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Synthesis of novel biotin anchors

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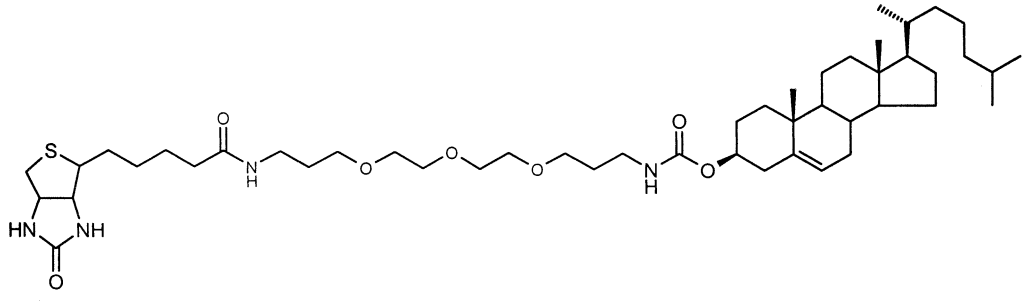
Abstract—Details are reported of the synthesis of biotinylated thiol and cholesteryl derivatives. The biotin derivative has been used to form multilayer films on gold through interaction with streptavidin.

1. Introduction

The bio-functionalisation of solid surfaces is one of the most challenging tasks of multidisciplinary research involving biology, chemistry, and physics. It has attracted a great deal of attention in recent years because it provides a bridge between man-made functional materials or devices and the supramolecular assemblies found in biology.[1] Potential applications range from the biocompatibilisation of implants for surgery, to novel biosensors.[2] The most important and commonly used solid surface is gold as it readily reacts with thiols, or disulfides to form a stable covalent bond.[3] We have reported a simple approach to the attachment of lipid bilayers to such gold surfaces. This employs self-assembled monolayers (SAMs) comprised of a mixture in which the anchoring component is an ethylenoxy oligomer, which is terminated with a cholesteryl group or phospholipid residue at one end and a thiol or disulfide group at the other. The cholesteryl moiety or the alkyl chain of the phospholipid penetrates into the hydrophobic region of the inner leaflet of the bilayer and acts as a 'hook'. The ethylenoxy part provides a hydrophilic space between the gold and the bilayer and the thiol or disulfide group covalently binds to the gold surface.[4,5] Extending our research interests in this field, we have now introduced a specific streptavidin/biotin interaction into our system to form multilayer films.

Streptavidin is an exceptionally stable tetrameric protein, each of the four subunits binds biotin with a free energy of binding which is comparable to that of a covalent bond.[6] Biotin can be attached to proteins and saccharides, as well other compounds and streptavidin then used as a molecular linker between the biotin-derivatized units.[7] Hence, biotinylated thiols have been synthesised and SAMs of these biotinylated thiols on gold used to bind streptavidin.[8–11] A particularly interesting point is that the four biotin binding sites have been shown to be arranged in two opposing pairs in each molecule such that two sites per streptavidin molecule are bound to the biotin on the surface and the remaining two sites are unoccupied. The bound streptavidin thus presents a matrix of well-ordered binding sites to the adjacent subphase, which can be employed as a template for further functionalisation. In this paper we report the synthesis of biotinylated thiol **3**, SAMs of which can be used to bind streptavidin to gold and



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Keywords: biotin cholesteryl; biomembranes; surfaces.

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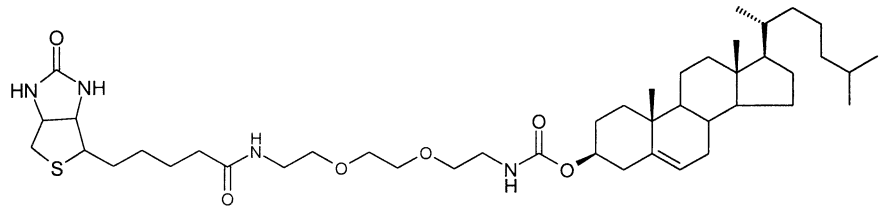

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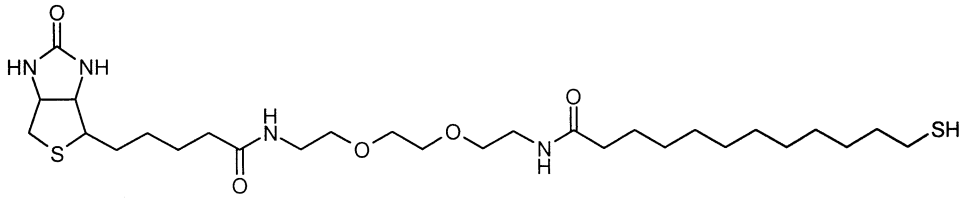

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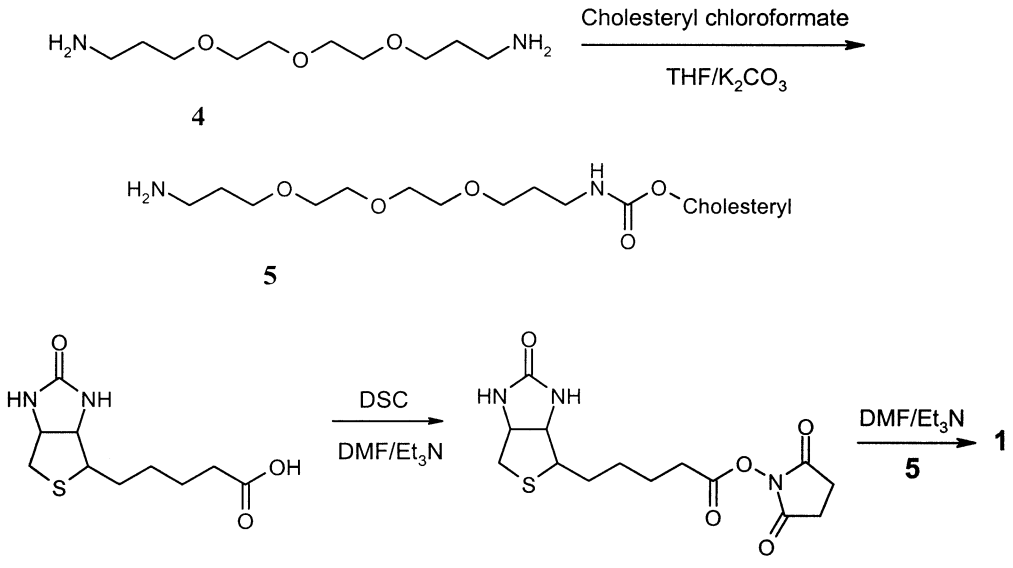
cholesteryl derivatives **1** and **2** which can then be used to react with the free sites to bind biomembranes on top of this.

Among various biotinylated thiols reported in the literature, the derivative **3** has been widely used.[10,11] However, this kind of compound is difficult to make. In the original report the analogue of **3** (11-mercaptododecanoic-(8-biotinoyl-amido-3,6-dioxaoctyl) amide) was made in poor yield and no characterisation data was given.[8] This restricts access to the field. A new synthesis route is reported here and the compounds involved are fully characterised. Biotinylated cholesteryl derivatives **1** and **2** have similar structures to the thiol cholesteryls we reported previously,[4] but the thiol group is replaced by biotin. They should prove useful for binding biomembranes to streptavidin immobilised on gold surfaces. Further more, it is known that streptavidin can crystallise in two-dimensional arrays on biotinylated phospholipids monolayers at an air/water interface. This is important for structural biology since the helical symmetry obtained facilitates the calculation of the three-dimensional protein structure.[12]

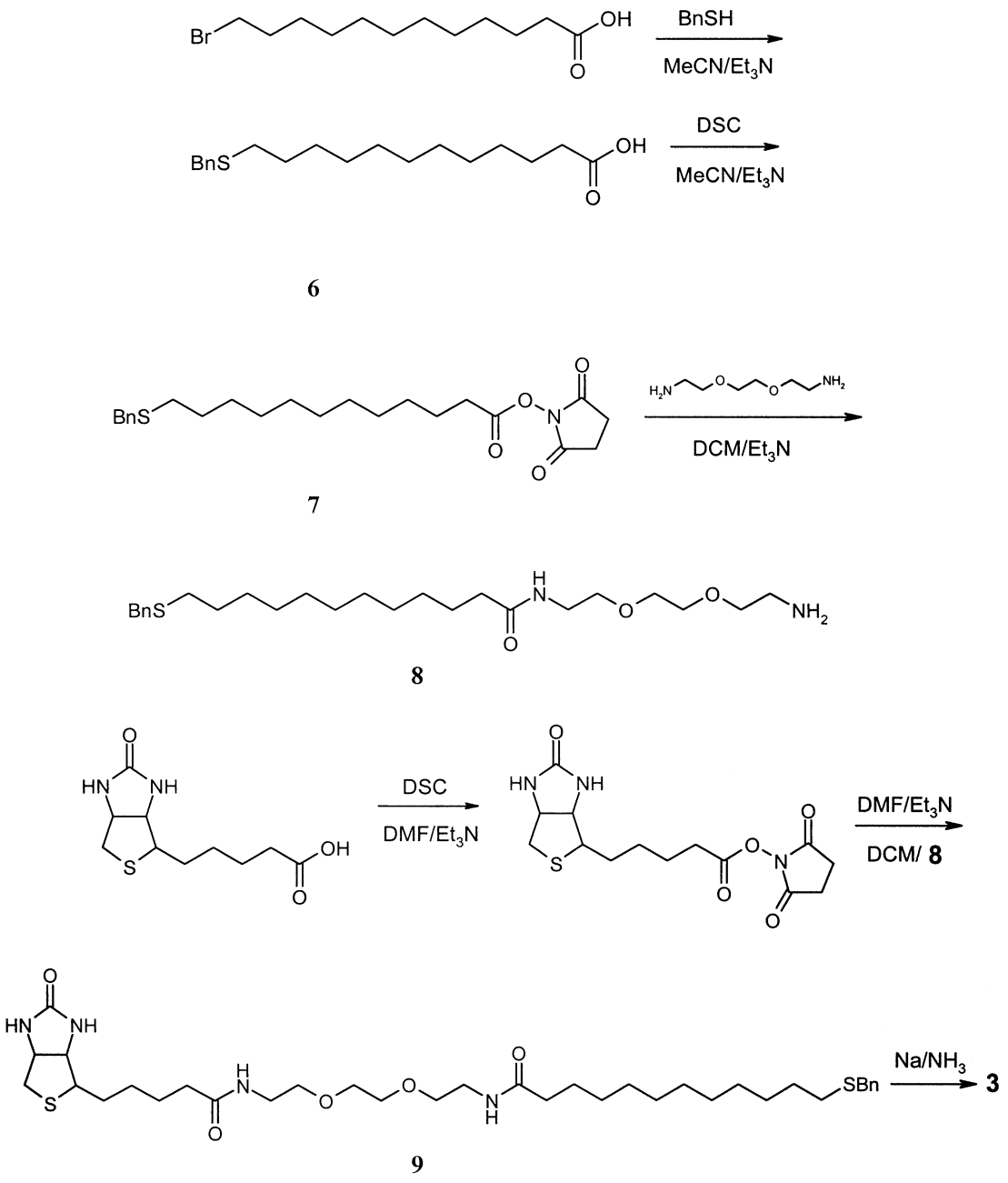
2. Results and discussion

2.1. Preparation of compounds 1, 2, and 3

The route for the synthesis of **1** started with the commercially available amine **4** as shown in Scheme 1. Cholesteryl chloroformate was reacted with an excess of the amine **4** in tetrahydrofuran using potassium carbonate as a base. After the reaction, inorganic salts were removed by filtration and the monocarbamate **5** separated by column chromatography. To form the amide from biotin and the monocarbamate **5**, the acid group of biotin was first activated by reacting with *N*,*N*′-disuccinimidyl carbonate (DSC)[13] in DMF. This gave the biotin *N*-hydroxysuccinimide ester which was reacted directly with **5** without purification to



Scheme 1.



Scheme 2.

give **1**. To simplify the purification procedure of **1**, a slight excess of biotin was used over the DSC to avoid the DSC reacting with **5**. The excess biotin can be easily removed in the final column chromatography since it has poor solubility in most solvents. However, the by-product *N*-hydroxysuccinimide is very difficult to remove and extensive column chromatography was needed. Compound **2** was made in a similar manner to **1**.

Compound **3** was made as shown in Scheme 2. The benzyl-protected thiol group was introduced in the first step in excellent yield. In the original report of synthesis of this kind of compound,[8] the bromo group was retained throughout reaction sequence until the final step where it was converted into the corresponding Bunte salt and subjected to acid hydrolysis to give the thiol. Considering that nucleophilic reagents are used or produced during these reactions (amine, *N*-hydroxysuccinimide), we argued that carrying a bromo group throughout would certainly reduce the yields and complicate the separation of products. Following the introduction of benzylthiol, 12-benzylthiododecanoic *N*-hydroxysuccinimide ester **7** was made using DSC reagent. The ester **7** was reacted with excess 2,2′-(ethylenedioxy)diethylamine to give monoamide **8** which reacted with biotin *N*-hydroxysuccinimide ester to generate **9**. The benzyl protecting group of **9** was readily removed using sodium in liquid ammonia to give the final product **3**.[4]

2.2. Surface characterisation of a biotinylated self-assembled monolayer

X-Ray photoelectron spectroscopy (XPS) was used to

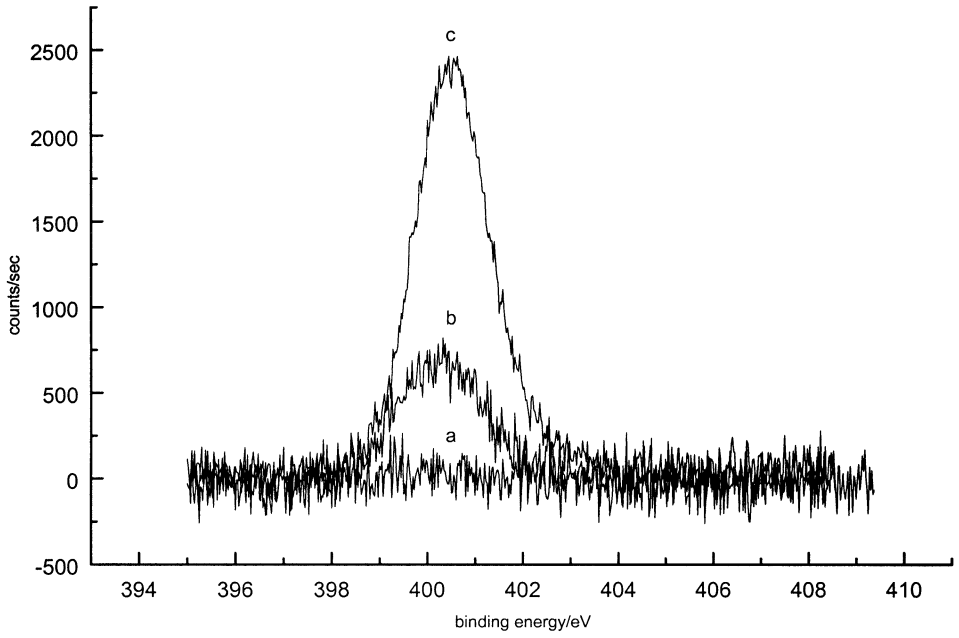


Figure 1. Detailed XPS scans of the nitrogen 1s peak for SAMs of: (a) 100% 11-mercaptoundecanol, (b) the mixed SAM used for streptavidin adsorption and (c) 100% biotin thiol **3**.

confirm the presence of biotin thiol **3** in a mixed monolayer used for streptavidin adsorption below. Spectra were recorded for SAMs of 100% 11-mercaptoundecanol, 100% biotin thiol **3** and a mixed SAM formed from a solution containing 20% biotin thiol **3** and 80% 11-mercaptoundecanol. All spectra were recorded at a take off angle of 90° and showed the presence of peaks corresponding to gold, carbon, oxygen and sulphur for all three SAMs. In addition, the SAMs containing biotin thiol **3** also had a nitrogen peak. Fig. 1 shows detailed scans of the nitrogen peak at 90° for each of the SAMs studied.

It is clear that there is virtually no nitrogen detected in the 100% 11-mercaptoundecanol SAM, as expected. For the 100% biotin thiol **3** SAM there is a relatively large nitrogen peak due to nitrogen in the urea and amide groups of the molecule. In the spectrum of the mixed monolayer, the nitrogen peak is reduced in intensity as there is a lower

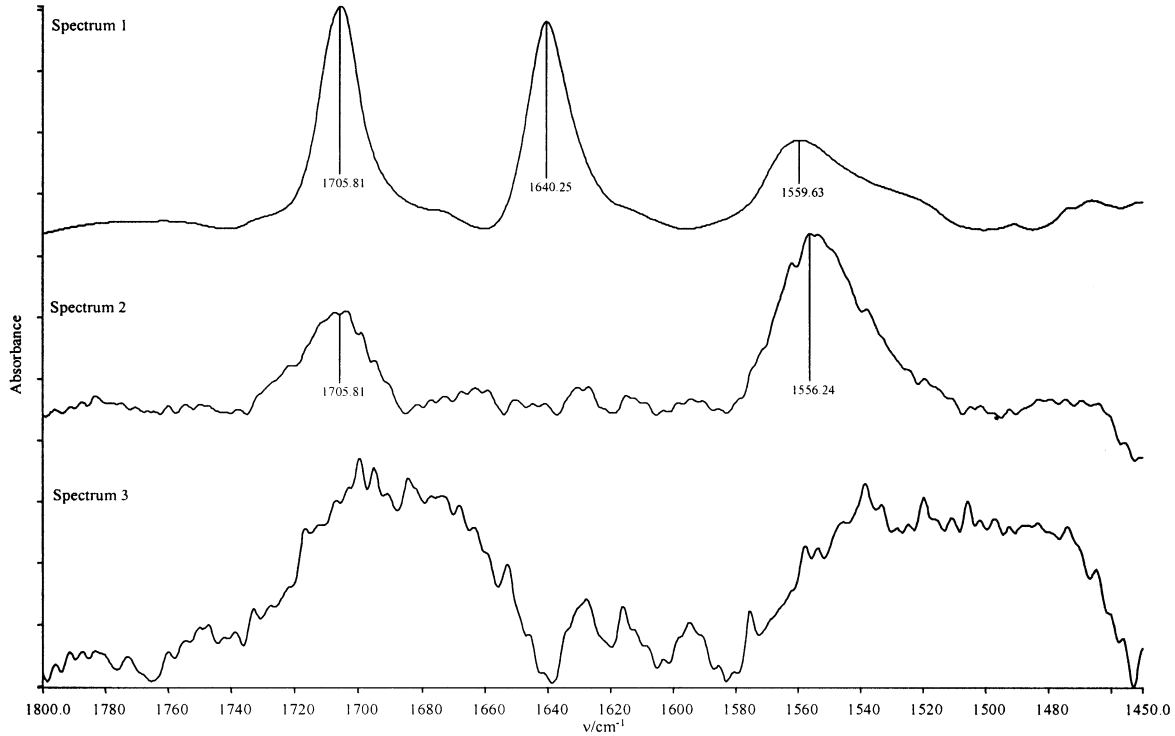


Figure 2. *Spectrum* 1: IR spectrum of a thin film of biotin thiol **3** on a calcium fluoride plate. *Spectrum* 2: IRAS of 100% biotin thiol **3** SAM. *Spectrum* 3: IRAS of the mixed SAM used for streptavidin adsorption.

proportion of biotin thiol **3** on the surface, but it confirms the presence of the biotinylated molecule in the monolayer. Detailed scans of the oxygen peaks also provide useful information on the composition of the SAMs. For the 11-mercaptoundecanol SAM the oxygen peak is composed of only one component centred at 533.2 eV, whereas for both the pure biotin thiol SAM and the mixed SAM, the oxygen peaks can be resolved into two components. The component at around 533 eV corresponds to the presence of oxygen–carbon single bonds, and the second component at 532 eV confirms the presence of oxygen–carbon double bonds in the monolayer.

Infrared reflection absorption spectroscopy (IRAS) was also used to study the SAMs. Fig. 2 shows the IR spectra in the amide region of a thin film of biotin thiol **3** on a calcium fluoride plate (spectrum 1), a SAM of biotin thiol **3** on gold (spectrum 2), and the mixed SAM formed from a solution containing 20% biotin thiol **3** and 80% 11-mercapto-undecanol (spectrum 3).

In the IR spectrum of the thin solid film of biotin thiol **3**, there are clearly three bands in the amide region. The band at 1706 cm^{-1} is assigned to the amide I vibration of the urea group,[16] which is predominantly the C═O stretching mode. The equivalent mode for the amide groups in the chain of the molecule occurs at 1640 cm^{-1}.[17,18] The weaker band at 1560 cm^{-1} is attributed to the amide II mode of these groups (i.e. the out of phase combination of N–H in plane bending and C–N stretching vibrations).[17,18] The position of these two peaks at 1640 and 1560 cm^{-1} is consistent with intermolecular hydrogen bonding interactions.[19]

Spectrum 2 shows the IRA spectrum of a SAM of biotin thiol **3** on gold. The amide I vibration of the urea group and the amide II vibration of the secondary amides in the chain are present in roughly the same positions as for the thin solid film, as expected. However, there is a significant difference here in that the amide I vibration of the amides in the chain of the molecule is no longer apparent. Since only vibrations which produce a change in dipole moment perpendicular to the substrate surface are active in the IRA spectrum, the absence of this band suggests that the C═O bonds in the chain are aligned parallel to the surface. In addition, the relatively high intensity of the amide II peak indicates that the dipole of the N–H bond oscillates nearly perpendicular to the substrate plane.[18] This orientation is consistent with the presence of lateral hydrogen bonding interactions between the amide containing chains of these molecules in the SAM.[20]

The IRA spectrum of the mixed SAM has two major bands in the amide region that are broad and less well defined, perhaps reflecting a lower degree of order in the mixed SAM as compared to the one component SAM. However, the position of the band at around 1706 cm^{-1} does suggest that it contains a contribution from the amide I vibration of the urea group, once again showing that the biotin thiol **3** molecule is present in the SAM.

2.3. Adsorption of streptavidin to a biotinylated self-assembled monolayers

The interaction of SAMs of biotin thiol **3** on gold surfaces and subsequent adsorption of streptavidin was investigated

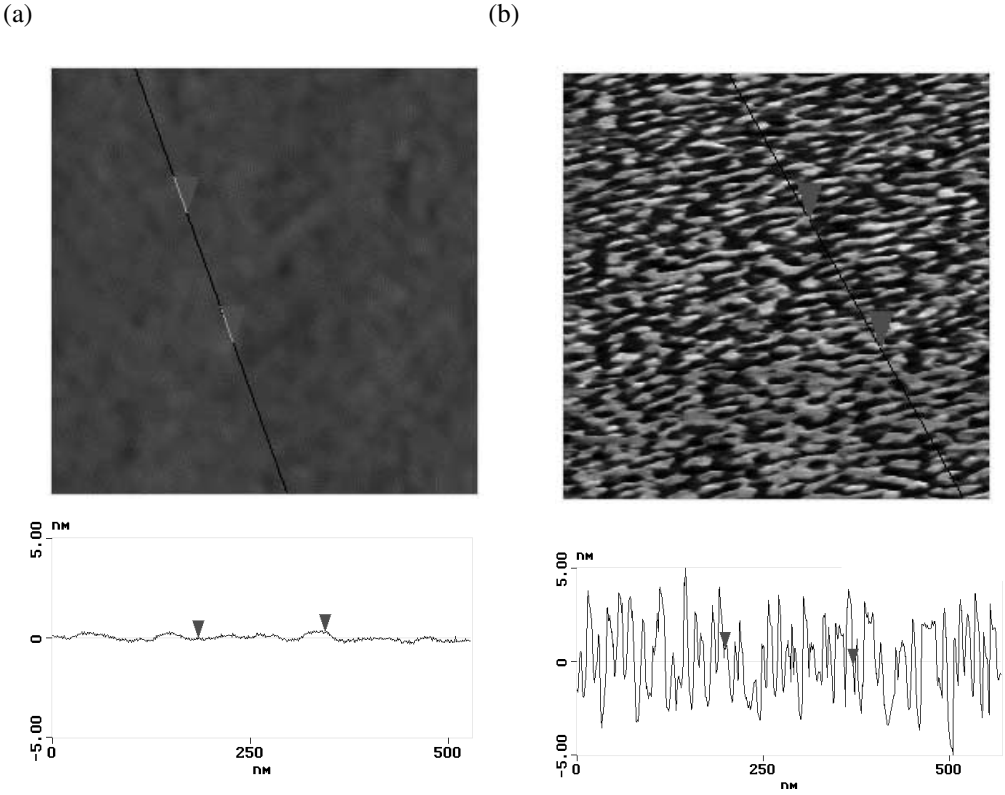


Figure 3. Atomic force microscopic images of biotinylated SAM: (a) before and (b) after protein adsorption. The images (scan size of 500 nm) were taken in tapping mode and under ambient conditions with a *Z* range of 10 nm.

by AFM. Fig. 3 shows AFM images of a biotinylated SAM, formed from a mixture of biotin thiol **3** and 11-mercaptoundecanol, and the result of exposure to streptavidin. The use of a mixed SAM, instead of a single component biotin thiol **3** SAM, reduces steric hindrance to protein binding.[11] The image for biotinylated SAMs before protein adsorption shows a smooth surface similar to that of unfunctionalised gold (Fig. 3). However, the image for biotinylated SAMs after exposure to streptavidin displays a layer of uniformly adsorbed material. The height of the features in this image are in the range 50–60 Å, which is consistent with the value of 45–50 Å obtained for two-dimensional crystals of streptavidin at the air/water interface.[14] Therefore, the AFM results indicate the binding of streptavidin on the biotinylated surfaces. It should be noted that no significant streptavidin adsorption was found in control experiments on SAMs formed from 100% 11-mercaptoundecanol, which is also in agreement with previous reports.[10,11] The observed adsorption is hence due to a specific interaction between biotin and streptavidin.

3. Conclusions

A biotinylated thiol has been made by an efficient new method. The SAMs of this biotinylated thiol and their interaction with steptavidin have been characterised by XPS, IRAS and AFM. Two biotin cholesteryls with different linking chains have also been made and ongoing work on construction of multilayer films using these compounds will be reported in due course.

4. Experimental

4.1. General procedures and instrumentation

Nuclear magnetic resonance spectra were recorded on a General Electric QE 300 spectrometer or a Bruker AM 400 spectrometer. Chemical shifts are expressed in parts per million (ppm) downfield of tetramethylsilane for ^{1}H resonances. Mass spectra were recorded on a VG Autospec mass spectrometer. Microanalyses were carried out at Leeds University Microanalytical Laboratory. All C, H, N, and S, analytical figures are percentage values. Some cholesteryl conjugated compounds were analysed as the corresponding hydrates.

Thin layer chromatography was carried out using precoated plastic-backed silica plates which were visualised using ultraviolet light, and permanganate stain. Flash chromatography signifies column chromatography on Merck silica gel (230–400). Petroleum ether refers to petroleum ether (bp 40–60°C) unless otherwise stated.

Preparation of pure and mixed monolayers of biotin thiol **3** *and* 11*-mercaptoundecanol for XPS, IRAS and AFM analysis*. Gold substrates were prepared by evaporation of approximately 10 nm chromium followed by 200 nm gold onto clean silicon wafer (for XPS) and glass microscope slides (for IRAS), using an Edwards 306 evaporator. For AFM studies SAMs were formed on uniformly flat gold substrates, prepared using the method described by Stamou et al.[15] The substrates were cleaned in an argon plasma, rinsed with solvent and dried under a stream of N_2 before immersion in solutions of 11-mercaptoundecanol, biotin thiol **3** and a mixed solution containing 80% 11-mercaptoundecanol with 20% biotin thiol **3** in ethanol (with total thiol concentration of 10^{-4} M) for overnight. On removal from solution, SAMs were rinsed with ethanol, milli-Q water and dried in a stream of N_2.

Protein adsorption. The binding of protein streptavidin was proceeded in a phosphate saline solution (pH 7.4) of streptavidin at a concentration of 30 mg ml^{-1}. One hour was allowed for binding to complete. The substrates were then removed, washed with milli-Q water and dried with a steam of N_2.

XPS was performed using a Vacuum Generators ML 500 instrument with an Al Kα source (1486.6 eV). All spectra were recorded using a constant pass energy of 30 eV and the residual pressure in the chamber remained in the order of 10^{-9} Torr. The peaks were resolved into a mixture of Gaussian and Lorentzian curves to fit the spectra.

Infrared reflection absorption spectra of SAMs were recorded on a Bruker IFS 48 system equipped with a grazing angle accessory (80°). Spectra were recorded using 2000 scans and 2 cm^{-1} resolution in the range 400–4000 cm^{-1}, whilst being purged with dry air at room temperature. The transmission IR spectrum of a thin film of biotin thiol **3** on a calcium fluoride plate was recorded using the same instrument.

For characterisation of binding of streptavidin onto biotinylated surfaces an atomic force microscope (Nanoscope IIIa, Digital Instruments) was used. The images were collected in tapping mode using a standard silicon tip under ambient conditions.

4.1.1. 13-Amino-4,7,10-trioxatridecaneamine cholesteryl carbamate (5). Cholesteryl chloroformate (2.04 g, 4.55 mmol) in dichloromethane (20 ml) was added dropwise to 4,7,10-trioxa-1,13-tridecanediamine (**4**) (5.00 g, 22.73 mmol) and potassium carbonate (3.14 g, 22.73 mmol) solution in tetrahydrofuran (100 ml). The reaction mixture was stirred at room temperature for 4 h. The inorganic salts were removed by filtration. The solution was concentrated and the product was separated by column chromatography on silica gel (ethyl acetate changed to ethyl acetate/methanol 9:1) to give the product (1.73 g, 2.73 mmol, 60%) as a pale yellow viscous oil. δ_H (300 MHz, $CDCl_3$) 5.37–5.32 (2H, m, vinyl cholesteryl, and N*H*), 4.50–4.46 (1H, m, C*H*O of cholesteryl), 3.64–3.49 (12H, m, 6×CH_2O), 3.31–3.25 (2H, m, CH_2NHCO), 2.85–2.81 (2H, m, CH_2NH$_2$), 2.36–2.25 (2H, m, cholesteryl). 2.01–1.72 (10H, m, NH_2, CH_2CH$_2$NH$_2$, CH_2CH$_2$NHCO, 4H of cholesteryl), 1.65–0.88 (m, 34H, cholesteryl), 0.67 (3H, s, cholesteryl). MS (EI) *m*/*z* 633 (M^+, 100%), 369 (47%), 265 (10%), 247 (7%), 221 (34%), 161 (34%) 147 (48%), 109 (29%), 95 (48%), 81 (56%). Calcd for $C_{38}H_{68}N_2O_5$: C, 72.11; H, 10.83; N, 4.43. Found: C, 72.15; H, 11.10; N, 4.30%.

4.1.2. 13-Biotinoylamido-4,7,10-trioxatridecaneamine cholesteryl carbamate (1). Biotin (112 mg, 0.46 mmol),

N,*N*′-disuccinimidyl carbonate (97 mg, 0.38 mmol), and triethylamine (192 mg, 1.90 mmol) in DMF (15 ml) were stirred at room temperature for 6 h. 13-Amino-4,7,10-trioxatridecaneamine cholesteryl carbamate (**5**) (240 mg, 0.38 mmol) was added and the reaction mixture was stirred overnight. Solvents were removed under vacuum and the product was separated by column chromatography on silica gel using ethyl acetate first then dichloromethane/methanol (9:1) as eluents to give the product (215 mg, 0.25 mmol, 66%) as a pale yellow solid. δ_H (300 MHz, $CDCl_3$) 6.61 (1H, br. N*H* of biotin), 6.10 (1H, br. N*H* of biotin), 5.38 (1H, m, vinyl cholesteryl), 5.29 (1H, br. N*H*), 5.26 (1H, br. N*H*), 4.53–4.48 (2H, m, C*H*O of cholesteryl and C*H*CH_2S of biotin), 4.34–4.30 (1H, m, C*H*CHS of biotin), 3.67–3.55 (12H, m, 6×CH_2O), 3.42–3.34 (2H, m, CH_2N), 3.29–3.21 (2H, m, CH_2N), 3.18–3.12 (1H, m, C*H*S of biotin), 2.92 (1H, dd, *J*=12.8, 4.9 Hz, one of CH_2S of biotin), 2.74 (1H, dd, *J*=12.8, 3.1 Hz, one of CH_2S of biotin), 2.35–2.21 (2H, m, cholesteryl), 2.20 (2H, t, *J*=7.4 Hz, CH_2CON), 2.01–1.92 (1H, m, cholesteryl), 1.87–0.88 (47H, m, cholesteryl, 2×C$H_2$$CH_2$N, and C$H_2CH_2CH_2$$CH_2$CON of biotin), 0.67 (3H, s, cholesteryl). MS (ES) *m*/*z* 882 (M^++Na, 97%), 865 (21%), 631 (17%), 611 (16%), 555 (100%), 517 (10%), 413 (18%), 322 (2%), 511 (3%). Calcd for $C_{48}H_{82}N_4SO_7$: C, 67.10; H, 9.62; N, 6.52; S, 3.73. Found: C, 66.80; H, 9.70; N, 6.25; S, 3.60%.

4.1.3. 8-Amino-3,6-dioxaoctaneamine cholesteryl carbamate. Cholesteryl chloroformate (2.00 g, 4.45 mmol) in dichloromethane (20 ml) was added dropwise to 2,2′-(ethylenedioxy)diethylamine (3.30 g, 22.25 mmol) and potassium carbonate (3.07 g, 22.25 mmol) in tetrahydrofuran (100 ml). The reaction mixture was stirred at room temperature for 4 h. The inorganic salts were removed by filtration. The solution was concentrated and the product was separated by column chromatography on silica gel (ethyl acetate changed to ethyl acetate/methanol 9:1) to give product (1.37 g, 2.45 mmol, 55%) as a pale yellow viscous oil. δ_H (300 MHz, $CDCl_3$) 5.36–5.34 (1H, m, vinyl cholesteryl), 5.31 (1H, m, N*H*), 4.48–4.51 (1H, m, C*H*O of cholesteryl), 3.63 (4H, s, OCH_2CH_2O), 3.58–3.51 (4H, m, 2×OC$H_2$$CH_2$N), 3.40–3.37 (2H, m, C$H_2$N), 2.90 (2H, t, *J*=5.3 Hz, C$H_2$$NH_2$), 2.36–2.25 (2H, m, cholesteryl), 2.00–1.78 (4H, m, cholesteryl), 1.67 (2H, m, NH_2), 1.65–0.88 (m, 34H, cholesteryl), 0.67 (3H, s, cholesteryl). MS (ES) *m*/*z* 583 (M^++Na, 25%), Calcd for $C_{34}H_{60}N_2O_4{\cdot}0.5H_2O$: C, 71.66; H, 10.79; N, 4.92. Found: C, 71.55; H, 10.55; N, 4.70%. Acc. Mass: Calcd for $C_{34}H_{60}N_2O_4Na$: 583.4451. Found. 583.4444.

4.1.4. 8-Biotinoylamido-3,6-dioxaoctaneamine cholesteryl carbamate (2). Biotin (0.11 g, 0.47 mmol), *N*,*N*′-disuccinimidyl carbonate (0.12 g, 0.47 mmol), and triethylamine (0.24 g, 2.35 mmol) in DMF (15 ml) were stirred at room temperature for 6 h. 8-Amino-3,6-dioxaoctaneamine cholesteryl carbamate (0.26 g, 0.47 mmol) was added and the reaction mixture was stirred over night. Solvents were removed under vacuum and the product was separated by column chromatography on silica gel using ethyl acetate first then dichloromethane/methanol (9:1) as eluents to give the product (0.25 g, 0.32 mmol, 67%) as a pale yellow solid. δ_H (300 MHz, $CDCl_3$) 6.58 (1H, br. N*H* of biotin), 6.25 (1H, br. N*H* of biotin), 5.38 (1H, m, vinyl cholesteryl), 5.29 (1H, br. N*H*), 5.27 (1H, br. N*H*), 4.53-4.48 (2H, m, C*H*O of cholesteryl and C*H*CH_2S of biotin), 4.34–4.30 (1H, m, C*H*CHS of biotin), 3.67–3.55 (8H, m, 4×CH_2O), 3.59–3.46 (2H, m, CH_2N), 3.44–3.37 (2H, m, CH_2N), 3.18–3.12 (1H, m, C*H*S of biotin), 2.92 (1H, dd, *J*=12.8, 4.9 Hz, one of CH_2S of biotin), 2.74 (1H, dd, *J*=12.8, 3.1 Hz, one of CH_2S of biotin), 2.35–2.21 (2H, m, cholesteryl), 2.24 (2H, t, *J*=7.4 Hz, CH_2CON), 2.01–1.92 (1H, m, cholesteryl), 1.87–0.88 (43H, m, cholesteryl and CH_2CH_2C$H_2$$CH_2$CON of biotin), 0.67 (3H, s, cholesteryl). MS (ES) *m*/*z* 809 (M^++Na, 62%), 322 (8%), 249 (8%). Acc. Mass: Calcd for $C_{44}H_{74}N_4O_6SNa$: 809.5227. Found. 809.5208.

4.1.5. 12-Benzylthiododecanoic acid (6). 12-Bromododecanoic acid (5.0 g, 17.92 mmol), benzyl mercaptan (5.5 g, 44.8 mmol), and triethylamine (9.0 g, 89.6 mmol) in acetonitrile (150 ml) were stirred in room temperature under N_2 for 48 h then dilute hydrogen chloride acid was added until the solution become acidic (pH=6). The product was extracted with dichloromethane, and the organic layer was dried ($MgSO_4$), filtered, and concentrated to give crude product which was purified by column chromatography on silica gel (petroleum spirit/ethyl acetate=4:1) to give the product (5.4 g, 16.7 mmol, 93%) as a white solid. δ_H (300 MHz, $CDCl_3$) 7.32–7.28 (5H, m, Ar*H*), 3.70 (2H, s, CH_2 of Bn), 2.43–2.32 (4H, m, CH_2S, CH_2COOH), 1.65–1.59 (4H, m, C$H_2$$CH_2S, CH_2$$CH_2$COOH), 1.32–1.25 (14H, m, CH_2). MS (EI) *m*/*z* 322 (M^+, 3%), 213 (18%), 123 (19%), 91 (100%). Calcd for $C_{19}H_{30}SO_2$: C, 70.81; H, 9.32, S, 9.94, Found: C, 70.70; H, 9.50, S, 10.00%.

4.1.6. 12-Benzylthiododecanoic-(8-amino-3,6-dioxaoctyl) amide (8). 12-Benzylthiododecanoic acid (5.25 g, 16.30 mmol), *N*,*N*′-disuccinimidyl carbonate (5.43 g, 21.20 mmol) and triethylamine (6.59 g, 65.20 mmol) in acetonitrile (50 ml) were stirred at room temperature for 6 h. Solvent was removed and the product 12-benzylthiododecanoic acid *N*-hydroxysuccinimide ester (**7**) (6.49 g, 15.49 mmol, 95%) separated by column chromatography on silica gel (petroleum spirit/ethyl acetate 1:3). The activated ester (3.00 g, 7.16 mmol) in dichloromethane (50 ml) was added dropwise to a mixture of 2,2′-(ethylenedioxy)-diethylamine (5.30 g, 35.80 mmol) and triethylamine (3.62 g, 35.8 mmol) in dichloromethane (100 ml). The reaction mixture was stirred at room temperature for 5 h. Solvent was removed and the product was separated by column chromatography on silica gel (ethyl acetate change to ethyl acetate/methanol 1:1) to give the product (2.20 g, 4.87 mmol, 68%) as a white solid. δ_H (300 MHz, $CDCl_3$) 7.32–7.23 (5H, m, Ar*H*), 6.24 (1H, br. N*H*), 3.70 (2H, s, CH_2 of Bn), 3.64–3.52 (8H, m, CH_2O), 3.49–3.45 (2H, m, CH_2N), 2.89 (2H, t, *J*=5.2 Hz, C$H_2$$NH_2$), 2.40 (2H, t, *J*=7.3 Hz, CH_2CH_2S), 2.17 (2H, t, *J*=7.3 Hz, CH_2CON), 1.70–1.51 (4H, m, CH_2), 1.32–1.16 (14H, m, CH_2); MS (ES) *m*/*z* 453 (M^++1, 100%), Calcd for $C_{25}H_{44}N_2SO_3$: C, 66.33; H, 9.80; N, 6.19; S, 7.08. Found: C, 66.45; H, 9.80; N, 6.05; S, 7.20%.

4.1.7. 12-Benzylthiododecanoic-(8-biotinoylamido-3,6-dioxaoctyl) amide (9). Biotin (0.36 g, 1.46 mmol), *N*,*N*′-disuccinimidyl carbonate (0.31 g, 1.22 mmol), and triethylamine (0.59 g, 5.84 mmol) in DMF (5 ml) were stirred at room temperature for 6 h. 12-Benzylthiododecanoic-(8-

amino-3,6-dioxaoctyl) amide (0.46 g, 1.02 mmol) in dichloromethane (10 ml) was added and the reaction mixture was stirred overnight. Solvents were removed under vacuum and the product was separated by column chromatography on silica gel using ethyl acetate first then dichloromethane/methanol (9:1) as eluents to give the product (0.50 g, 0.74 mmol, 72%) as a white solid. δ_H (300 MHz, $CDCl_3$) 7.32–7.23 (5H, m, Ar*H*), 6.28 (1H, br. N*H*), 6.27 (1H, br. N*H*), 5.67 (1H, br. N*H*), 4.76 (1H, br. N*H*), 4.54–4.50 (1H, m, C*H*CH_2S of biotin), 4.37–4.31 (1H, m, C*H*CHS of biotin), 3.70 (2H, s, CH_2 of Bn), 3.64–3.56 (8H, m, CH_2O), 3.50–3.46 (4H, m, CH_2NHCO), 3.18–3.13 (1H, m, C*H*S of biotin), 2.93 (1H, dd, J=5.1, 12.4 Hz, one of CH_2S of biotin), 2.74 (1H, d, J=12.4 Hz, one of CH_2S of biotin), 2.40 (2H, t, J=7.3 Hz, CH_2CH_2S), 2.27–2.17 (4H, m, CH_2CON), 1.77–1.21 (24H, m, CH_2); MS (ES) m/z 702 (M^++Na, 100%), Calcd for $C_{35}H_{58}N_4S_2O_5{\cdot}0.5H_2O$: C, 61.10; H, 8.64; N, 8.14; Found: C, 60.90; H, 8.70; N, 7.95%.

4.1.8. 12-Mercaptododecanoic-(8-biotinoylamido-3,6-dioxaoctyl) amide (3). About 50 ml liquid ammonia was condensed into a 250 ml 2-neck flask. Sodium (0.10 g) was added and a dark blue colour observed. 12-Benzylthiododecanoic-(8-biotinoylamido-3,6-dioxaoctyl) amide (0.22 g, 0.32 mmol) in 1:1 mixture of tetrahydrofuran and 1,4-dioxane (30 ml) was added and the reaction mixture stirred at −78°C for 1 h and refluxed for 2 h. Powdered ammonium chloride (1.0 g) and tetrahydrofuran (80 ml) was added after the reaction mixture was cooled to −78°C and the ammonia was left to evaporate over night. The inorganic salts were removed by filtration and washed with dichloromethane. The product was separated by column chromatography on silica gel (10% methanol in dichloromethane) to give product (0.12 g, 0.20 mmol, 65%) as a pale yellow glass. δ_H (300 MHz, $CDCl_3$) 6.81 (1H, br. N*H*), 6.80 (1H, br. N*H*), 6.50 (1H, br. N*H*), 5.81 (1H, br. N*H*), 4.54–4.50 (1H, m, C*H*CH_2S of biotin), 4.37–4.31 (1H, m, C*H*CHS of biotin), 3.64–3.56 (8H, m, CH_2O), 3.50–3.46 (4H, m, CH_2NHCO), 3.18–3.13 (1H, m, C*H*S of biotin), 2.93 (1H, dd, J=5.1, 12.4 Hz, one of CH_2S of biotin), 2.74 (1H, d, J=12.4 Hz, one of CH_2S of biotin), 2.52 (2H, q, J=7.2 Hz, CH_2SH), 2.27–2.17 (4H, m, CH_2CON), 1.77–1.21 (24H, m, CH_2); MS (ES) m/z 611 (M^++Na, 100%), Acc Mass Calcd for $C_{28}H_{52}N_4NaS_2O_5$: 611.3277; Found: 611.3276.

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