Spatially-Addressable Immobilization of Macromolecules on Solid Supports

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Abstract: A method is described for immobilization of receptors, antibodies, or other macromolecules at precise locations on solid substrates. We have combined photolithographic techniques with the use of a "caged" biotin analogue that has been covalently linked to the substrate surface. Exposure to near UV light through a photolithographic mask yields biotin sites for streptavidin binding. Biotinylated macromolecules are then immobilized via a biotin−streptavidin−biotin bridge. Molecules may be attached at selected locations by carrying out repeated rounds of exposure, streptavidin binding, and application of the biotinylated reagent. We have demonstrated the immobilization of fluorescein−streptavidin molecules in 500 μm × 500 μm sites, and the localization of two biotinylated antibodies at different sites on a planar substrate surface. We anticipate that the technique will prove useful in drug screening, diagnostics, and biosensor applications.

Introduction

Over the last five years, there has been growing scientific and commercial interest in the development of techniques for selectively immobilizing macromolecules at specific locations on solid supports. Potential applications for these techniques occur in fields ranging from biosensor design to diagnostic product development and the emergence of new technologies for drug discovery. Desirable features of any practical immobilization method for forming arrays of biopolymers include applicability to a wide range of biological macromolecules under conditions that retain the activity of the biomolecule, a high degree of spatial resolution for creating patterned arrays of molecules, and the ability to control the surface coverage, orientation, and site density of immobilized molecules. Numerous examples of techniques for immobilizing macromolecules on solid supports have been reported in the literature. Traditional covalent cross-linking techniques and properties of support materials have been summarized by Weetall et al.[1] and Royer.[2] Recent studies have focused on the use of deep ultraviolet exposure of organosilanes to create patterns of alternating hydrophobic/hydrophilic surfaces to which proteins may be adsorbed or covalently coupled.[3,4] Other groups have utilized Langmuir−Blodgett techniques and the properties of biotinylated lipid or alkanethiol self-assembled monolayers to immobilize antibodies[5] and create patterned arrays of proteins and cells.[6,7] Direct photochemical cross-linking of proteins to support materials has been employed as well.[8,9] These approaches are subject to a variety of limitations. Immobilization techniques involving nonspecific adsorption may suffer from lack of control over the site density or orientation of the adhered molecules, and immobilization of different biomolecules at distinct locations on the same surface may be difficult if not impossible to achieve. Photochemical cross-linking, on the other hand, may require the use of reaction conditions that degrade or denature biological molecules (e.g., photochemical degradation of tryptophan residues), while low yields may necessitate the use of large quantities of reagent.

In this paper, we describe a novel approach to immobilizing receptors, antibodies, or other macromolecules at precise locations on solid supports. We combine photolithographic techniques with the use of photodeprotectable biotin analogues ("caged biotin")[10] covalently linked to a solid substrate to permit spatially-localized attachment of biotinylated macromolecules via high-affinity biotin−streptavidin−biotin binding interactions. The success of this approach in practical applications will depend on a number of experimental factors, including the following: (i) synthesis of biotin analogues with greatly reduced binding affinity for streptavidin until after they have undergone photodeprotection; (ii) efficient photolysis of the protecting group under aqueous conditions; (iii) uniform derivatization of the substrate surface, using linkers that allow tethered biotin molecules to access the deep binding sites of the streptavidin molecule; (iv) optimization of substrate surface properties to minimize nonspecific binding of streptavidin or biotinylated

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(10) Our use of the terms photodeprotectable and caged biotin to describe biotin analogues in which the imidazole ring nitrogen atom(s) is (are) acylated with photolabile protecting groups should not be confused with the carboxyl-protected "photoactivatable biotins" described by Forster et al. (*Nucleic Acids Res.* **1985**, *13*, 745−761) and commercially available from Calbiochem (San Diego, CA).

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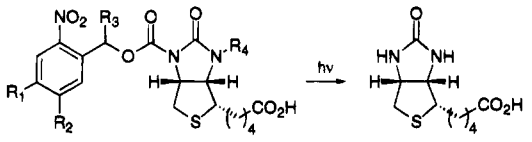
macromolecules; (iv) effective masking techniques to provide efficient photodeprotection and good contrast relative to unexposed regions (vi) finally, sensitive detection methods for assessing the extent and precision of immobilization will be required. Here, we describe initial results using nitroveratryloxycarbonyl (NVOC)- and other *o*-nitrobenzyl-protected biotin analogues that address some of these issues. The photolabile



Caged Biotin **Biotin**

R_1	R_2	R_3	R_4	Name
OMe	OMe	H	H	Nvoc-biotin-OH
OMe	OMe	Me	H	Menvoc-biotin-OH
OMe	OMe	Me	Menvoc	$(Menvoc)_2$-biotin-OH
OCH_2O		Me	H	Menpoc-biotin-OH
OCH_2O		Me	Menpoc	$(Menpoc)_2$-biotin-OH

NVOC protecting group has been described previously by Patchornik et al.[11] See the review by McCray and Trentham[12] for a general discussion of the properties of photoreactive caged compounds and their uses. We chose to synthesize *N*-1′- and *N*-3′-biotin derivatives since crystallographic data for the biotin–streptavidin complex indicate that polar interactions between the biotin ureido group and the protein molecule are extensively involved in stabilization of the complex.[13] The presence of bulky, photolabile protecting groups at these positions was therefore anticipated to cause a significant reduction in binding affinity. We have made use of these novel compounds to demonstrate spatially-directed immobilization of streptavidin and antibody molecules on derivatized glass surfaces. Part of this work has been reported previously.[14,15]

Materials and Methods

Synthesis of Biotin Methyl Ester (Biotin-OMe, 3). Biotin methyl ester was prepared by adding 2.0 g (8.19 mmol) of D-biotin to a methanolic HCl solution prepared from 2.5 mL of acetyl chloride in 40 mL of anhydrous methanol. After stirring for 15 h, the solvent was removed under reduced pressure to afford 2.11 g of biotin-OMe as a white solid, with a melting point of 116–118 °C (100% yield).

Synthesis of *N*-1′-(6-Nitroveratryloxycarbonyl)biotin Methyl Ester (NVOC-Biotin-OMe, 4). Methods for the preparation of acylimidazolidinones, such as biotin derivatives, are well known.[16,17] Treatment of biotin methyl ester (biotin-OMe, **3**) with methyl chloroformate in refluxing chloroform (no base) for 72–80 h affords a mixture heavily favoring the *N*-1′-derivative. Under similar conditions, the use of 6-nitroveratryloxycarbonyl chloride (NVOC-Cl, **1**)[18] gave *N*-1′-(6-nitroveratryloxycarbonyl)biotin methyl ester (NVOC-biotin-OMe) in 47% yield after chromatography and crystallization. A solution of 1.0 g (3.87 mmol) of biotin-OMe and 1.6 g (5.81 mmol) of NVOC-Cl in 10 mL of chloroform was heated to reflux for 50 h. The product was purified via flash-column chromatography on silica gel (3% methanol, 3% acetone, 94% chloroform as eluent) and recrystallized from methylene chloride/ether to afford 0.90 g of NVOC-biotin-OMe as a yellow solid (47% yield, 84% yield based on unreacted starting biotin-OMe recovered (0.44 g, 44% recovery)): mp 199–203 °C; IR (KBr) 1740, 1705 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 1.38–1.62 (m, 2 H), 1.62–1.77 (m, 4 H), 2.35 (d, J = 6.5 Hz, 2 H), 3.05 (dd, J = 6.3, 13.0 Hz, 1 H), 3.16–3.27 (m, 2 H), 3.68 (s, 3 H), 3.96 (s, 3 H), 4.04 (s, 3 H), 4.22–4.29 (m, 1 H), 4.90–4.97 (m, 1 H), 5.05 (br s, 1 H), 5.73 (s, 2 H), 7.77 (s, 1 H), 7.86 (s, 1 H); MS (FAB) *m/z* 498 (MH^+). Anal. Calcd for $C_{21}H_{27}N_3O_9S$: C, 50.69; H, 5.47; N, 8.45. Found: C, 50.28; H, 5.28; N, 8.23.

Synthesis of NVOC-Biotin *p*-Nitrophenyl Ester (NVOC-biotin-ONP, 2). A solution of 340 mg (0.930 mmol) of biotin-ONP and 450 mg (1.63 mmol) of NVOC-Cl in 9 mL of $CHCl_3$ (hydrocarbon as preservative) was heated to reflux for 60 h. The reaction mixture was cooled, concentrated under reduced pressure, and chromatographed on silica gel (3% acetone, 3% MeOH, 94% $CHCl_3$) to afford 0.20 g of recovered biotin-ONP (59%) and 0.24 g of the product NVOC-biotin-ONP as an orange solid. The product was recrystallized from EtOAc/hexanes to give 0.21 g (37% yield, 91% yield based on recovered starting material) of NVOC-biotin-ONP as a pale yellow solid: mp 200–201 °C; IR (KBr) 1735, 1710 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) 1.50–1.65 (m, 2 H), 1.67–1.85 (m, 4 H), 2.64 (t, J = 7.0 Hz, 2 H), 3.06 (dd, J = 6.5, 14.0 Hz, 1 H), 3.17–3.29 (m, 2 H), 3.96 (s, 3 H), 4.03 (s, 3 H), 4.23–4.30 (m, 1 H), 4.90–4.98 (m, 1 H), 5.22 (br s, 1 H), 5.72 (s, 2 H), 7.25–7.31 (m, 2 H), 7.75 (s, 1 H), 7.80 (s, 1 H), 8.23–8.31 (m, 2 H); MS (FAB) *m/z* 605 (MH^+). Anal. Calcd for $C_{26}H_{28}N_4O11S$: C, 51.65; H, 4.67; N, 9.27. Found: C, 51.47; H, 4.70; N, 9.17.

Synthesis of *N*-1′-(6-Nitroveratryloxycarbonyl)biotin (NVOC-biotin-OH, 5). A solution of NVOC-biotin-OMe (1.40 g, 2.814 mmol) in 40 mL of THF and 20 mL of 3 N HCl was heated to reflux for 4 h. The reaction mixture was cooled and the solvent removed under reduced pressure on a rotary evaporator. The crude residue was recrystallized from EtOAc to afford 1.20 g of the product NVOC-biotin-OH as an off-white solid (88% yield): mp 219–221 °C; ^1H NMR (300 MHz, $CDCl_3$ plus 3 drops of DMF-d_7) δ 1.44–1.56 (m, 2 H), 1.61–1.74 (m, 2 H), 1.74–1.88 (m, 2 H), 2.33 (t, J = 7.3 Hz, 2 H), 3.04 (dd, J = 5.2, 12.7 Hz, 1 H), 3.15 (br d, J = 12.7 Hz, 1 H), 3.20–3.27 (m, 1 H), 3.97 (s, 3 H), 4.03 (s, 3 H), 4.25–4.32 (m, 1 H), 4.86–4.94 (m, 1 H), 5.69 (s, 2 H), 7.04 (s, 1 H), 7.76 (s, 1 H), 7.88 (s, 1 H), 8.02 (s, 1 H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 24.3, 27.6, 27.8, 33.2, 38.0, 55.0, 55.9, 56.4, 57.2, 62.1, 64.8, 107.3, 110.1, 127.5, 138.1, 147.3, 151.2, 153.8, 154.7, 175.0; MS (ESI) *m/z* 484 (MH^+). Anal. Calcd for $C_{20}H_{25}N_3O_9S$: C, 49.68; H, 5.21; N, 8.69. Found: C, 49.33; H, 5.16; N, 8.45.

Synthesis of *p*-[(α-Methyl-6-nitroveratryloxycarbonyl)oxy] nitrobenzene (Menvoc-ONP, 6). A solution of 1.95 g (9.68 mmol) of 4-nitrophenyl chloroformate, 2.00 g (8.80 mmol) of α-methyl-6-nitroveratryl alcohol, and 0.85 mL (10.5 mmol) of pyridine in 35 mL of CH_2Cl_2 was stirred for 16 h at room temperature. An additional 200 mg (1.0 mmol) of chloroformate and 0.20 mL (2.5 mmol) of pyridine were added, and the stirring was continued for 48 h. The reaction mixture was partitioned between CH_2Cl_2 and 1 N HCl. The combined organic phase was washed (0.1 N NaOH), dried ($MgSO_4$), and concentrated to give a white solid. Chromatography on silica gel (100% CH_2Cl_2) afforded 3.36 g (97% yield) of pure product as a white solid: mp 145–147 °C; ^1H NMR (300 MHz, $CDCl_3$) δ 1.77 (d, J = 6.4 Hz, 3 H), 3.96 (s, 3 H), 4.03 (s, 3 H), 6.55 (q, J = 6.4 Hz, 1 H), 7.13 (s, 1 H), 7.32–7.38 (m, 2 H), 7.52 (s, 1 H), 8.23–8.28 (m, 2 H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 22.0, 56.4, 56.6, 73.7, 107.7, 107.8, 121.6, 125.3, 131.3, 153.9. This compound was used without further purification.

Synthesis of *N*-1′,*N*-3′-Bis(α-methyl-6-nitroveratryloxycarbonyl)-biotin Methyl Ester ($(Menvoc)_2$-biotin-OMe, 7). A suspension of NaH (42 mg of 60% in oil, 1.05 mmol) was added to a solution of biotin-OMe (250 mg, 0.968 mmol) and Menvoc-ONP (463 mg, 1.18 mmol) in 10 mL of $CHCl_3$, generating a white precipitate. The slurry was stirred for 14 h at room temperature, and TLC analysis indicated no reaction had taken place. DMF (3 mL) and an additional 40 mg (1.0 mmol) of NaH suspension were added, and the resultant solution was heated to reflux for 4.5 h. The reaction mixture was cooled and partitioned between $CHCl_3$ and saturated NaCl, dried ($MgSO_4$), and

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evaporated to a light yellow oil. Chromatography on silica gel (1% MeOH to 5% MeOH in $CHCl_3$) gave 55 mg (11% yield) of the monoprotected Menvoc-biotin-OMe and 350 mg (47% yield) of the diprotected $(Menvoc)_2$-biotin-OMe as a colorless oil: ^1H NMR (300 MHz, $CDCl_3$) δ 1.04−1.57 (m, 4 H), 1.60−1.77 (m, 8 H), 2.15−2.24 (m, 1 H), 2.27−2.37 (m, 1 H), 2.88−3.27 (m, 2 H), 3.38−3.52 (m, 1 H), 3.63 (s, 1/4 × 3 H), 3.64 (s, 1/4 × 3 H), 3.67 (s, 1/4 × 3 H), 3.68 (s, 1/4 × 3 H), 3.85−4.06 (m, 12 H), 4.62−4.78 (m, 2 H), 6.59−6.76 (m, 2 H), 7.36−7.67 (m, 4 H). The compound was carried on to the next step without further characterization.

Synthesis of *N*-1′,*N*-3′-Bis(α-methyl-6-nitroveratryloxycarbonyl)-biotin ($(Menvoc)_2$-biotin-OH, 8). A solution of 273 mg (0.357 mmol) of $(Menvoc)_2$-biotin-OMe in 10 mL of THF and 8 mL of 3 N HCl was heated to reflux for 5 h. After cooling, the reaction mixture was partitioned between $CHCl_3$ and saturated NaCl and the organic phase was dried ($MgSO_4$) and concentrated to give a colorless oil. Chromatography on silica gel (5% MeOH in $CHCl_3$) gave 222 mg (83% yield) of $(Menvoc)_2$-biotin-OH as a pale yellow solid: mp 120−125 °C; ^1H NMR (300 MHz, $CDCl_3$) δ 1.05−1.66 (m, 6 H), 1.66−1.78 (m, 6H), 2.22−2.28 (m, 1 H), 2.33−2.42 (m, 1 H), 2.88−3.04 (m, 1 H), 3.08−3.29 (m, 1 H), 3.38−3.52 (m, 1 H), 3.84−4.05 (m, 12 H), 4.62−4.77 (m, 2 H), 6.58−6.76 (m, 2 H), 7.35−7.66 (m, 4 H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 22.1, 22.3, 22.4, 24.1, 24.4, 27.5, 27.5, 27.8, 28.0, 28.3, 28.5, 33.7, 33.8, 36.2, 36.5, 36.6, 36.9, 52.8, 53.1, 53.4, 54.2, 56.6, 56.8, 56.8, 59.0, 59.0, 59.1, 59.3, 60.0, 60.2, 60.4, 60.5, 72.5, 72.6, 107.7, 107.8, 108.0, 108.0, 108.8, 109.0, 109.3, 109.4, 109.5, 109.7, 131.9, 132.2, 132.2, 132.3, 132.4, 139.6, 139.6, 148.4, 148.4, 148.6, 148.8, 148.8, 150.8, 151.0, 151.0, 151.2, 151.4, 154.0, 154.1, 154.2, 154.2, 178.7, 178.8; MS (ESI) *m/z* 749 $(M + Na)^+$. Anal. Calcd for $C_{32}H_{38}N_4O_{15}S$: C, 51.20; H, 5.10; N, 7.46. Found: C, 51.37; H, 5.29; N, 7.30.

Synthesis of *p*-[(α-Methyl-6-nitropiperonyloxycarbonyl)oxy]-nitrobenzene (Menpoc-ONP, 9). A solution of 2.15 g (10.7 mmol) of 4-nitrophenylchloroformate, 2.00 g (9.47 mmol) of α-methyl-6-nitropiperonyl alcohol, and 0.92 mL (11.4 mmol) of pyridine in 35 mL of CH_2Cl_2 was stirred for 16 h at room temperature. An additional 200 mg (1.0 mmol) of chloroformate and 0.20 mL (2.5 mmol) of pyridine were added, and the stirring was continued for 48 h. The reaction mixture was partitioned between CH_2Cl_2 and 1 N HCl. The combined organic phase was washed (0.1 N NaOH), dried ($MgSO_4$), and concentrated to give a white solid. Chromatography on silica gel (100% CH_2Cl_2) afforded 3.32 g (93% yield) of pure product as a white solid: mp 157−163 °C dec; ^1H NMR (300 MHz, $CDCl_3$) δ 1.75 (d, J = 7.0 Hz, 3 H), 6.15 (s, 2 H), 6.42 (q, J = 7.0 Hz, 1 H), 7.14 (s, 1 H), 7.33−7.38 (m, 2 H), 7.51 (s, 1 H), 8.22−8.28 (m, 2 H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 22.0, 73.7, 103.2, 105.4, 105.6, 121.6, 125.2, 125.5, 133.6, 145.4, 147.7, 151.4, 152.6, 155.3. This compound was used without further purification.

Synthesis of *N*-1′,*N*-3′-Bis(α-methyl-6-nitropiperonyloxycarbonyl)biotin Methyl Ester ($(Menpoc)_2$-biotin-OMe, 10). A suspension of NaH (60% in oil, 42 mg, 1.05 mmol) was added to a solution of biotin-OMe (250 mg, 0.968 mmol) and Menpoc-ONP (435 mg, 1.16 mmol) in 10 mL of $CHCl_3$, generating a white precipitate. The slurry was stirred for 14 h at room temperature, and TLC analysis indicated no reaction had taken place. DMF (3 mL) and an additional 40 mg (1.0 mmol) of NaH suspension were added, and the resultant solution was heated to reflux for 4.5 h. The reaction mixture was cooled and partitioned between $CHCl_3$ and saturated NaCl, dried ($MgSO_4$), and evaporated to a light yellow oil. Chromatography on silica gel (1% MeOH to 5% MeOH in $CHCl_3$) gave 44 mg (9% yield) of the monoprotected Menpoc-biotin-OMe and 320 mg (45% yield) of the diprotected $(Menpoc)_2$-biotin-OMe as a colorless oil: ^1H NMR (300 MHz, $CDCl_3$) δ 1.08−1.39 (m, 3 H), 1.42−1.67 (m, 3 H), 1.67−1.77 (m, 6 H), 2.18−2.27 (m, 1 H), 2.27−2.35 (m, 1 H), 2.84−3.00 (m, 1 H), 3.19−3.30 (m, 1 H), 3.39−3.52 (m, 1 H), 3.64 (s, 1/4 × 3 H), 3.65 (s, 1/4 × 3 H), 3.66 (s, 1/4 × 3 H), 3.67 (s, 1/4 × 3 H), 4.55−4.72 (m, 2 H), 6.08−6.20 (m, 4 H), 6.48−6.58 (m, 2 H), 7.26−7.32 (M, 2 H), 7.49−7.54 (m, 2 H). The compound was carried on to the next step without further characterization.

Synthesis of *N*-1′,*N*-3′-Bis(α-methyl-6-nitroveratryloxycarbonyl)-biotin ($(Menpoc)_2$-biotin-OH, 11). A solution of 239 mg (0.326 mmol) of $(Menpoc)_2$-biotin-OMe in 15 mL of THF and 5 mL of 3 N HCl was heated to reflux for 6 h. After cooling, the reaction mixture was partitioned between $CHCl_3$ and saturated NaCl and the organic phase was dried ($MgSO_4$) and concentrated to give a colorless oil. Chromatography on silica gel (5% MeOH in $CHCl_3$) gave 172 mg (74% yield) of $(Menpoc)_2$-biotin-OH as a pale yellow solid: mp 132−137 °C; ^1H NMR (300 MHz, $CDCl_3$) δ 1.10−1.45 (m, 2 H), 1.45−1.68 (m, 2 H), 1.68−1.77 (m, 6 H), 2.25−2.33 (m, 1 H), 2.33−2.41 (m, 1 H), 2.85−2.97 (m, 1 H), 3.20−3.32 (m, 1 H), 3.40−3.53 (m, 1 H), 4.55−4.72 (m, 2 H), 6.09−6.16 (m, 4 H), 6.48−6.58 (m, 2 H), 7.24−7.31 (m, 2 H), 7.49−7.55 (m, 2 H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 22.2, 22.3, 22.4, 22.4, 24.3, 24.4, 27.5, 27.6, 28.0, 28.0, 33.8, 33.9, 36.2, 36.4, 52.4, 52.6, 58.8, 60.5, 72.4, 72.4, 103.4, 103.4, 105.5, 105.6, 106.2, 106.3, 106.7, 106.7, 106.8, 134.3, 134.3, 134.4, 134.5, 134.7, 141.7, 141.7, 147.7, 150.6, 151.0, 151.0, 151.1, 152.8, 152.8, 152.9, 178.8; MS (ESI) *m/z* 741 $(M + Na)^+$. Anal. Calcd for $C_{30}H_{30}N_4O_{15}S$: C, 50.14; H, 4.21; N, 7.80. Found: C, 50.32; H, 5.39; N, 7.64.

HPLC Analysis of Caged Biotin Photolysis Kinetics. Reaction conditions for photolysis of NVOC-biotin-OH and other caged biotin analogues were studied by exposing 0.10 mM solutions in an appropriate solvent or buffer to UV light (363 nm, 10 mW/cm^2) for varying lengths of time, followed by injection of samples into a Beckman Instruments (Palo Alto, CA) System Gold HPLC system and monitoring the loss of starting material by measuring absorption at 365 nm. Analysis of the amount of remaining starting material as a function of exposure time allows one to determine the photolysis half-time, i.e., the exposure time required for 50% loss of starting material.

Covalent Attachment of Caged Biotin to Glass Microscope Slides. Commercially-available glass microscope slides (Erie Scientific, Portsmouth, NH) were cleaned by sonication in Micro detergent (Baxter Scientific, McGaw Park, IL; 10% in water, 70 °C), followed by rinsing in deionized water, a 3 min immersion in 10% (w/v) NaOH (70 °C), more rinsing in deionized water, a 1 min immersion in 1% HCl, and further rinsing in deionized water. After a final ethanol rinse, the slides were dried under a stream of nitrogen and inspected for spots or scratches.

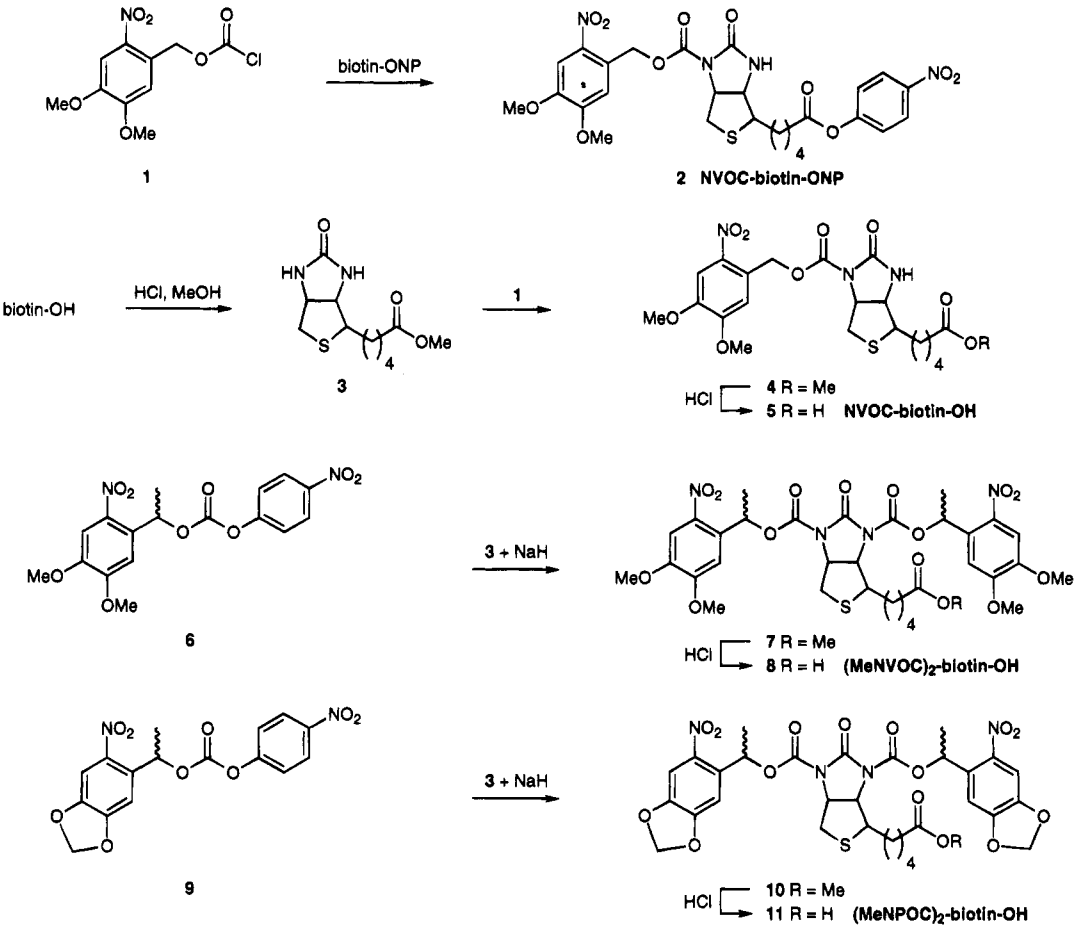
Freshly-cleaned glass slides were placed in plastic staining jars and immersed in a 1% solution of 1:10 mole ratio *tert*-butyloxycarbonyl (tBOC)-(3-aminopropyl)triethoxysilane/methyltriethoxysilane in methylene chloride overnight. The tBOC-protected (3-aminopropyl)-triethoxysilane was prepared from (3-aminopropyl)triethoxysilane (Petrarch, Bristol, PA) with di-*tert*-butyl dicarbonate in methylene chloride at 0 °C. After pouring off the silation solution, the slides were rinsed with methylene chloride and toluene, and dried under a stream of nitrogen. The slides were then placed in glass drying racks, allowed to sit on the bench for 30 min, and finally baked at 100 °C for 1 h.

The tBOC-3-aminopropyl slides were deprotected by a 30 min immersion in 50% trifluoroacetic acid/methylene chloride, followed by rinsing in methylene chloride, and neutralization of surface amines by immersing in 5% *N,N*-diisopropylethylamine/methylene chloride (2×; 5 min each). The slides were then rinsed in methylene chloride and ethanol, and dried under a stream of nitrogen.

NVOC-protected 6-aminocaproic acid was coupled onto the freshly-deprotected 3-aminopropyl slides[19] using (benzotriazol-1-yloxy)tris-(dimethylamino)phosphonium hexafluorophosphate (BOP) activation chemistry. NVOC-6-aminocaproic acid, 1-hydroxybenzotriazole hydrate (HOBT), and *N,N*-diisopropylethylamine (DIEA) (1:1.1:2 final mole ratio) were dissolved in a small volume (1/10 of the final volume) of *N,N*-dimethylformamide (DMF) or 1-methyl-2-pyrrolidinone (NMP). Similarly, BOP reagent was dissolved in a 1/10 volume of DMF or NMP. The two solutions were mixed and allowed to activate for 10

(19) tBOC-protected 3-aminopropyl slides and NVOC-6-aminocaproic acid derivatized slides were used in these studies because they were routinely produced for other purposes at the time, and were stored with the amine protected. In subsequent biotin−streptavidin binding experiments, we have streamlined the glass derivatization procedure and obtained better consistency in our results by eliminating the use of detergent, NaOH, and HCl in the cleaning step (we currently use a Nochromix solution (Godax Labs, New York, NY)), we have eliminated the tBOC protection and subsequent deprotection steps entirely, we are using neat (3-aminopropyl)triethoxysilane (2% (v/v) in 95% ethanol/water; 15 min immersion) for the silylation step, and we couple Fmoc-protected 6-aminocaproic acid or other spacer molecules directly to the freshly prepared 3-aminopropyl slides before covalently attaching biotin analogues.

Scheme 1. Synthesis of Caged Biotin Analogues



min, followed by dilution to the final concentration (100 mM NVOC-aminocaproic acid). The slides were arranged on a bench top, and 0.4 mL of activated NVOC-6-aminocaproic acid solution was layered onto each. The reaction was allowed to proceed for 2 h at room temperature, followed by rinsing in DMF, methylene chloride, and ethanol. In order to acetylate any residual unreacted amines, the slides were then capped by immersion for 1 h in 25% acetic anhydride/pyridine/catalytic 4-(dimethylamino)pyridine (DMAP), followed by rinsing in DMF, methylene chloride, and ethanol, and dried under a stream of nitrogen.

NVOC-6-aminocaproic slides were deprotected by photolysis in 5 mM H_2SO_4/dioxane using a large area illuminator (OAI, Inc., Milpitas, CA; 500 W mercury arc lamp; 350 −450 nm dichroic reflector; 12 mW/cm^2) for 12 min. The slides were then neutralized by two successive immersions in 5% DIEA/DMF for 5 min each, followed by rinsing in DMF, methylene chloride, and ethanol and drying under a stream of nitrogen. Caged biotin analogues having a carboxy-terminal activated ester were coupled to derivatized glass surfaces by layering a 50−100 mM solution in 5% DIEA/DMF onto the surface and reacting overnight. Caged biotin analogues having a free carboxyl group were coupled onto freshly-deprotected slides using BOP activation chemistry as described above (50−100 mM final concentration) and reacted for 2 h. Following the coupling reaction, the slides were rinsed in DMF, methylene chloride, and ethanol and dried under nitrogen.

Photodeprotection of Caged Biotin Derivatized Slides and Immobilization of Biological Macromolecules. Selected regions of NVOC-biotin-OH derivatized slides were deprotected by exposing the active surface to near UV light (OAI, Inc., Milpitas, CA; 500 W mercury arc lamp; 350−450 nm dichroic reflector; 10−12 mW/cm^2) through a photolithographic mask (Photo Sciences, Torrance, CA). Photolysis was carried out either in dioxane or in phosphate-buffered saline, pH 7.4 (PBS). Exposure times were typically 15−30 min. After photolysis, slides were rinsed with PBS/0.05% Tween 20, blocked for 30 min to 1 h using 1% BSA in PBS/Tween, and then incubated with streptavidin (Molecular Probes, Eugene, OR; 10−50 μg/mL in PBS/BSA/Tween) for 30 min to 1 h, rinsed thoroughly with PBS/Tween, and exposed to biotinylated antibodies (Vector Laboratories, Burlingame, CA; 50 μg/mL in PBS/BSA/Tween) for 1 h. After further rinsing, the slides were stained with fluorescein-labeled secondary antibodies (100 μg/mL in PBS/BSA/Tween) for 1 h, rinsed, dried, and imaged using the confocal fluorescence microscope described below. All protein solutions were filtered through 0.2 μm syringe filters (Gelman Sciences, Ann Arbor, MI) before application to the surface. In experiments involving multiple exposure and immobilization steps, the surface was treated with streptavidin (10 μg/mL in PBS/BSA/Tween) for 30 min and then a biotin solution (1 mM in PBS/Tween) for 10 min following each primary immobilization in order to block excess biotin moieties attached to the primary molecule.

Fluorescence Imaging of Macromolecule Binding on Derivatized Surfaces. Fluorescence imaging of treated surfaces was carried out using the stage-scanning confocal fluorescence microscope described previously.[20] Briefly, this instrument consists of a Zeiss Axioskop epifluorescence microscope equipped with a Spectra-Physics (Mountain View, CA) Model 2020 argon ion laser as the excitation source, a fluorescein filter set, and a Hamamatsu (Bridgewater, NJ) Model R-923 photon-counting photomultiplier in a thermoelectrically-cooled housing as the detector. Fast data acquisition rates are possible by virtue of the 250 MHz input signal bandwidth and 100 MHz counter of a Stanford Research (Sunnyvale, CA) Model SR430 multichannel scaler used for data acquisition. Fluorescence intensity is monitored as a function of $X-Y$ position as the slide is moved under the focused laser beam using a Newport (Irvine, CA) Model PM-500 micropositioning system to generate a high-resolution image of the surface. Slides were either imaged in air or placed on a flow cell and imaged through the glass slide under appropriate buffers. Laser power was kept sufficiently low to avoid saturation of the fluorophore duty cycle.

Results

Caged Biotin Structural Characterization and Photolysis Kinetics in Solution. Compounds **2**−**11** were synthesized as shown in Scheme 1. Structural assignments were based on

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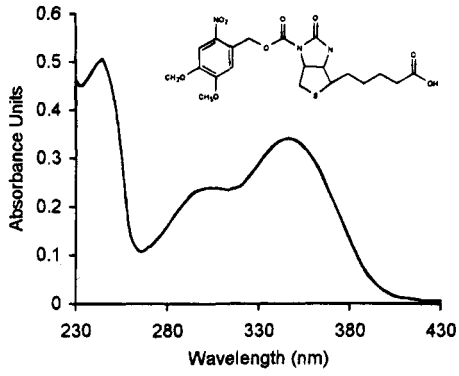

Figure 1. Absorption spectrum of NVOC-biotin-OH in 5 mM H_2SO_4/dioxane.

synthetic precedent[17,21] as well as spectroscopic properties.[22] The ^{1}H-NMR spectrum of NVOC-biotin-OH readily differentiated the ring fusion protons bearing (i) a urea nitrogen (ca. 4.2 ppm) and (ii) an imide nitrogen (ca. 4.8 ppm). COSY spectra indicated that the former is vicinal to a methine adjacent to sulfur and that the latter is vicinal to a methylene adjacent to sulfur. The absorption spectrum of NVOC-biotin-OH (Figure 1) exhibits a near UV absorption maximum that is characteristic of the nitroveratryloxycarbonyl group. The molecule has a formula weight of 485.6 and an extinction coefficient of 9400 M^{-1} cm^{-1} at 346 nm in dioxane.

The photolysis mechanism of NVOC-biotin-OH is thought to follow the general reaction scheme proposed by Patchornik et al.[11] Exposure of NVOC-biotin-OH to near UV light results in an intramolecular rearrangement that culminates in release of a nitroso aldehyde molecule and the free biotin moiety. Solution-phase photolysis rates of NVOC-biotin-OH and other caged biotin analogues were studied using HPLC analysis of residual starting material. Dilute samples in organic solvents or aqueous buffers were exposed to UV light for varying lengths of time, and residual starting material was monitored by measuring absorbance at 365 nm. NVOC-biotin-OH photolysis rates were generally faster in organic solvents ($t_{1/2} \cong 5$ min in dioxane; 363 nm illumination at 10 mW/cm^2) than in aqueous solution ($t_{1/2} \cong 30$ min in phosphate buffered saline, pH 7.4), and showed substantial pH dependence (Figure 2A). Photolysis rates were significantly enhanced by addition of 10 mM bisulfite or dithiothreitol (DTT) ($t_{1/2} \cong 6$ min at pH 7.5). Even in the absence of scavengers, Menvoc- or Menpoc-protected biotin molecules exhibited significantly faster photolysis rates in aqueous solution ($t_{1/2} \cong 10-13$ min) than the NVOC-protected compounds (Figure 2B).

A Generic Scheme for Spatially-Addressable Immobilization of Macromolecules on Solid Supports. Figure 3 illustrates a general scheme for the use of caged biotin compounds to immobilize macromolecules on solid supports in a spatially-addressable manner. In the figure, NVOC-biotin-OH is covalently coupled to a solid support. Masked exposure to near UV light (step 1) removes the photolabile protecting groups in the selected area, thus making biotin sites (B) available for streptavidin (A) binding and subsequent immobilization of a biotinylated macromolecule (R1) (step 2). The process may be repeated (steps 3 and 4) to immobilize a second molecule (R2) in another location on the surface. After each immobilization step, incubation with streptavidin to "cap" multiple biotin moieties on macromolecules, followed by washing with a biotin solution to saturate the remaining streptavidin sites, reduces the likelihood of cross-contamination during subsequent rounds.

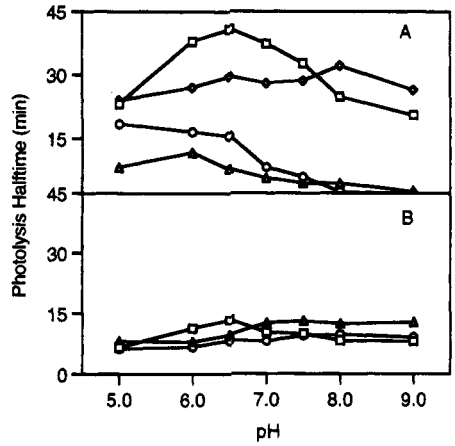

Figure 2. Photolysis half-times of caged biotins in aqueous solution: (A) 0.1 mM NVOC-biotin-OH in aqueous buffers (squares, 100 mM buffer; diamonds, 10 mM buffer; circles, 10 mM buffer + 10 mM bisulfite; triangles, 10 mM buffer + 10 mM DTT); (B) 0.05 mM $(Menvoc)_2$-biotin-OH or $(Menpoc)_2$-biotin-OH in 10 mM aqueous buffers (squares, $(Menvoc)_2$-biotin-OH; triangles, $(Menvoc)_2$-biotin-OH + 10 mM DTT; circles, $(Menpoc)_2$-biotin-OH). Buffers are acetate (pH 5.0), phosphate (pH 6.0−7.5), and borate (pH 8.0−9.0).

Demonstration of Light-Dependent, Spatially-Addressable Immobilization of Fluorescein−Streptavidin on a Solid Support. In order to demonstrate spatially-localized immobilization of streptavidin, glass microscope slides were covalently derivatized with NVOC-biotin-OH as described in the Materials and Methods section, mounted on a custom-made flow cell, and exposed to near UV light (365 nm; 13 mW/cm^2) through a 500 μm × 500 μm checkerboard mask for 30 min in phosphate-buffered saline, pH 7.4. The slide was then blocked with PBS, 1% BSA, and 0.05% Tween 20, treated with fluorescein−streptavidin (10 μg/ml) for 60 min, rinsed, and dried. An example of the fluorescence images obtained is shown in Figure 4, which demonstrates the localized binding of streptavidin to areas of the surface which have been exposed to light. The contrast ratio in this image (the ratio of signal in the bright squares to that in the dark squares) is approximately 3:1 (before background subtraction). Similar results were obtained using Menvoc- and Menpoc-protected biotins. The image is asymmetric (the bright squares are larger than the dark squares) due to diffraction effects resulting from the 0.7 mm separation (the thickness of the microscope slide) between the photolithographic mask and the active surface during the exposure.

The specificity of streptavidin binding was investigated by carrying out a pseudocompetition binding experiment using biotin as a competitive inhibitor. NVOC-biotin-OH slides were prepared as described above. One slide was exposed to near UV light (365 nm; 13 mW/cm^2) for 30 min in phosphate-buffered saline, pH 7.4. The other was kept in the dark as a control. The slides were then clamped in a template that forms discrete wells, blocked with PBS/1% BSA/0.05% Tween 20, and treated with samples of fluorescein−streptavidin (10 μg/mL) or fluorescein−streptavidin preincubated with excess biotin (1 mM). After rinsing and drying, the slides were imaged using a stage-scanning confocal fluorescence microscope. Background fluorescence was measured for wells exposed to buffer only and subtracted from the total signal. As can be seen in Figure 5, exposure of the derivatized surface to near UV light resulted in a dramatic increase in the amount of fluorescein−streptavidin bound. The signal was blockable by free biotin, indicating that increased binding results from specific interactions between the deprotected biotinylated surface and the biotin-binding pocket of the streptavidin molecule. A small amount of streptavidin binding was observed even in the dark control.

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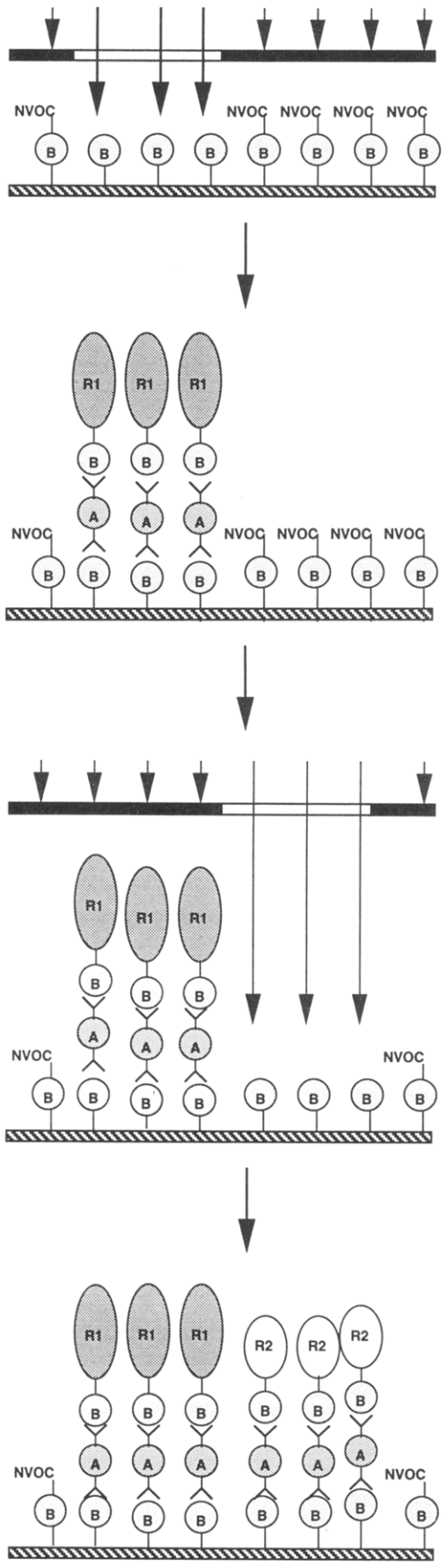

Figure 3. Strategy for spatially-addressable immobilization of macromolecules on solid supports using NVOC-biotin-OH covalently coupled to the support surface. Repeated cycles of selective illumination, streptavidin treatment, and incubation with biotinylated macromolecules allow one to create arrays of immobilized molecules. A = avidin or streptavidin, B = biotin, R1 = macromolecule no. 1, and R2 = macromolecule no. 2.

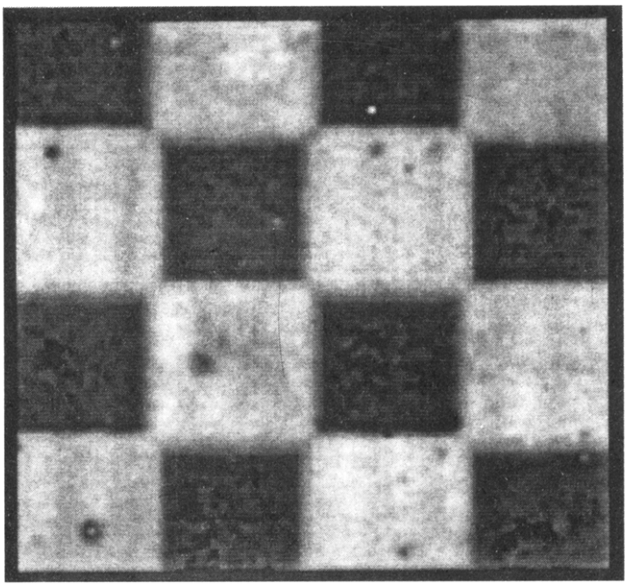

Figure 4. Demonstration of spatially-addressable immobilization of fluorescein−streptavidin on a solid support. An NVOC-biotin-OH derivatized microscope slide was mounted on a custom-made flow cell and illuminated for 30 min through a 500 μm × 500 μm checkerboard mask using 365 nm light from a mercury arc lamp (13 mW/cm^2, in PBS, pH 7.4). The slide was then blocked with PBS, 1% BSA, and 0.05% Tween 20 and treated with fluorescein−streptavidin (10 μg/mL) for 60 min. After rinsing and drying, the slide was imaged using a stage-scanning confocal fluorescence microscope. The contrast ratio in this image (the ratio of signal in the bright squares to that in the dark squares) is approximately 3:1.

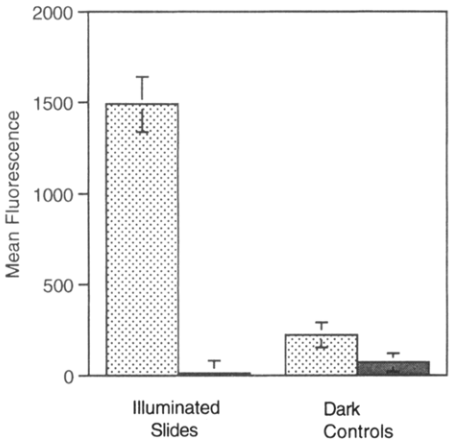

Figure 5. Demonstration of light-dependent, specific immobilization of fluorescein−streptavidin on a solid support. Fluorescence intensities were measured from images of glass slides derivatized with NVOC-biotin-OH and treated with fluorescein-streptavidin solutions (10 μg/mL) after exposure to light. Binding specificity was demonstrated by incubating some areas of the derivatized surface with fluorescein−streptavidin solutions that had been preincubated with free biotin (1 mM final concentration). Background fluorescence was measured for glass surfaces exposed to buffer only and subtracted from the total fluorescence signal. Light shading indicates surfaces treated with fluorescein−streptavidin. Dark shading indicates surfaces treated with fluorescein−streptavidin preincubated with excess biotin.

Immobilization of Two Antibodies at Different Sites on a Glass Surface. In order to demonstrate site-selective immobilization of two different macromolecules on the same surface, an NVOC-biotin-OH derivatized microscope slide was mounted on a flow cell and carried through the sequence of immobilization steps described above. The slide was exposed to UV light using a horizontal stripe mask, and biotinylated rabbit IgG molecules were immobilized via a biotin−streptavidin−biotin bridge. The slide was then retreated with strepta-

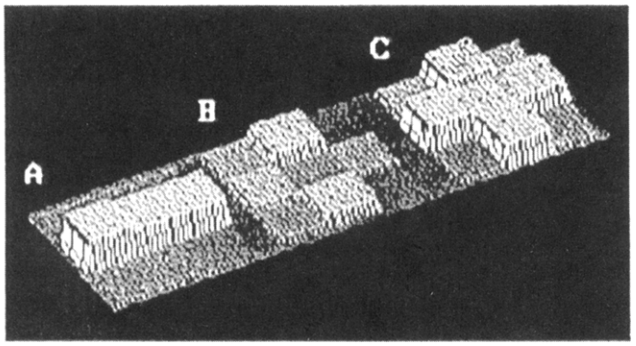

Figure 6. Immobilization of two different antibody molecules at specific locations on a glass surface. An NVOC-biotin-OH derivatized microscope slide was mounted on a custom-made flow cell and illuminated for 15 min through a 1 mm wide horizontal striped mask (365 nm light; 13 mW/cm^2; in dioxane). The slide was then blocked with PBS, 1% BSA, and 0.05% Tween 20, treated with streptavidin (10 μg/mL) for 30 min, and incubated with biotinylated rabbit IgG (50 μg/mL) for 60 min. After rinsing, the slide was retreated with streptavidin and washed with a 1 mM biotin solution to cap any remaining biotin moieties on the macromolecule and saturate free streptavidin biotin-binding sites. The slide was then reexposed to light (365 nm light; 13 mW/cm^2; 30 min; in PBS, 0.05% Tween 20, pH 7.4) using a mask having vertical stripes, treated with streptavidin, and incubated with biotinylated mouse IgG. After rinsing and drying briefly, the slide was divided into sections, treated with fluoresceinated secondary antibodies (100 μg/mL), rinsed, dried, and imaged using a stage-scanning confocal fluorescence microscope. Fluorescence intensity (vertical axis) was plotted as a function of position on the surface in pseudo-three-dimensional plots: (A) surface treated with fluorescein−anti-rabbit IgG; (B) surface treated with fluorescein−anti-mouse IgG; (C) surface treated with a mixture of fluorescein−anti-rabbit and fluorescein−anti-mouse antibodies.

vidin and washed with a biotin solution to "cap" any remaining biotin moieties on the IgG molecules and to saturate any free streptavidin biotin-binding sites. The slide was then reexposed to light using a vertical stripe mask, and the immobilization steps were repeated using biotinylated mouse IgG. After rinsing and briefly drying, the slide was divided into sections and treated with fluoresceinated secondary antibodies. The fluorescence images presented in Figure 6 demonstrate immobilization of the two different macromolecules at specific locations on the glass surface. Fluorescence intensity (vertical axis) was plotted versus position on the surface in a pseudo-three-dimensional plot. The contrast ratios in these images range from 3:1 to 9:1 (before background subtraction).

Discussion

We have demonstrated the use of a "caged" biotin compound to achieve light-dependent, specific immobilization of macromolecules on solid supports. The approach allows us to exploit the spatial precision and miniaturization made possible by photolithographic techniques while simultaneously taking advantage of the high affinity (ca. 10^{-15} M)[23,24] and specificity of the streptavidin−biotin interaction.

The technique offers several potential advantages for site-selective tethering of biomolecules over some of the methods reported previously in the literature. It should have broad application due to the widespread availability of biotinylated molecules and methods for producing them. Because the irradiation and immobilization steps are temporally separate, protein molecules to be immobilized need never be directly exposed to the intense near UV irradiation (365 nm) used for photolysis, as is the case in some techniques,[8,35] and therefore are not subjected to possible photochemical damage. Nor are they subjected to drying, as in other photochemical cross-linking methods that have been described.[9] The caged biotin approach provides the possibility of controlling the surface density of binding sites by carrying out a partial photolysis step. Also, the method should be applicable to a variety of support materials provided that (i) the material carries suitable functional groups for covalent derivatization with a linker molecule and/or caged biotin and (ii) a front-surface photolithographic printing technique can be adopted in the case of opaque support materials. One limitation of the approach is its reliance on serial rounds of photodeprotection and immobilization, which may restrict its application to the creation of fairly simple arrays of biomolecules.

We chose to use nitroveratryloxycarbonyl as a protecting group in this study in part because of our laboratory's prior experience with this molecule,[20,27] and because the synthetic procedures are straightforward and provide good yields (50−70%) from the corresponding biotin esters. One of the interesting observations to come out of this work is the drastic reduction in photolysis rate of NVOC-biotin-OH when water is used as the solvent. This is an important point when one considers the overall procedure for immobilization of multiple receptors on a surface. The first round of photolysis can take place in an organic solvent because there are no biological materials on the surface, but subsequent rounds of photolysis must be done under biologically-compatible conditions. Optimization of photolysis reaction conditions in aqueous buffers is therefore a desirable step in the development of this approach for practical applications.

As a first step in this direction, we examined the influence of buffer capacity and pH on photolysis rates. Significant differences in photolysis half-time were observed as ionic strength and pH were varied. Walker et al.[25] have previously reported that DTT acts as a scavenger for photobyproducts of *o*-nitrobenzyl caging groups. The use of bisulfite as a scavenger has also been reported.[26] Addition of either 10 mM bisulfite or DTT significantly reduced photolysis half-times for the NVOC group. Bisulfite ion was also observed to react with NVOC-biotin-OH in the absence of light to form an adduct. The reversibility of adduct formation is still in question, but it is apparent that it is both time- and pH-dependent (data not shown), and thus we have chosen DTT as the scavenger of choice in ongoing experiments.

An additional concern that arose during the course of these studies was the stability of caged biotin at different pH values since the nitrobenzyl carbamate was anticipated to be base-sensitive. The stability of NVOC-biotin-OH as a function of pH was monitored for 30 h by HPLC. No detectable decomposition of NVOC-biotin-OH was observed in buffers with pH < 9.0 (data not shown). At pH 9.0, 7.2% of the caged biotin was hydrolyzed to free biotin and nitroveratryl alcohol after 30 h, for a rate of decomposition of 0.23% per hour.

In the immobilization experiments described in this paper, NVOC-biotin-OH was covalently coupled to glass slides which had been derivatized using a (3-aminopropyl)silane, followed by addition of an 6-aminocaproic acid linker molecule to provide distance between the biotin moiety and the surface. In general, use of NVOC-biotin-OH free acid and BOP/HOBT activation chemistry[28] gave better coupling yields than use of the corresponding *p*-nitrophenyl ester, as indicated by the fluorescence

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signal levels and quality of the images obtained in immobilization experiments. The demonstration of light-dependent, specific binding of streptavidin to a glass surface derivatized with caged biotin indicates that illumination of NVOC derivatives of biotin leads to the removal of the NVOC protecting group and consequent availability of the tethered biotin molecule for interaction with the streptavidin binding site. A dramatic increase in the affinity of NVOC-biotin-OH for streptavidin binding following exposure to light was confirmed in separate experiments using [^{3}H]biotin as a competitive inhibitor (data not shown). In some of our experiments, surfaces derivatized with NVOC-biotin-OH showed a slight affinity for streptavidin even before exposure to light. This may have been due either to trace contaminants of free biotin in our NVOC-biotin-OH preparation or to weak interactions between streptavidin and other surface components, e.g., aminocaproic acid linker molecules remaining on the surface if the NVOC-biotin-OH coupling reaction was incomplete. Avidin, another biotin-binding protein, has been shown to bind molecules resembling fragments of the biotin molecule.[29]

We have demonstrated the immobilization of fluorescein–streptavidin in 500 μm × 500 μm squares as an example of spatially-localized tethering of a macromolecule. The dimensions of the features in this array are limited only by the contrast and resolution of the lithographic process used. In this case, the relatively simple scheme of contact printing through the backside of the microscope slide gave a limiting useful resolution of approximately 200 μm due to the diffraction of light as it passes through the 0.7 mm thick glass. Higher resolution should be obtainable by using thinner glass slides or by adopting a front-surface printing scheme.

Immobilization of different antibodies at specific locations on the surface was demonstrated by using the multivalent biotin-binding properties of streptavidin to create a biotin–streptavidin–biotin bridge between the surface and the biotinylated molecules. A complication arose in these feasibility experiments due to our use of antibodies having multiple biotin moieties attached, which gave rise to cross-contamination during subsequent immobilization cycles unless we first "capped" the residual biotin moieties with excess streptavidin, and then blocked the additional biotin-binding sites by flooding the surface with a biotin solution. More elegant procedures would incorporate the use of site-selective biotinylation of the macromolecule either through chemical means,[30] or by engineering a biotinylation site into the gene encoding the macromolecule of interest.[31]

Areas of ongoing research interest in our laboratory include alternative photolabile protecting groups for use in combinatorial synthetic strategies involving light, and control of surface properties that influence the performance of tethered ligands in solid-phase bioassays. We have synthesized and begun testing a number of biotin derivatives that may prove useful for immobilization of macromolecules, e.g., Menpoc-biotin that exhibits faster photolysis kinetics and generates fewer reactive byproducts (our unpublished results). One could also prepare caged forms of biotin analogues such as iminobiotin or dethiobiotin that have lower affinities for streptavidin than biotin itself.[32] Such compounds may be useful in applications requiring spatially-selective immobilization of molecules followed by subsequent release using biotin as an eluting agent.

Surface parameters that will undoubtably influence the performance of caged biotin for immobilization of macromolecules include the choice of linker molecule used for tethering, the surface density of biotin groups, and the hydrophilicity of the resulting surface. The biotin-binding site of the streptavidin molecule is known to lie in a deep pocket of the protein molecule, as indicated by the large changes in binding energy observed for biotin analogues having shortened alkyl chains[29,32] and recent crystallographic data.[13] The structure of the linker molecule is therefore likely to influence the accessibility of the tethered biotin molecule to the streptavidin binding site. Recently, Spinke et al.[33] have demonstrated increased streptavidin binding to self-assembled monolayers deposited on gold surfaces when the surface density of biotin groups was reduced by using binary mixtures of biotinylated and nonbiotinylated thiol molecules. One approach to controlling the surface density of binding sites using caged biotin is to carry out a partial photolysis step, as mentioned above. Alternatively, one could dope the reaction mixture used to derivatize the surface with an inert molecule, e.g., at the step in which the linker molecule is coupled to the support, to increase the average distance between primary amines available for coupling to caged biotin. In addition to allowing optimization of specific binding interactions, the latter approach may permit simultaneous modification of other surface properties to minimize nonspecific adsorption of streptavidin and other macromolecules. Numerous studies have demonstrated the correlation between the hydrophilicity of surfaces and their resistance to nonspecific protein adsorption.[34] The choice of linker molecule, or mixtures of linkers and other diluent molecules used to control the density of biotin sites on the surface, is likely to influence surface wettability as well. Experiments to vary these parameters and examine their influence on the immobilization of macromolecules using caged biotin compounds are currently underway.

Acknowledgment. We thank Drs. Lubert Stryer and Stephen Fodor for valuable discussion and technical assistance.

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