Facile solid-phase synthesis of biotinylated alkyl thiols

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Abstract

Biotinylated alkyl thiols with the capacity to graft avidin proteins are in increasing demand for the development of self-assembled monolayers on gold. Here we propose 2-Chlorotrityl Chloride solid-phase resin as a new platform to produce these functionalized alkyl thiols. Biotinylated alkyl thiols of non-obvious solution synthesis were obtained rapidly using this method and without previous purification steps.

Keywords: Solid-phase; Biotinylated alkyl thiols; Self-assembled mono-layers.

Abbreviations: AFM, atomic force microscope; BAT, biotinylated alkyl thiols; DCM, dichloromethane; DIPEA, N,N´ -diisopropylethylamine; DIPCDI, N,N´-diisopropylcarbodiimide; DMF, N,N´-dimethylformamide; HOAt, 1-hydroxy-7-azabenzotriazole (3-hydroxy-3H-1,2,3-triazolo-[4,5-b] pyridine); MALDI-TOF, matrix assisted laser desorption/ionization time of flight (mass spectrometry); MeCN, acetonitrile; MeOH, methanol; MHDA, mercaptohexadecanoic acid; NMR, nuclear magnetic resonance; PDMS, polydimethylsiloxane; PEG, polyethylene glycol; SAM, self-assembled monolayers; TFA, trifluoroacetic acid; TES, triethylsilane.

Introduction

Self-assembled monolayers (SAMs) are a popular tool for tailoring the reactive properties of surfaces. In order to produce these monolayers, molecules may be physisorbed from solution or more tightly grafted by covalent bond formation with gold substrates and alkyl thiols. Because of the features of the functional group of alkyl thiols, surface properties can be easily modified by simply changing the chemical nature of the terminal groups. Alkyl thiols are widely used, mainly in biological applications.[1] Gold surfaces can be derivatized to bind proteins,[2] carbohydrates, peptides,[3] DNA,[4] haptenes[5] or to produce new surfaces for cell culture studies such as cell attachment, differentiation or proliferation.[6] For all these purposes, rapid and efficient access to a diversity of functional alkyl thiols is required. In an attempt to minimize the difficulty of access to these molecules, researchers introduce the desired functionalities (biotins, haptenes, polyglycols) by reaction over amino- or acid-terminating SAMs. Reactions over a previously formed SAM do not ensure perfect derivatization because the processes between surfaces and solution are not kinetically well afforded. This problem is exacerbated when the gold surface to be derivatized is a fragile gold AFM tip or a micro(nano)electrode because the manipulation and the solvent rinsing procedures required to remove the undesired reagents may cause damage. Consequently, a thiol with the desired functionality is required. Given that dip pen nanolithography, the AFM-based soft-lithography developed by Mirkin et al. in 1999,[7] involves the direct deposition of thiols in nanometer scale using an AFM probe, the development of functional alkyl thiols is crucial for this technique.

Here we propose a novel solid-phase strategy for the development of biotin alkyl thiol (BAT) derivates. BAT structures are formed by the thiol, the aliphatic chain (n=11, 16), a PEG linker, and the biotin group (Fig. 1).

BATs are useful for the development of biosensors as they allow the production of well-defined biotinylated surfaces (Fig 2a). Biotin surfaces are one of the most used tools to immobilize antibodies (i.e., antigens, enzymes or DNA) onto surfaces through the biotin-streptavidin (i.e., neutravidin, avidin) pair (K_dw10^{-15} M) as building block. Streptavidin has four equivalent sites for biotin (two on one side and two on the opposite). Streptavidin vacancies for biotin can be used to link the protein almost irreversibly to the surface and to create well-oriented free biotin sites that are exposed to the surface (Fig. 2b). These exposed sites allow the grafting of biotinylated biomolecules for the preparation of biosensors and have minimal impact on biological activity (Fig. 2c). BAT structures must include PEG groups in order to avoid the non-specific adsorption of streptavidin and other proteins onto surfaces, and to allow a good orientation of the streptavidin molecule.

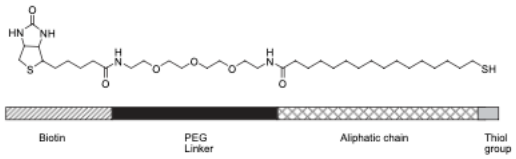


Figure 1. BAT structure

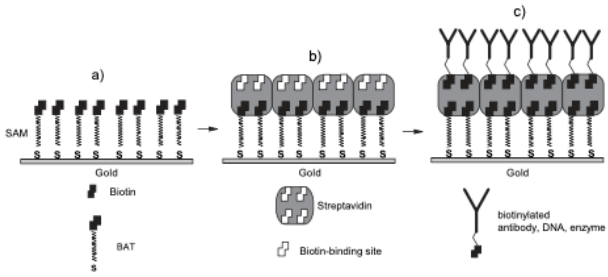


Figure 2. Streptavidin–biotin procedure to immobilize biomolecules: (a) biotinylated gold surface, (b) streptavidin-grafted surface through biotin groups and formation of biotin vacancies on the surface, (c) grafting of biotinylated biomolecules over the surface.

To date, the synthesis of BATs has been described through solution-phase.[8] In the original report, BAT was attained in poor yield and no characterization data were given. In this case, the molecule was constructed by the reaction of the PEG linker with the biotin molecule, followed by attachment of the thiol-protected acid-activated alkyl chain. New syntheses in solution have recently been reported but the procedure is still long, and tedious silica sol–gel purification work-up steps are required in each step of synthesis.[9,10] In all solution-phase syntheses, the protection and deprotection of the thiol cannot be avoided during the procedure and consequently large amounts of starting products and reagents are needed to obtain sufficient amounts of the thiols of interest.

Here we describe a new solid-phase approach, based on the 2-Chlorotrityl Chloride (CTC) resin, for the development of BATs. CTC resin is a polymer support functionalized with chlorotrityl groups that graft nucleophiles, such as thiols,[11] amines[12] or carboxylates,[13] thereby allowing cleavage of the final product under acidic conditions. One of the advantages of this resin is that it can be regenerated several times.[14] Moreover, it offers a new platform to obtain alkyl thiols of interest.[15] The solid-phase method reported here allows the synthesis of high purity BATs in only a few days. The number of carbons of the alkyl chain, the presence or number of glycols, in the case of BAT with PEG linkers, and the functional group can be chosen freely. In solution- phase synthesis, modifications in molecule design change the purification procedures, which must be optimized for any new molecule. We obtained two BATs, with and without PEG, using the same solid-phase synthetic procedure, which indicates the robustness of the new method. The development of this method allows the production of custom- designed BATs or other functionalized alkyl thiols.

Results and discussion

Solid-phase synthesis has been widely used for the synthesis of biopolymers such as peptides, oligonucleotides, and polysaccharides and has dramatically improved yield, purity, and length of synthesis over traditional solution-phase methodology. Solid-phase approaches have evolved exponentially in recent decades because they provide a means to develop small bioactive organic molecules. Moreover, these procedures allow the parallel synthesis of analog families with high purity.[16]

The structure of functionalized alkyl thiols as consecutive building blocks assembled by amide, ester, and ether bonds make this kind of molecule highly suitable for synthesis by solid-phase approaches. Here we focused on the development of BATs in compounds 1 and 2 (Fig. 3).

BAT structures have a long chain with a thiol group at one end and convenient functional group at the other. This strategy is based on chain growth from thiol to functional group. The functional group is added in the last step. This methodology facilitates the generation of compound families with a range of functional groups (Scheme 1). The cornerstone of this strategy is the use of CTC resin as solid-support; (i) CTC resin is functionalized with a chlorotrityl group, which can be attached by nucleophile groups such as amines, carboxylates, and thiols; (ii) cleavage of final compound is under very mild acidic conditions, thereby recovering the initial functional group; (iii) the capacity of CTC resin for regeneration reduces the cost of synthesis.

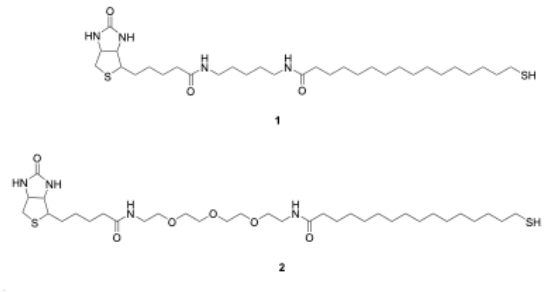
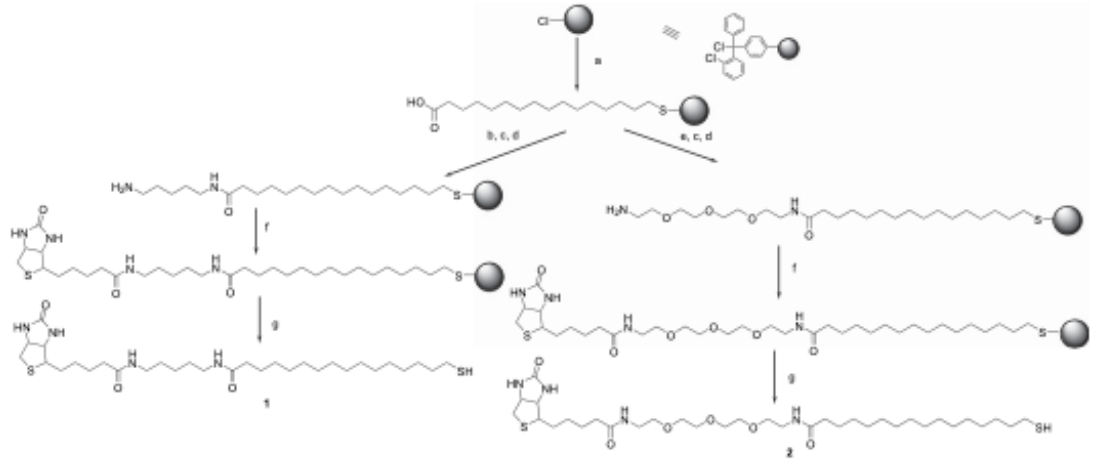


Figure 3. Bat structures



Scheme 1. Solid-phase of 1 and 2. (a) MHDA (2 equiv), DCM, overnight, rt; (b) Fmoc-diaminopentane hydrochloride (3 equiv), DIPCDI (3 equiv), HOAt (3 equiv), DIPEA (3 equiv), DMF, overnight, rt; (c) MeOH (2 mL/g resin), DIPEA (0.3 mL/g resin), DCM, 10 min, rt; (d) piperidine–DMF (1:4, v/v), 1x1 min, 2x10 min, rt; (e) Fmoc-1-amino-4,7,10-trioxa-13-tridecanamine hydrochloride (3 equiv), DIPCDI (3 equiv), HOAt (3 equiv), DIPEA (3 equiv), DMF, overnight, rt; (f) Biotin (5 equiv), DICPDI (5 equiv), HOAt (5 equiv), DMF, overnight, rt; (g) TFA 65% in DCM/TES (95:5, v/v).

Chain elongation is through available protected building blocks using the standard coupling reagents, as in peptide solid-phase synthesis. On solid-support, coupling reaction with quantitative yields greatly facilitates the removal of the excess of reagents and soluble side-products by simple filtration and washings.

The solid-phase resin protects the thiol group and prevents the formation of non-desired compounds via reaction with the thiol instead of acid group. Several attempts were made before achieving the attachment of the 16-mercapto- hexadecanoic acid through its thiol group instead of the carboxylic group. We performed qualitative colorimetric tests,[17] such as Ellman's test for thiol groups and Malachite green for carboxylic groups, to identify the group that was not attached to the resin. Thus, a two-fold molar excess of 16-mercaptohexadecanoic acid and less than an equimolar amount of diisopropylethylamine (DIPEA) was left to react with the resin (156 mg) for 2 h in DCM–DMF (1:1, v/v).[18,13,19] However, in our case, under these conditions, the attachment of mercapto acid through the carboxylic group was partial. The same result was observed when the reaction time was extended overnight. The experiment was repeated without DIPEA. Attachment was not complete through the thiol group. A fourth attempt was performed with DMF only, with no positive results. Finally, the expected result was achieved using DCM without base overnight. The absence of base makes the thiol group more reactive than the carboxylic one and the DCM solvates the aliphatic long chain better than DMF. The carboxylic acid attached to the CTC resin was split into two in order to obtain two BAT-modifying linkers. One half was treated with protected diamino compound Fmoc-diaminopentane hydrochloride and DIPEA to release the free amine, and DIPCDI–HOAt was used as coupling reagent. This inverse solid-phase amide formation (activation of the supported carboxylic groups, which react with excess of the solution of the amino component) requires a powerful coupling reagent such as DIPCDI–HOAt.[20,21] Coupling was performed by mixing the amino compound with all reagents prior to adding to the resin. The remaining active sites (free chloride groups of the resin) were capped by addition of MeOH (200 mL/100 mg resin) and DIPEA (29 mL) for 10 min. The Fmoc group was removed by treatments with piperidine–DMF (1:4, v/v) for 10min, and absorbance (l 290 nm, 3 5800 L/mol/cm) of washes was measured. A good resin

loading (0.85 mmol/g resin) was achieved. The second fraction of 16-mercaptohexadecanoic acid–CTC resin was treated with PEG linker (Fmoc-1-amino-4,7,10- trioxa-13-tridecanamine hydrochloride) neutralized by DIPEA, and DIPCDI–HOAt was used as coupling reagents. Coupling and Fmoc removal were performed as described above. Biotin was attached to the two resin fractions in a parallel way. Coupling was made with a five molar excess of biotin and using DIPCDI (5 equiv), HOAt (5 equiv), and DIPEA (5 equiv) as coupling reagents. The poor solubility of the biotin derivative makes the solid-phase synthesis the strategy of choice for the incorporation of the biotin into the macromolecule.[22] Thus, excess of the derivative can be used to drive the reaction to completion and excesses can be removed by single filtration and washings. To afford compounds 1 and 2, the two resin fractions were treated with TFA–TES–DCM (65:2:33, v/v/v, 3x10 min). Compounds 1 (41 mg, 80% yield) and 2 (61 mg, 96% yield) were obtained as white solids. A 1H NMR spectrum revealed traces of TES in the final product (See Supplementary data). Modified cocktail cleavage should be addressed in further studies to avoid the use of TES.

To test the functionality of these compounds synthesized on solid-phase, micro-contact printing was used. As an example, the interaction of streptavidin with BAT with PEG linker (compound 2, without purification) was examined on gold surfaces. BAT (4 mM) in acetonitrile was used to ink a 2.5 mm PDMS-positive stamp. Before thiol inking, the PDMS stamp was oxidized with O2 plasma to ensure good coverage of the stamp surface with the thiol. After keeping the stamp in contact with gold surface, the samples were immersed in PEG-terminated alkyl thiol, 1 mM diluted ethanol solution for 24 h. This PEG thiol prevented the non-specific adsorption of proteins on the non-functionalized gold areas. Incubation of the samples with streptavidin labeled with a fluorescent dye (Texas Red) and inspection of the surface with fluorescent microscopy showed the patterned areas of proteins over the gold surface and indicated the functionality of the BAT (Fig. 4).

Figure 4. Fluorescent picture of a micropatterned gold surface with BAT showing the interaction between streptavidin labeled with a fluorescent dye and biotin.

Conclusions

CTC resin is a rapid, efficient, and versatile tool for the production of BATs. The solid-phase method described here improves the synthesis of BATs compared with solution-phase syntheses reported to date. This methodology contributes to the development of molecules that are increasingly required for nanobiotechnology applications.

Experimental

General procedures and instrumentation

CTC was a gift from Rohm and Haas, USA. 16-Mercapto-hexadecanoic acid and Biotin was obtained from Aldrich (Milwaukee, WI, USA). Fmoc-diaminopentane hydrochloride was supplied by Bachem AG (Bubendorf, Switzerland). Fmoc-1-amino-4,7,10-trioxa-13-tridecanamine hydrochloride was from Neosystem (Strasbourg, France). DIPCDI and TES were obtained from Fluka Chemika (Buchs, Switzerland). HOAt was supplied by Applied Biosystems, DIEA was from Merck Schuchardt (Hohenbrunn, Germany). TFA was supplied by Scharlau (Barcelona, Spain). DCM, DMF, MeOH, MeCN, piperidine, and tert-butanol were obtained from SDS (Peypin, France). $(EG)_6$-terminated alkyl thiol was purchased from Prochimia (Gdansk, Poland).

Mass spectra were recorded on a MALDI-TOF Applied Biosystems 4700 Proteomics Analyzer.

^{1}H NMR spectroscopy was performed on a Nuclear Magnetic Resonance Varian: Mercury-400 (^{1}H de 400 MHz, $CDCl_3$) spectrophotometer.

UV detection was performed at 290 nm on a UV–vis Shimadzu UV mini 1240 spectrophotometer.

Elastomeric stamp fabrication for mCP has been described previously.[23] Here we use positive stamps, fabricated from polydimethylsiloxane (PDMS; Sylgard 184, Dow Corning), where the structures protrude from the bulk PDMS surface. The PDMS stamps were replicated from silicon-based molds that had previously been structured using deep reactive ion etching (DRIE).

General synthesis

The solid-phase synthesis was performed in 5-mL polypropylene syringes fitted with polyethylene porous disks. Solvents and soluble reagents were removed by suction. After a second coupling, a MeOH capping (200mL MeOH–29 mL DIPEA–100 mg resin) in DCM was carried out (10 min). Next, Fmoc was removed with piperidine– DMF (1:4, v/v) (1x1 min, 2x10 min). Resins were washed before use and between deprotection, and coupling and subsequent deprotection steps were done with DMF (3x30 s) and DCM (3x30 s) using 10 mL of solvent/g of resin each time. Cleavage was performed using TFA 65% in DCM– TES (95:5, v/v) (3x10 min) followed by washing with DCM (3x1 min); to avoid the high concentration of TFA in the balloon, 200 mL of water was added before cleavage. Given the high insolubility of the compounds, once evaporated, lyophilization was performed under tert-butanol. To change the contra ion, a second lyophilization was done under tert-butanol and 100 mL of HCl 1 N.

Loading calculation

Deprotection with piperidine gave the fulvene–piperidine adduct, which was determined by quantitative spectrophotometry at l 290 nm, 3 5800 L/mol/cm.

Synthesis of compound 1

A two-fold molar excess of 16-mercaptohexadecanoic acid (MHDA) (51 mg, 0.18 mmol) was left to react with CTC resin (78 mg, 0.086 mmol) in DCM overnight. Then the Fmoc-di- amminopentane hydrochloride (93 mg, 0.26 mmol) coupling was performed using DIPCDI (40 mL, 0.26 mmol), HOAt (39 mg, 0.28 mmol) as coupling reagents, and addition of DIPEA (44 mL, 0.26 mmol) in DMF overnight at rt. The amino compound was mixed with all reagents prior to adding to the resin. A capping step with methanol was then performed before treatment with piperidine, with several washings between steps (the loading was 0.85 mmol/g of resin). The next coupling was done with biotin (108 mg, 0.44 mmol), DIPCDI (67 mL, 0.44 mmol), HOAt (66 mg, 0.48 mmol), and DIPEA (73 mL, 0.43 mmol), in DMF overnight as mentioned above. After cleavage, compound 1 was obtained as a white solid, the yield was 80% (41 mg). The product was characterized by ^{1}H NMR d_H (400MHz, $CDCl_3$–CD_3OD (1:1)) 4.34–4.31 (2H, m, $CHCH_2S$ of biotin), 3.20–3.16 (4H, m, CH_2NHCO of biotin) and (1H, m, CHS of biotin) both overlapped, with correct integration, 2.94 (1H, dd, CH_2S of biotin), 2.74 (1H, d, CH_2S of biotin), 2.52 (2H, t, CH_2SH), 2.20–2.15 (4H, m, CH_2CON), 1.41–1.24 (36H, m, CH_2) and MALDI-TOF m/z calcd for $C_{31}H_{58}N_4O_3S_2$ 598.95, found 599.43 $[M+H]^+$, 621.42 $[M+Na]^+$, 637.39 $[M+K]^+$ (See Supplementary data).

Synthesis of compound 2

The first attachment of the 16-mercaptohexadecanoic acid (53 mg, 0.18 mmol) to the CTC resin (78 mg, 0.086 mmol) was done as explained above, then Fmoc-1-amino-4,7,10- trioxa-13-tridecanamine hydrochloride (13 mg, 0.27 mmol) coupling was performed using DIPCDI (40 mL, 0.26 mmol), HOAt (37 mg, 0.27 mmol) as coupling reagents and DIPEA (44 mL, 0.26 mmol) in DMF overnight. The amino compound was mixed with all reagents prior to adding to the resin. Next, a capping step with methanol was carried out before treatment with piperidine, with several washings between steps. The loading was 0.45 mmol/g of resin. The next coupling was done with Biotin (113 mg, 0.46 mmol), DIPCDI (66 mL, 0.43 mmol), HOAt (63 mg, 46 mmol), and DIPEA (72 mL, 0.42 mmol) in DMF overnight as mentioned above. After cleavage, compound 2 was obtained as a white solid, the yield was 96% (61 mg). The product was characterized by ^{1}H NMR[3,4] d_H (400 MHz, CD_3OD) 4.49– 4.47 (1H, m, $CHCH_2S$ of biotin), 4.31–4.28 (1H, m, $CHCH_2S$ of biotin), 3.63–3.52 (12H, m, CH_2O), 3.20 (4H, td, CH_2NHCO), 3.19–3.17 (1H, m, CHS of biotin), 2.92 (1H, dd, CH_2S of biotin), 2.69 (1H, d, CH_2S of biotin), 2.48 (2H, t, CH_2SH), 2.21–2.19 (4H, m, CH_2CON), 1.47– 1.30 (34H, m, CH_2) and MALDI-TOF m/z calcd for $C_{36}H_{68}N_4O_6S_2$ 716.46, found 717.62 $[M+H]^+$, 739.59 $[M+Na]^+$, 755.56 $[M+K]^+$ (See Supplementary data).

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