution of T-Tween 20 ($0.1\,mg\,mL^{-1}$) overnight and characterized the resulting SAMs by ellipsometry and IR spectroscopy. The thickness of the SAMs was measured to be about 23 Å. The IR spectrum showed characteristic peaks of T-Tween 20 after the formation of the SAMs (Figure 1a). The CH_2 stretching vi-

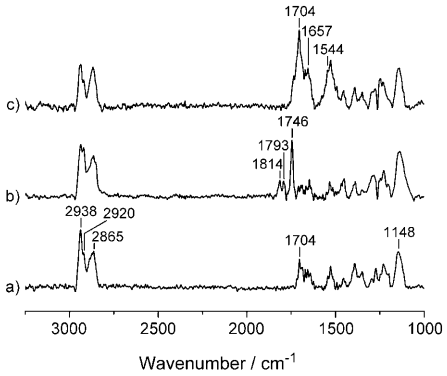

Figure 1. IR spectra of a) intact SAMs of T-Tween 20 on Au and the SAMs after b) the activation with DSC and c) the subsequent coupling with biotin–NH_2. DSC = *N,N′*-disuccinimidyl carbonate.

bration peaks were observed at around 2938 (asymmetric CH_2 stretching from EG), 2920 (asymmetric CH_2 stretching from alkyl chain), and 2865 cm^{-1} (symmetric CH_2 stretching from EG). The CH_2 stretching vibrations of the alkyl chain were used to determine the crystallinity of the SAMs as the peak position was very sensitive to the presence of gauche defects. The peak at 2920 cm^{-1} indicated that the decyl carbon chains had a crystalline, well-ordered structure on gold, although it was not well differentiated from the broad and strong band from the EG groups.[42] This peak position was unexpected because we had thought that the EG part of T-Tween 20 was too spatially demanding to tolerate such a well-ordered structure of alkyl chains during the formation of SAMs. We hypothesized that mutual interpenetration of the flexible EG chains led to effective molecular overlaps and a diminished steric effect of the large EG groups. We also observed a peak from the carbonyl group at 1704 cm^{-1} (C=O; stretching of ester) and a peak from the ether group at 1148 cm^{-1} (C–O–C; stretching).

For further applications, it would be crucial to maintain the stability of the SAMs of T-Tween 20. Therefore, we investigated the desorption behavior of T-Tween 20 in DMF (a solvent used in the activation of hydroxy groups) and water (a solvent used in common biological experiments). The T-Tween 20-coated substrates were immersed in DMF or water, and the changes in film thicknesses were monitored (Figure 2). The T-Tween 20 layer remained almost

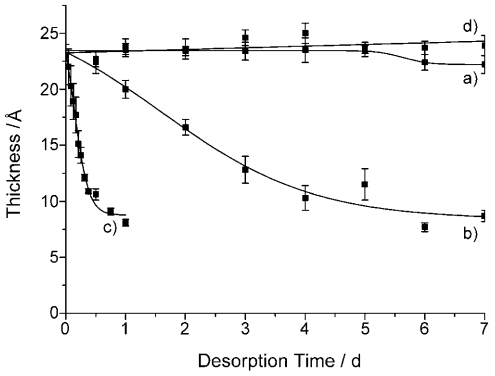

Figure 2. Desorption of T-Tween 20 in DMF a) at room temperature, b) at 40 °C, and c) at 50 °C. d) Desorption of T-Tween 20 in pure water at 60 °C.

intact in DMF for one week at room temperature, as verified by the unchanged film thicknesses. The desorption rate increased as temperature increased (Figures 2a–c). The long-term thermal instability would not be problematic in surface functionalization reactions that used organic reagents; for example, the activation of hydroxy groups with DSC was completed within several hours at room temperature.[43] The T-Tween 20 layer remained stable in water for one week even at 60 °C, indicating that the SAMs of T-Tween 20 on gold are suitable for common biological experiments (Figure 2d).

Non-Biofouling Properties of T-Tween 20

We investigated whether the SAMs of T-Tween 20 on gold inhibited nonspecific adsorption of proteins effectively. The amount of the adsorbed protein was quantified by ellipsometric measurements, and four different proteins (ribonuclease A, bovine serum albumin (BSA), fibrinogen, and lysozyme) were used as model proteins. The ellipsometric technique particularly offers an accurate way to measure the amount of proteins adsorbed strongly and irreversibly onto surfaces since weakly bound proteins are eliminated from the surfaces by washing processes.[9,17] The selected model proteins present different characteristics and have generally been used to study the protein-resistant properties of surfaces. Ribonuclease A is a globular protein with molecular weight of approximately 13.7 kDa and a pI value of 9.6,[44,45] and BSA (~66 kDa, pI = 4.7–4.9) is a popular blocking agent used in western blotting, enzyme-linked immunosorbent assay, and conventional protein microarrays.[46,47] Fibrinogen (~340 kDa, pI = 6) is a large and sticky protein from blood plasma that is adsorbed onto a broad range of surfaces. Lysozyme (~14 kDa, pI = 11) is commonly used for the studies of electrostatic adsorption of proteins onto surfaces as it possesses strong positive charges under the commonly used conditions.[9]

Figure 3 shows the changes in the film thicknesses with immersion of the substrates for 2 h in 10 mM sodium phosphate buffer (pH 7.5) solution of each protein (1 mg/mL) at

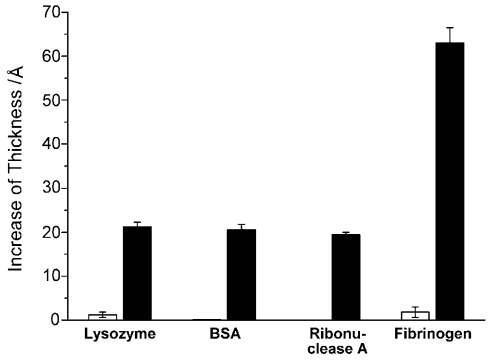

Figure 3. Ellipsometric thickness changes of the T-Tween 20/Au (white bar) and DDT/Au surfaces (black bar) after protein adsorption experiments.

room temperature. The SAMs of dodecanethiol (DDT) were used as a comparison. The thickness increases for the T-Tween 20/Au surface were negligible (white bars) compared with those for the DDT/Au surface (black bars). The thickness increases for the T-Tween 20/Au and DDT/Au surfaces were 1.2 and 21.2 Å (for lysozyme), 0.1 and 20.6 Å (for BSA), 0.0 and 19.4 Å (for ribonuclease A), and 1.8 and 63.0 Å (for fibrinogen), respectively. These values indicated that the decreases in the nonspecific adsorption of proteins were about 94% for lysozyme, 99% for BSA, 100% for ribonuclease A, and 97% for fibrinogen. In other words, the T-Tween 20/Au surface diminished the nonspecific adsorption of proteins by more than 94% for all the proteins studied in comparison with the DDT/Au surface. In addition, the non-biofouling capability of the T-Tween 20/Au surface was greater than that of the surface prepared by the hydrophobic adsorption of intact Tween 20,[28] which may have resulted from a relatively higher density of EG groups on the T-Tween 20/Au surface owing to the strong and effective adsorption of T-Tween 20.

Preparation of Biotin-Presenting Surfaces and Surface Plasmon Resonance (SPR) Spectroscopy

To attach an amine-derivatized biotin compound, (+)-biotinyl-3,6,9-trioxaundecanediamine (biotin–NH_2), onto the T-Tween 20/Au surface, the terminal hydroxy groups were activated by immersing the substrate in a dry DMF solution of DSC (0.1 M) and 4-(dimethylamino)pyridine (DMAP; 0.1 M) for 3 h at room temperature under an argon atmosphere. After treatment, new IR absorption peaks were observed at 1746 (asymmetric C=O stretching of imide), 1793 (symmetric C=O stretching of imide), and 1814 cm^{-1} (C=O stretching of ester bond; Figure 1b). The shapes and positions of the IR peaks were very similar to those obtained from the surfaces presenting *N*-hydroxysuccinimide-activated carboxylic acid groups.[16,48,49] The IR spectrum, therefore, confirmed the successful activation of hydroxy groups into the succinimidyl ester groups. The covalent attachment of biotin–NH_2 was performed by soaking the DSC-activated substrate in an ethanolic solution of biotin–NH_2 (1 mg mL^{-1}) for 1 h at room temperature, and the substrate was subsequently soaked in a 10 mM ethanolic solution of EG_2–NH_2 for 1 h at room temperature to deactivate any remaining reactive ester groups. After attachment of biotin–NH_2, the IR bands from the succinimidyl ester groups disappeared completely. In addition, we observed strong new peaks at 1704 (C=O stretching from the coupling between the succinimidyl ester group and the primary amine), 1657 (amide I of biotin–NH_2), and 1544 cm^{-1} (amide II of biotin–NH_2; Figure 1c). The ellipsometric thickness also increased to 31 Å from 23 Å, which, along with the IR spectrum, indicated the successful immobilization of biotin–NH_2.

We used SPR spectroscopy to characterize binding specificity and capacity of the T-Tween 20 films. The SAMs of HS–EG_5–COOH were used as a comparison.[24] SPR is an optical technique that monitors localized differences in the reflectivity of incident light from the interface between the prism and the gold substrate. Because the localized reflectivity differences are caused by the adsorption/desorption of molecules, the SPR technique has shown great potential for affinity biosensors, allowing real-time analysis of biospecific interactions without the use of labeled molecules.[50] In this work, we investigated the binding specificity and capacity of the biotinylated, T-Tween 20 film for streptavidin as well as the amount of nonspecific adsorption of fibrinogen and lysozyme. The binding capacity of the biotinylated, T-Tween 20/Au surface was measured to be 3261 RU (326.1 ng cm^{-2}) for streptavidin, while it was 8 RU (0.8 ng cm^{-2}) in the case of the intact T-Tween 20/Au surface (Figures 4a and b). The

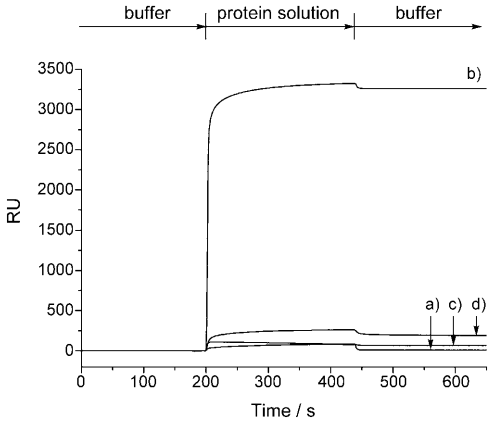

Figure 4. SPR sensorgrams for binding of streptavidin a) onto the intact T-Tween 20/Au surfaces, and for binding of b) streptavidin, c) lysozyme, and d) fibrinogen onto the biotinylated, T-Tween 20/Au surfaces.

value was larger than 2556 RU (255.6 ng cm^{-2}), which was obtained from the biotinylated SAMs of HS–EG_5–COOH,[24] indicating of a 28% enhanced binding capacity for streptavidin. The nonspecific adsorption of lysozyme and fibrinogen was comparable between the two biotinylated surfaces (66 vs 82 RU for lysozyme; 188 vs 103 RU for fibrinogen; Figures 4c and d).

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Covalent Immobilization of M-Tween 20 on Si/SiO_2

The SAMs of alkylalkoxysilanes and alkylchlorosilanes have been prepared on various substrates, including silicon oxide, glass, aluminum oxide, quartz, mica, and germanium oxide.[51] The driving force for the formation of the SAMs is thought to be covalent bonding between surface hydroxy groups and silane moieties. The formation of the SAMs of commercially available alkoxysilanes and chlorosilanes is generally followed by organic functionalization on the SAMs in the practical applications. For example, in the development and construction of biochips and sensors, one has widely used the covalent bond formation between the amine groups of biomolecules and the organic functional groups (aldehyde, epoxide, carboxylic acids, and hydroxy groups) on silanized glass substrates.[6,52] It might be an important strategy for the functionalization of Si/SiO_2 surfaces to synthesize other desired silanes and subsequently form SAMs, but the functionality incompatibility makes it cumbersome or 'undoable' to synthesize some desired silanes. For example, practically, it was difficult to synthesize silane compounds with the Tween 20 moiety because of a compatibility problem between the silane groups and the hydroxy groups. There were several strategies to introduce both EG moieties and reactive functional groups on SiO_2 surfaces: for example, surface-initiated polymerization of poly(ethylene glycol) methacrylate,[22,24] the coupling of star PEG prepolymers,[34] and the synthesis of random copolymers of poly(ethylene glycol) methyl ether methacrylate, 3-(trimethoxysilyl)propyl metharcylate, and *N*-acryloxysuccinimide and the subsequent formation of SAMs.[53] In this work, we used the strategy of post-functionalization for generating the SAMs of Tween 20 on Si/SiO_2: thiol-terminated SAMs were formed on Si/SiO_2, and M-Tween 20 was subsequently attached onto the surface by Michael addition. The Michael addition reactions between maleimides and sulfhydryl groups have been used for chemoselective coupling of biomolecules, such as sugars and oligonucleotides, which have a variety of complex functional groups.[37–40] It was thought that M-Tween 20 would be immobilized easily onto thiol-presenting surfaces through Michael addition, with another three terminal hydroxy groups kept intact for further uses.

We optimized the conditions for forming the SAMs of (3-mercaptopropyl)trimethoxysilane (MPTMS) on Si/SiO_2, and obtained the SAMs by immersing a substrate in a 1% (v/v) solution of MPTMS (solvents: DMF/1% (v/v) aqueous solution of acetic acid = 95:5 (v/v)) for 24 h at 100 °C. The thickness of the formed SAMs was measured to be about 4 Å. The XPS spectrum confirmed the formation of the SAMs: 166.9 (S $2p_{3/2}$) and 162.5 (S $2p_{1/2}$) eV. The MPTMS-coated substrate was then immersed in a sodium phosphate buffer solution (10 mM, pH 7.0) of M-Tween 20 (0.1 mg mL^{-1}) for 24 h at room temperature. After the reaction, the film thickness increased to 17 Å. The C1s and the N1s peaks were observed at 285.7 eV (C–*C*–O) and 398.8 eV, respectively, in the narrow-scan XPS spectrum (Figure 5). The reaction yield was calculated from the N/S atomic ratios; it was about 20%.

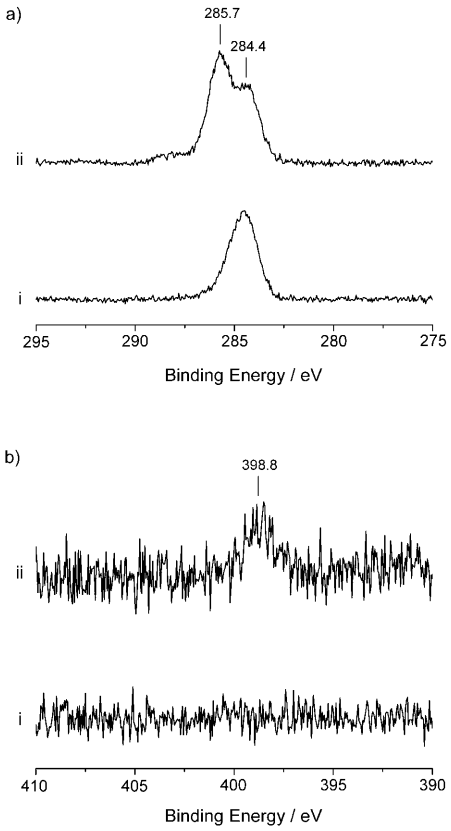

Figure 5. High-resolution XPS spectra of a) C1s and b) N1s regions acquired from the SiO_2 surfaces with the SAMs of MPTMS i) before and ii) after the coupling of M-Tween 20. MPTMS = (3-mercaptopropyl)trimethoxysilane.

Non-Biofouling Properties of M-Tween 20

The ellipsometric measurements were used for investigating the non-biofouling effect of the Si/SiO_2 surface that presented M-Tween 20 (M-Tween 20/SiO_2 surface) with ribonuclease A, BSA, fibrinogen, and lysozyme as model proteins. The SAMs of octadecyltrichlorosilane (OTS/SiO_2 surface) were used as a comparison. The thickness increases for the M-Tween 20/SiO_2 and OTS/SiO_2 surfaces were 3.0 and 18.8 Å (for lysozyme), 2.7 and 20.0 Å (for BSA), 2.7 and 15.3 Å (for ribonuclease A), and 5.0 and 51.2 Å (for fibrinogen), respectively (Figure 6). Therefore, the decreases in the nonspecific adsorption of proteins were 84% for lysozyme, 87% for BSA, 82% for ribonuclease A, and 90% for fibrinogen, compared with the OTS/SiO_2 surface. The non-biofouling effect of the M-Tween 20/SiO_2 surface was slightly lower than that of the T-Tween 20/Au surface, which may have been caused by a relatively lower EG density on the M-Tween 20/SiO_2 surface caused by the imperfect reaction between M-Tween 20 and the SAMs of MPTMS. Of interest, the M-Tween 20/SiO_2 surface prevented the nonspecific adsorption of sticky fibrinogen most effectively among the pro-

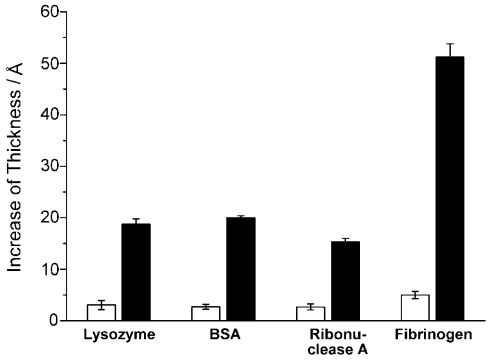

Figure 6. Ellipsometric thickness changes of the M-Tween 20/SiO_2 (white bars) and OTS/SiO_2 surfaces (black bars) after protein adsorption experiments.

teins studied. We reasoned that this characteristic behavior was caused by both the large size of fibrinogen and the low density of M-Tween 20. It was reported that star-shaped PEG molecules generated open spaces at their intermediate grafting density, and the sizes of the open spaces significantly affected the nonspecific adsorption of proteins, depending on the sizes of the proteins.[33] Briefly, the adsorption of proteins was prevented when the size of proteins was larger than that of the open space; smaller proteins were adsorbed onto the open space easily. It was thought that the low surface density of M-Tween 20 would generate open spaces and that the adsorption of the largest fibrinogen would be prevented most effectively.

Micropatterning of Streptavidin on the M-Tween 20/SiO_2 Surface

As a demonstration of the applicability of the M-Tween 20-modified surface to the selective immobilization of biomolecules, we generated micropatterns of biotin on Si/SiO_2 by microcontact printing. The same reaction conditions were employed for the activation of the terminal hydroxy groups of M-Tween 20 as T-Tween 20, and biotin–NH_2 was contact-printed with a poly(dimethylsiloxane) (PDMS) stamp that had relief features with lateral dimensions of 50 μm (width of lines, Figure 7a). After passivation of the non-contacted areas with EG_2–NH_2, the substrate was incubated in a phosphate-buffered saline (PBS) solution (pH 7.4) of TRITC-conjugated streptavidin (0.1 mg mL^{-1}) for 1 h. Figure 7b shows distinct 50-μm-wide lines of red fluorescence, which confirmed that the terminal hydroxy groups were coupled with biotin–NH_2 in a spatioselective manner by the DSC activation and microcontact printing. In addition, the effective suppression of the nonspecific adsorption of streptavidin was verified by the dark background regions.

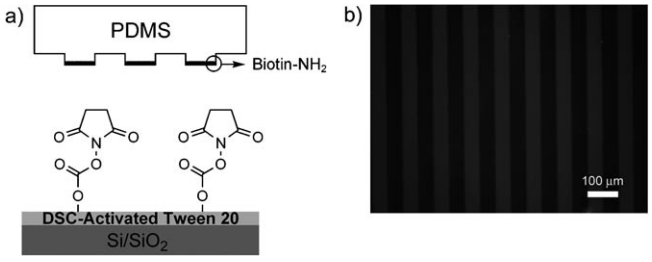

Figure 7. a) Simplified scheme for microcontact printing of biotin–NH_2 on the SiO_2 surface with DSC-activated M-Tween 20. b) Fluorescence image of TRITC-conjugated streptavidin bound to the resulting micropatterns of biotin. TRITC = tetramethylrhodamine isothiocyanate.

Conclusions

In summary, we designed and formed the stable SAMs of Tween 20 derivatives on gold and Si/SiO_2. The Tween 20 derivatives were asymmetrically functionalized, four-armed, PEG compounds in which the intrinsic non-biofouling property of Tween 20 was maintained and one arm of Tween 20 was derivatized for forming stable SAMs. The thiol-modified Tween 20 was used directly for gold surfaces, and the maleimide-modified Tween 20 was anchored to Si/SiO_2 through Michael addition. The resulting SAMs were stable in both water and organic solvents, and showed resistance against nonspecific adsorption of proteins. Further functionalization was achievable though the activation of the terminal hydroxy groups and subsequent coupling with biologically active molecules, which was demonstrated by the selective binding of streptavidin to the surface-attached biotin.

It is a first but important step in the improvement of biodevice performances to design and construct surfaces that possess both non-biofouling properties and chemical functionalizability. The formation of organic monolayers is an intensively studied strategy to construct molecularly-controlled, functional surfaces. The Tween 20 derivatives (T-Tween 20 and M-Tween 20) would be promising candidates as organic coating materials for the fabrication of man-made bioactive surfaces. We believe that the method demonstrated herein would provide a simple but versatile strategy for the functionalizable, non-biofouling coating of solid substrates and be a crucial starting point in the development of biomaterial-based devices.

Experimental Section

Materials

Si<100> wafers were obtained from Prolog Semicor, Ltd., Ukraine. 29-Mercapto-3,6,9,12,15,18-hexaoxanonacosanoic acid ($HS(CH_2)_{11}(OCH_2CH_2)_5OCH_2COOH$, HS-$EG_5$-COOH; COS Biotech, Inc., Korea), (3-mercaptopropyl)trimethoxysilane (MPTMS, 95%; Aldrich), *m*-dPEG_{12}-MAL (Quanta Biodesign, Ltd.), mPEG-maleimide (M.W. 5000; Shearwater), *N*-ethylmaleimide (98%; Sigma), *N,N′*-disuccinimidyl carbonate (DSC; Aldrich), 4-(dimethylamino)pyridine (DMAP, 99%, Fluka), 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC, 98+%; Aldrich), *N*-hydroxysuccinimide (NHS; 97%; Aldrich), (+)-biotinyl-3,6,9-trioxaundecanediamine (biotin–NH_2; Pierce), 2-(2-aminoethoxy)ethanol (EG_2–NH_2, 98%; Aldrich), dodecanethiol (DDT, 98%; Aldrich), octadecyltrichlorosilane (OTS; 90%; Aldrich), absolute ethanol (EtOH, 99.8+%; Merck), *N,N*-dimethylformamide (DMF; 99.8%), fibrinogen (fraction I: from human plasma; Sigma), lysozyme

(grade III: from chicken egg white; Sigma), ribonuclease A (type III-A from bovine pancrease; Sigma), bovine serum albumin (BSA; Sigma), TRITC-conjugated streptavidin (TRITC-SA; Pierce), sulfuric acid (H_2SO_4, 95.0+%; Junsei), acetic acid (99.0+%; Junsei), and hydrogen peroxide (H_2O_2, 30–35%; Junsei) were used as received. Ultrapure water (18.3 MΩ cm) from the Human Ultra Pure System (Human Corp., Korea) was used.

Synthesis of T-Tween 20 and M-Tween 20

Hydrolysis of Tween 20: NaOH (652 mg) was added to an aqueous solution (100 mL) of Tween 20 (5.0 g) at room temperature, and the resulting mixture was stirred for 12 h at room temperature. After removal of H_2O by vacuum evaporation, DMF (100 mL) was added, and the resulting mixture was stirred for three additional hours at room temperature. The product was then filtered through celite, and the filtrate was concentrated in vacuo to afford the product (**1**; 3.66 g, 86%).

Synthesis of T-Tween 20: The hydrolyzed Tween 20 (**1**, 1 g) and 11-mercaptoundecanoic acid (MUA, 230 mg) were dissolved in CH_2Cl_2 (30 mL). N,N′-Dicyclohexylcarbodiimide (DCC, 217 mg) was added to the solution, and the resulting mixture was stirred for 12 h at room temperature. The precipitated white solid was filtered off, and then the filtrate was evaporated under reduced pressure. The crude product was purified by flash column chromatography to afford the oily compound (536 mg, 45%).

Synthesis of M-Tween 20: Triethylamine (TEA, 20 μL) was added to a CH_2Cl_2 (30 mL) solution of the hydrolyzed Tween 20 (**1**, 100 mg) and 6-maleimido-1-hexanoic acid succinimidyl ester (MHASE, 33 mg) at room temperature, and the resulting mixture was stirred for 12 h at room temperature. The reaction mixture was concentrated in vacuo and purified by flash silica column chromatography to afford the product (83 mg, 52%).

Preparation of SAMs of T-Tween 20 on Gold

The gold substrates were prepared by thermal evaporation of 5 nm of titanium and 100 nm of gold onto silicon wafers. Prior to use, gold substrates were cleaned for 1 min in piranha solution (3:7 by volume of 30% H_2O_2 and H_2SO_4, *Caution: piranha solution reacts violently with most organic materials and must be handled with extreme care*), rinsed with H_2O and ethanol, and dried under a stream of argon. The SAMs of T-Tween 20 were prepared by immersing the gold substrates in a 0.1 mM ethanolic solution of T-Tween 20 overnight. After the formation of SAMs, the substrates were rinsed with ethanol several times and then dried under a stream of argon.

Introduction of M-Tween 20 on Si/SiO$_2$ via Michael Addition

Prior to the chemical modification of the Si/SiO_2 surfaces, the substrates were treated with an O_2 plasma for 10 min to clean the surfaces as well as to generate OH groups on SiO_2 surfaces (Harrick PDC-002, medium setting). To form thiol-terminated SAMs on Si/SiO_2, the substrates were immersed in a 1% (v/v) solution of MPTMS in a mixed solvent composed of DMF and 1% (v/v) aqueous solution of acetic acid (95:5, v/v) for 24 h at 100 °C. After formation of the SAMs, the substrates were rinsed with DMF, ethanol, and water several times, dried under a stream of argon, and then baked for 10 min at 100 °C. After baking, the substrates were dipped into a 10 mM sodium phosphate buffer solution (pH 7.0) containing M-Tween 20 (0.1 mg mL^{-1}) for 24 h at room temperature, and then washed with the used buffer solution, distilled water, and ethanol. The unreacted thiol groups were blocked by dipping the substrates into a 10 mM sodium phosphate buffer solution (pH 7.0) containing *m*-dPEG$_{12}$-MAL and *N*-ethylmaleimide at room temperature, and washing with the sodium phosphate buffer, distilled water, and ethanol.

Study of Non-Biofouling Properties

Four kinds of proteins (fibrinogen, lysozyme, ribonuclease A, and BSA) were selected for investigating degrees of nonspecific protein binding. The substrates presenting Tween 20 moieties were immersed in 10 mM sodium phosphate buffer solutions (pH 7.5) of proteins (1 mg mL^{-1}) for 2 h at room temperature. The amount of nonspecifically adsorbed proteins on the surfaces was determined by measuring ellipsometric thickness changes after rinsing the substrates with water.

Activation of Hydroxy Groups with N,N′-Disuccinimidyl Carbonate (DSC)

To activate terminal hydroxy groups of Tween 20, the films were immersed in the dry DMF solution containing 0.1 M DSC and 0.1 M DMAP for 3 h at room temperature under an argon atmosphere.[43] The DSC-activated substrates were rinsed with DMF and ethanol, and then dried in a stream of argon.

Surface Plasmon Resonance (SPR) Spectroscopy

The gold substrates with DSC-activated T-Tween 20 were soaked in an ethanolic solution of biotin–NH_2 (1 mg mL^{-1}) for 1 h at room temperature. After the reaction, the substrates were washed with ethanol, and then dried in a stream of argon. Subsequently, to deactivate the unreacted reactive ester groups, the substrates were immersed in a 10 mM ethanolic solution of EG_2–NH_2 for 1 h at room temperature. After treating with the blocking agent, the substrates were rinsed with ethanol, and then dried in a stream of argon. SPR measurements were performed with a Biacore instrument (model: Biacore X, Sweden) to investigate specific and nonspecific binding capacity of proteins on the gold surfaces with intact T-Tween 20 and biotinylated T-Tween 20. The specific binding of streptavidin, the target protein (0.1 mg mL^{-1}; PBS solution (pH 7.4)) and the nonspecific binding of model proteins, fibrinogen and lysozyme (1 mg mL^{-1}; PBS solution (pH 7.4)), were carried out by one injection of 20 μL of a protein solution with a constant flow of the solution (5 μL min^{-1}). After the elution of the protein solution for 4 min, the surface was washed with a constant flow rate of PBS buffer solution (5 μL min^{-1}). Protein binding resulted in a shift in the resonance angle that was reported in resonance units (RU; 10000 RU = 1.0°).[54] As a control experiment, we also prepared biotinylated substrate with the SAM of HS–EG_5–COOH through EDC/NHS activation and subsequent coupling of biotin–NH_2.

Microcontact Printing (μCP)

A PDMS stamp was prepared according to the literature method by using Sylgard 184 silicon elastomer (Dow Corning).[55] Before use, the PDMS stamp was oxidized by an oxygen plasma cleaner (Harrick PDC-002 at medium setting) for 1 min. The oxidized PDMS stamp was coated with biotin–NH_2 by spin casting a 5 mM ethanolic solution of biotin–NH_2 at 1000 rpm for 1 min. A micropattern of biotin was generated by μCP by using the PDMS stamp, which had relief features with lateral dimensions of 50 μm (width of line), on the Si/SiO_2 surface presenting DSC-activated M-Tween 20. After μCP of biotin–NH_2, the sample was immersed immediately in a 10 mM ethanolic solution of EG_2–NH_2, and after 1 h the sample was rinsed with ethanol. For the visualization of the pattern, the sample was incubated in a solution of TRITC-conjugated streptavidin (0.1 mg mL^{-1}) in PBS buffer solution (pH 7.4) at room temperature. After 1 h, the sample was removed and washed several times with PBS buffer solution and distilled water. Fluorescence images were acquired on an IX 71 fluorescence microscope (Olympus, Japan) to investigate the streptavidin-bound surface.

Instrumentation

Polarized infrared external reflectance spectroscopy (PIERS) spectra were obtained in a single reflection mode by using a dry N_2-purged Thermo Nicolet Nexus FT-IR spectrophotometer equipped with the smart SAGA (Smart Apertured Grazing Angle) accessory. The *p*-polarized light was incident at 80° relative to the surface normal to the substrate and a narrow band mercury–cadmium–telluride (MCT) detector cooled with liquid nitrogen was used to detect the reflected light. We averaged 4000 scans to yield the spectrum at a resolution of 2 cm^{-1} and all spectra were reported in the absorption mode relative to a clean gold surface. The thicknesses of the monolayer and polymer films were measured with a Gaertner L116 s ellipsometer (Gaertner Scientific Corporation, IL) equipped with a He–Ne Laser (632.8 nm) at a 70° angle of incidence. A refractive index of 1.46 was used for all films. The XPS study

was performed with a VG-Scientific ESCALAB 250 spectrometer (UK) with monochromatized $Al_{K\alpha}$ X-ray source. Emitted photoelectrons were detected by a multi-channel detector at a take off angle of 90° relative to the surface. During the measurements, the base pressure was 10^{-9}–10^{-10} Torr. Survey spectra were obtained at a resolution of 1 eV from three scans and high-resolution spectra were acquired at a resolution of 0.05 eV from 5–20 scans.

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