Pergamon

Tetrahedron Letters 42 (2001) 2677–2680

TETRAHEDRON LETTERS

# Dithio-phospholipids for oriented immobilization of proteins to gold surfaces

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Received 14 February 2001; accepted 15 February 2001

**Abstract**—We report the syntheses of phospholipids containing a dithio-group on their hydrophobic end and a choline or a biotin-group on their hydrophilic end. As demonstrated by surface plasmon resonance, a monolayer of these dithiolipids on gold affords specific binding of streptavidin and of biotinylated molecules in further steps. © 2001 Elsevier Science Ltd. All rights reserved.

Lipid monolayers and bilayers supported on solid substrates have attracted great interest in the past decade.[1] A variety of applications have been developed, such as design of biosensors,[2] immobilization of DNA,[3] and 2D-crystallization of proteins.[4] The choice of support is dominated by the measuring technique applied. Noble metal surfaces are suitable for surface plasmon resonance (SPR) and atomic force microscopy (AFM). This choice determines the type of chemistry required for surface modification which is needed for optimal conditions of self assembly and attachment of further biomolecules to the support.[5] Metal surfaces can easily be modified by sulfur-bearing components. Long-chain alkylthiols, derivatized with different functional groups,[6,7] or phospholipids and cholesterol[8] with thiol headgroups have been adsorbed from solution onto gold surfaces, yielding densely packed self assembled monolayers (SAMs). However, the composition of a SAM generated by coadsorption of more than one component is usually different from that in solution, due to the influence of molecular structure on the kinetics of chemisorption.[9]

One way to solve this problem is spreading surface-active molecules at the air–water interface, which is only possible with hydrophobic molecules, such as phospholipids. In addition, the resulting phospholipid monolayers are an ideal matrix for 2D-crystallization of proteins when 10–50% of the phospholipid headgroups contain a specific anchoring site for the protein of interest.[10–13] Subsequently, the 2D-crystal can be transferred to an electron microscopy (EM)-grid[4] or to highly oriented pyrolytic graphite (HOPG, for AFM studies[13]), due to the strong hydrophobic interaction between lipid tails and solid substrate.

In order to apply the same strategy to gold substrates, we designed phospholipids with a disulfide element at their hydrophobic end. These dithio-phospholipids can be spread at the air–water interface and the resulting monolayer can be transferred to a gold substrate. There it covalently binds to the surface, which is of particular advantage for AFM experiments where pulling forces are applied. The detergent resistance of this monolayer is essential when it comes to binding of integral membrane proteins from detergent solutions. In all experiments, the predominant lipid ('host') was the dithio-derivative of phosphatidylcholine (**5**). For the biospecific capture of streptavidin (or of $His_6$-tagged proteins), a small fraction ('guest') of the analogous phosphatidylethanolamine with a biotin-group (**8**) (or with a nitrilotriacetic acid function, NTA, currently under study) was included. The surface density of the 'guest' lipids was easily adjusted during spreading at the air–water interface.

The synthetic steps are shown in Scheme 1. ω-Hydroxyhexadecanoic acid (**1**) was converted into ω-bromohexadecanoic acid with hydrobromic acid.[6] ω-Mercaptohexadecanoic acid (**2**) was formed by converting ω-bromohexadecanoic acid into its thiouronium hydrobromide, followed by cleavage of the complex with NaOH and precipitating compound **2** with sulfuric acid.[14] Reaction with propylmethanethiosulfonate gave propyldithiohexadecanoic acid (**3**) in good yield (about

*Keywords*: phospholipid; disulfide; gold; streptavidin; atomic force microscopy; surface plasmon resonance.

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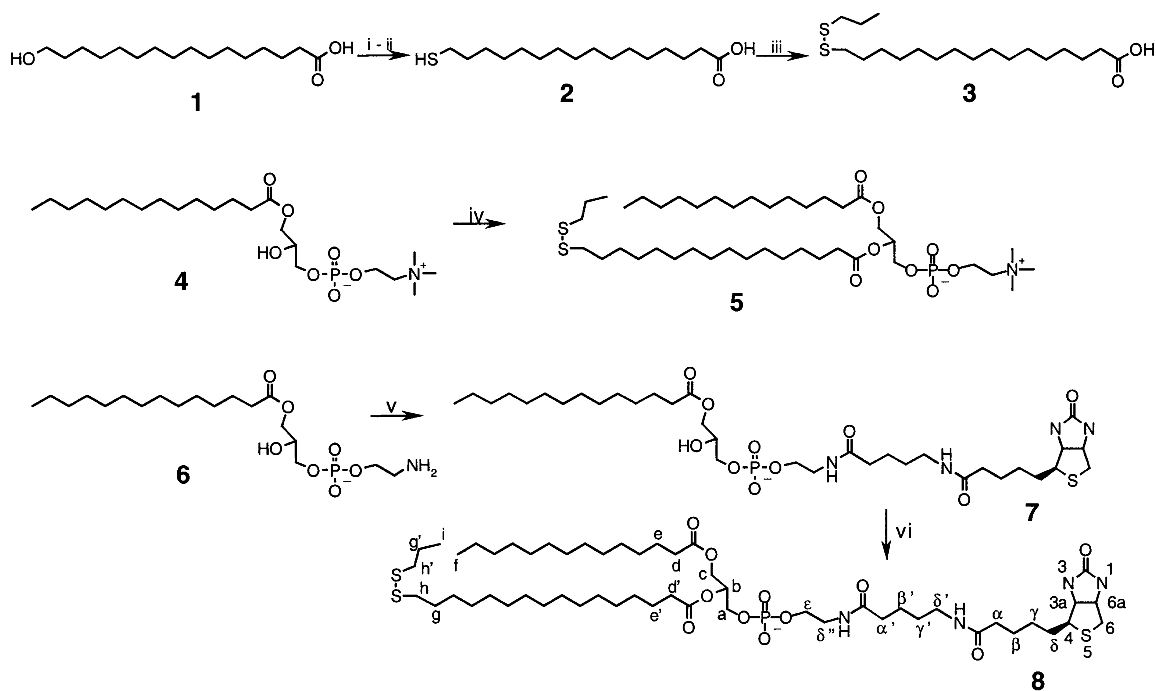
PII: S0040-4039(01)00261-1




**Scheme 1.** *Reagents and conditions*: (i) 1:1 mixture of 48% HBr and 98% acetic acid; (ii) thiourea in 98% ethanol followed by NaOH and sulfuric acid treatment; (iii) propylmethanethiosulfonate/diisopropylethylamine in methanol; (iv) DCC/DMAP in $CH_2Cl_2$ followed by silica gel chromatography; (v) biotin-cap-NHS/diisopropylethylamine in $CHCl_3$/methanol; (vi) DCC/DMAP in $CH_2Cl_2$ followed by silica gel chromatography.

60%). The purity was confirmed by NMR spectrometry.[15]

The 'host'-lipid **5** was obtained by esterification of propyldithiohexadecanoic acid (**3**) with 1-myristoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (**4**) (Avanti Polar Lipids Inc., Alabaster, AL) using equimolar DCC and *N*,*N*-dimethylaminopyridine (DMAP).[16–18] Lipid **5** was purified by silica gel column chromatography (35% yield) and characterized by NMR spectrometry.[19]

For synthesis of the 'guest'-lipid **8**, 1-myristoyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine (**6**) (Avanti Polar Lipids) was reacted with *N*-hydroxysuccinimidyl-biotinamidocaproate (biotin-cap-NHS) to give the biotin–lysolipid **7**. Finally, esterification of propyldithiohexadecanoic acid (**3**) with the biotin–lysolipid gave molecule **8**, which was purified by silica gel column chromatography and characterized by NMR spectrometry.[20]

For AFM experiments, 1 mg/mL 'host' lipid **5** in chloroform/hexane 1:1 (v/v) was spread on a small round

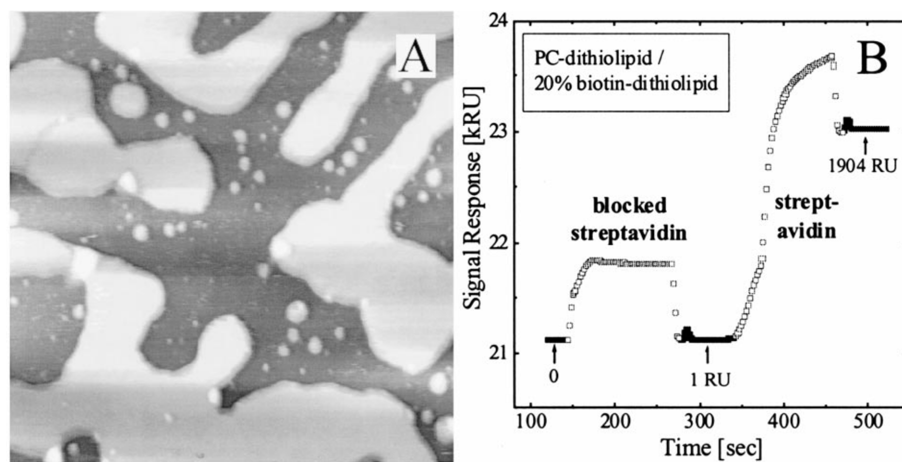



**Figure 1.** (A) MAC-mode™ AFM image of a monolayer of dithiolipid **5** (1 µm in square, height of the lipid film between 2.5 and 3 nm) scanned in PBS buffer solution; (B) BIACORE® sensorgram showing the specific binding of streptavidin to a dithiolipid monolayer (containing biotin-dithiolipid **8** as a scavenger) adsorbed on an untreated gold chip. Enhanced signal response during incubation is due to different protein buffer and transient weak adsorption. Only the solid lines after the washing step reflect specifically bound protein.

Teflon trough (4 mm diameter) filled with a drop of water.[21] The thiolipid monolayer was then transferred to a plate of ultraflat gold (Molecular Imaging Corp., Phoenix, AZ), glued to a steel disc and mounted on the AFM device (MAC-mode™ PicoSPM, Molecular Imaging).[13] Topographic images clearly revealed lipid monolayer patches up to micrometer size (Fig. 1A) which significantly differed from the much smaller atomically flat gold island on underivatized substrate ($\leq$100 nm, data not shown). Moreover, there was no measurable adhesive force between the hydrophobic $Si_3N_4$ tip and the hydrophilic surface of the dithiolipid monolayer, in contrast to the strong adhesion of the tip to untreated gold (measured by AFM force distance cycles, data not shown).

For SPR experiments we used a BIACORE®J (Biacore AB, Uppsala, Sweden) instrument. A monolayer of a 4:1 (*w*/*w*) mixture of 'host'-lipid **5** and biotin–lipid **8** was applied to an untreated gold chip (Pioneer J1, Biacore AB) as described above. In the negative control, streptavidin preblocked with *d*-biotin showed no binding to the biotin-derivatized chip (Fig. 1B). In the absence of *d*-biotin however, streptavidin effectively bound to the biotin–lipid containing monolayer (Fig. 1B, 30–40% calculated surface coverage). In the next step we observed specific binding of biotinylated proteins (biotin–BSA, biotin–ferritin) on the streptavidin layer in SPR experiments. $His_6$-tagged proteins could be bound after treating the streptavidin layer with biotin–NTA (synthesized as described[22]). Currently, we are synthesizing a dithiophospholipid with an NTA moiety for the direct binding of $His_6$-tagged proteins onto the dithiolipid monolayer.

Functionally similar mercaptolipids had been synthesized before. Samuel et al.[23] used oxidation of ω-mercaptolipids for polymerization of vesicle membranes. Linhardt et al.[24] prepared a phosphatidylethanolamine derivative with a 14-mercaptomyristoyl chain for the attachment of polymeric nanotubes, formed from giant unilamellar vesicles, to a thin layer of gold. Finally, Schütterle et al.[25] designed a phosphatidylcholine with a pyridyldithio-group at the end of one acyl chain for SAM formation on gold. While the latter lipid closely resembles our propyldithio analogue **5**, the present study aims at biospecific binding of proteins by inclusion of proper anchor lipids, such as **8**, as demonstrated in Fig. 1B.

## Acknowledgements

The authors would like to thank Dr. Steffi Bachem from Biacore AB for help with BIACORE measurements. Helpful advice from Prof. K. Grubmayr and Dr. T. G. Dax is gratefully acknowledged. This work was supported by the Austrian Science Foundation project P12801-MED.

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15. $^1$H NMR of compound **3** (200 MHz, $CDCl_3$) $\delta$ (ppm): 2.68 (m, 4H; -C$H_2$-S-S-C$H_2$-); 2.35 (t, 2H, $J$=7.4 Hz; -C$H_2$-COOH); 1.55–1.81 (m, 6H; -C$H_2$-$CH_2$-S-S-$CH_2$-C$H_2$-, C$H_2$-$CH_2$-COOH); 1.27 (s, 22H; -C$H_2$-); 1.00 (t, 3H, $J$=7.3 Hz; C$H_3$-).
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19. $^1$H NMR of compound **5** (500 MHz, $CDCl_3$) $\delta$ (ppm): 5.20 (m, 1H; CH); 4.39 (m, 1H; 1-myristoyl-COO-C$H_2$(I)); 4.32 (m, 2H; P-O-$CH_2$-C$H_2$-$N^+$); 4.12 (m, 1H; 1-myristoyl-COO-C$H_2$(II)); 3.94 (m, 2H; CH-C$H_2$-O-P); 3.81 (m, 2H; P-O-C$H_2$-$CH_2$-$N^+$); 3.36 (s, 9H; $N^+$-C$H_3$); 2.67 (m, 4H; -C$H_2$-S-S-C$H_2$-); 2.28 (m, 4H; -C$H_2$-COO); 1.54-1.75 (m, 8H; -C$H_2$-$CH_2$-S-S-$CH_2$-C$H_2$-, C$H_2$-$CH_2$-COO); 1.25 (s, 42H; -C$H_2$-); 0.99 (t, 3H, $J$=7.4 Hz; C$H_3$- of propyldithio); 0.88 (t, 3H, $J$=6.8 Hz; C$H_3$- of myristoyl).
20. $^1$H NMR of compound **8** (200 MHz, $CDCl_3$) $\delta$ (ppm): 5.19 (m, 1H; *b*); 4.50 (m, 2H; *c*(*I*) and 6*a*); 4.30 (m, 1H; 3*a*); 4.1–3.8 (m, 5H; *c*(*II*), *a*, ε); 3.44 (m, 1H; 4); 3.1–3.3 (m, 4H; δ′ and δ″); 2.94 (q, 1H; 6(*I*)); 2.70 (m, 5H; 6(*II*), *h*, *h*′); 2.35 (m, 4H; *d* and *d*′); 2.21 (m, 4H; α and α′); 1.55–1.75 (m, 8H; *e*, *e*′, *g*, *g*′); 1.55–1.25 (m, 10H; β, β′, γ, γ′, δ); 1.25 (s, 42H; -C$H_2$-); 0.99 (t, 3H; *i* ); 0.88 (t, 3H, *f* ).

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