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Pragmatic Studies on Protein-Resistant Self-Assembled Monolayers

Christoph D. Hahn[1], **Ali Tinazli**[2], **Martin Hölzl**[1], **Christa Leitner**[1], **Filip Frederix**[3], **Bernd Lackner**[4], **Norbert Müller**[4], **Christian Klampfl**[5], **Robert Tampé**[2], and **Hermann J. Gruber**[1,*]

[1] Institute of Biophysics, University of Linz, Linz, Austria
[2] Institute of Biochemistry, Johann Wolfgang Goethe-University, Frankfurt a.M., Germany
[3] IMEC, MCP-ART, Leuven, Belgium
[4] Institute of Organic Chemistry, University of Linz, Linz, Austria
[5] Institute of Analytical Chemistry, University of Linz, Linz, Austria

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Summary. The present study describes new synthetic routes to oligo(ethylene glycol)-terminated alkanethiols (*OEG-AT*s), starting from α,ω-dibromoalkanes, which are reacted either with *OEG* or with trityl mercaptan in the first step. In addition to these ether conjugates of *OEG* and *AT*, analogous ester and amide conjugates were prepared by established procedures. All thiols were used to form self-assembled monolayers (*SAM*s) on cleaned gold surfaces and these were stored for 1–2 weeks under water at 4°C before the extent of nonspecific protein adsorption was tested with *IgG*, *BSA*, and lysozyme at 1 mg cm^{-3} protein concentration in phosphate-buffered saline. Under these practice-oriented testing conditions, *SAM*s with tri(ethylene glycol) chains (EG_3) exhibited nonsatisfactory protein resistance, in sharp contrast to EG_4 or longer *OEG* chains. The effectiveness of EG_3 was partially restored when they were linked to a long acyl chain (16-mercaptohexadecanoic acid) instead of 12-mercaptododecane or 11-mercaptoundecane. Furthermore it was found that (i) *SAM* formation at 20 μM thiol *versus* 500 μM *OEG-AT* gave identical results, (ii) gel-filtered proteins were much less adsorbed than the unpurified commercial products, and (iii) the method for gold-precleaning was very critical. In conclusion, this study offers convenient synthetic routes to *OEG-AT* and helps to choose molecules and procedures for reliable preparation of protein-resitant *SAM*s with prolonged stability during storage.

Keywords. Adsorption; Alkanethiol; Biosensors; Monolayers; Proteins.

* Corresponding author. E-mail: hermann.gruber@jku.at

Introduction

Dense brushes of poly(ethylene glycol) chains (*PEG*) on solid surfaces or lipid membranes have long been known to afford high resistance to nonspecific protein adsorption [1, 2]. Long *PEG* chains are usually preferred because short *PEG* chains require high grafting site densities which are difficult to achieve in practice [1, 2]. In contrast, self-assembled monolayers (*SAM*s) of oligo(ethylene glycol)-terminated alkanethiols (*OEG-AT*s) are easily prepared with high *OEG* density, in which case even short tri(ethylene glycol) chains (EG_3) afford complete protein resistance [3, 4]. Later, it was shown that the ether bonds between the hydrophobic tail and the polar *OEG* chain could be replaced by ester or amide linkages (see Fig. 1) while retaining high resistance to protein adsorption [5, 6].

Protein-resistant *OEG*-terminated *SAM*s have widely been used for suppression of nonspecific adsorption in biosensing [6–13], for passivation of the area which surrounds the active spots of capture molecules on microarrays [14, 15], as well as on nanoarrays which had been prepared by dip-pen nanolithography [14–16] or nanografting [17]. Moreover they represent a clean, ultraflat surface for single molecule microscopy [11, 18] or for buildup and imaging of large nanoassemblies [19].

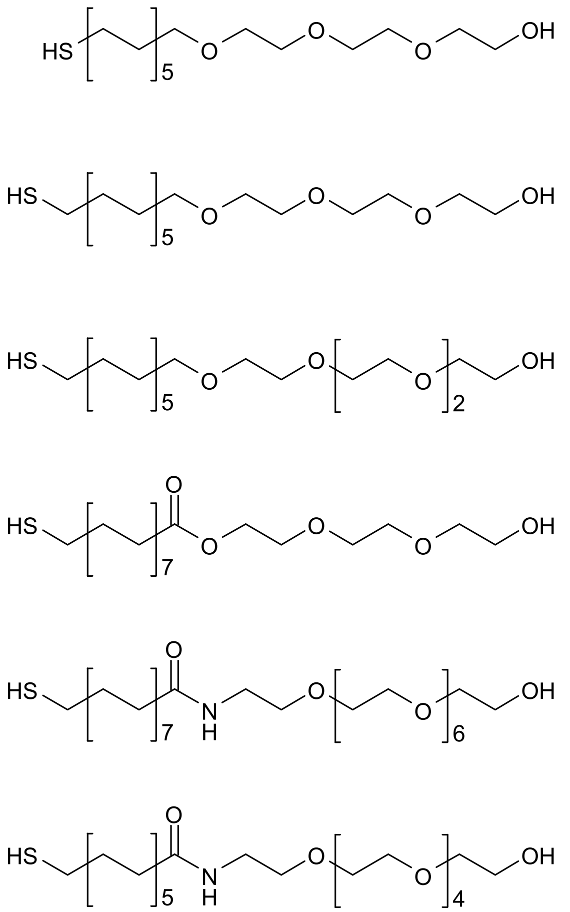

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Fig. 1. Molecular structures of the *OEG*-terminated alkanethiols examined in this study; for convenience, the term HS–C_{11}–EG_3 is used for 20-sulfanyl-3,6,9-trioxa-1-icosanol (**1**), HS–C_{12}–EG_3 for 21-sulfanyl-3,6,9-trioxa-1-heneicosanol (**2**), HS–C_{12}–EG_4 for 24-sulfanyl-3,6,9,12-tetraoxa-1-tetracosanol (**3**), HS–C_{15}–COO–EG_3 for 8-hydroxy-3,6-dioxaoctyl 16-sulfanylhexadecanoate (**4**), HS–C_{15}–CONH–EG_8 for *N*-(23-hydroxy-3,6,9,12,15,18,21-heptaoxatricosanyl)-16-sulfanylhexadecanoylamide (**5**), and HS–C_{11}–CONH–EG_6 for *N*-(17-hydroxy-3,6,9,12,15-pentaoxaheptadecyl)-12-sulfanyldodecanoylamide (**6**)

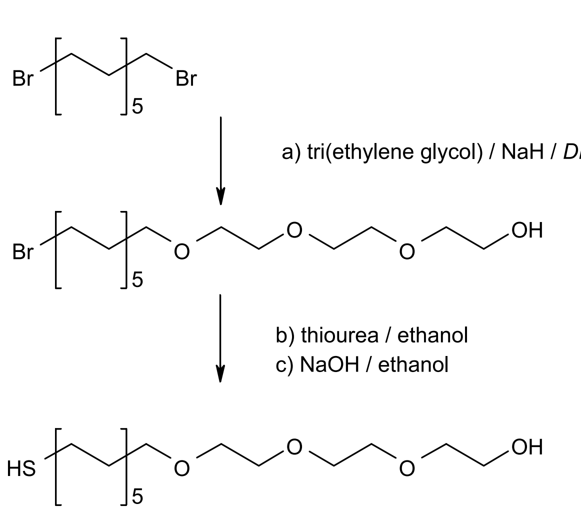
The purpose of the present study was to examine a representative variety of *OEG-AT* (see Fig. 1) with respect to the following criteria: (i) High protein resistance should be retained over several weeks so that larger batches of chips can be prepared at once and used on demand. (ii) *SAM*s formed at low thiol concentration were hoped to show full protein resistance in order to save precious thiols. Finally, (iii) shorter *OEG* chains were much preferred because a typical application is the parallel study of protein–protein interaction, both in the ensemble (by surface plasmon resonance, SPR) and on the single molecular level (by atomic force microscopy, AFM) [11, 18]. Longer *OEG* chains are known to form more loosely packed *SAM*s on gold [20], and such soft "cushions" appear less favorable as support for AFM studies on single molecules than thin, compact *SAM*s with shorter *OEG* chains.

Results and Discussion

Synthesis of OEG-Terminated Alkanethiols from α,ω-Dibromoalkanes

The conventional synthesis route to *OEG-AT* starts from 11-bromoundecene, the first step being ether conjugation with *OEG* by substitution of the bromine atom, followed by addition of thioacetic acid to the terminal C=C double bond which requires catalysis by both UV light and azobisisobutyronitrile [4]. A major advantage of this route is that it starts from a heterobifunctional module which is commercially available. Practical problems, however, are the slow turnover of thioacetic acid addition and the need for a suitable photochemical setup which is not ubiquitous.

The present study reports two alternative synthetic schemes for *OEG-AT* both of which start from an α,ω-dibromoalkane. Scheme 1 [21] is a combination of previously described synthetic steps. In the first step, 1,11-dibromoundecane was reacted with tri(ethylene glycol), followed by isolation of the asymmetric ether conjutate [22]. In the second step, the thiol group was introduced by reaction with thiourea, alkaline hydrolysis, and acidic extraction [23]. Scheme 2 [24] describes the opposite strategy, *i.e.* replacement



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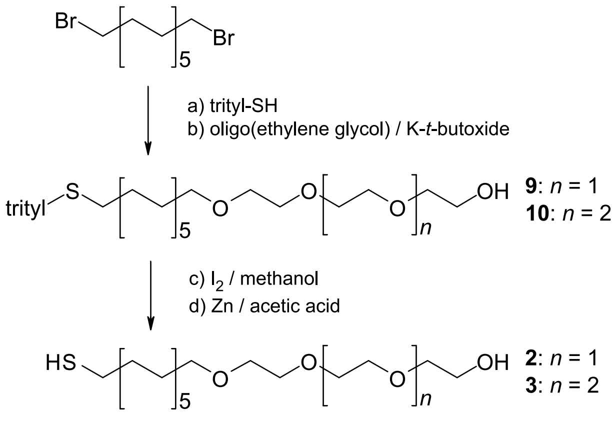
Scheme 1



Scheme 2

of bromine by a protected thiol function in the first step [25], followed by ether conjugation with tri- or tetra(ethylene glycol), cleavage of the *S*-trityl group and reduction of the disulfidic intermediate.

Although Schemes 1 and 2 start from symmetric α,ω-dibromoalkanes, the isolation of the initially generated asymmetric intermediates from unreacted educt and from disubstituted byproduct was straightforward, due to the widely differing physical properties of the components. Meanwhile, intermediate **9** proved to be a generally useful module because the stability of the *S*-trityl group towards strong bases allowed for extension of the terminal hydroxyl group with a propionic acid function and introduction of further coupling/ligand functions [26]. In conclusion, Schemes 1 and 2 represent valuable alternatives to the conventional synthesis route, depending on circumstances, preferences, and the possible need for further functional groups in the basic *OEG-AT* structure. Moreover, the *OEG*-terminated dodecanethiol derivatives **2** and **3** synthesized *via* the route in Scheme 2 have not been reported before.

Protein Resistance of OEG-Terminated SAMs after Prolonged Storage

In several previous studies [3–6], *SAM*s from various *OEG-AT*s have been examined for their protein resistance. In case of unitary *SAM*s, *i.e. SAM*s with an *OEG* chain on every alkanethiol, even EG_2 chains gave perfect protein resistance [4]. In mixed *SAM*s consisting of simple alkanethiols and *OEG-AT*s, however, longer *OEG* chains were shown to allow for a progressively larger molar fraction of the *OEG*-lacking alkanethiol before protein resistance was measurably reduced [4], in other words, longer *OEG* chains obviously provide for more robustness of protein resistance.

The intention of the present study was to apply this concept for the preparation of *SAM*s with extended lifetime of protein resistance when stored under water. Storage under water has the advantage that the *SAM* is fully protected from atmospheric influences and is not manipulated between *SAM* preparation and its use. By analogy with the above cited study [4], the expected trend was clear: longer *OEG* chains should provide for more robustness and long term stability. Nevertheless it was essential to find out which particular *OEG* chain length actually provides for uncompromised protein resistance on the time scale of 2 weeks. Moreover, little was known about the possible influence of the length of the hydrophobic tail and of the type of linkage between *OEG* chain and alkanethiol.

We therefore selected the different types of *OEG-AT*s depicted in Fig. 1 to prepare *SAM*s the protein resistance of which was examined by SPR after 1–2 weeks of storage under water. The chips with the *SAM*-covered gold surfaces were mounted in a BIAcore® X setup and superfused with phosphate-buffered saline (*PBS*) at a flow of $10\,mm^3\ min^{-1}$.

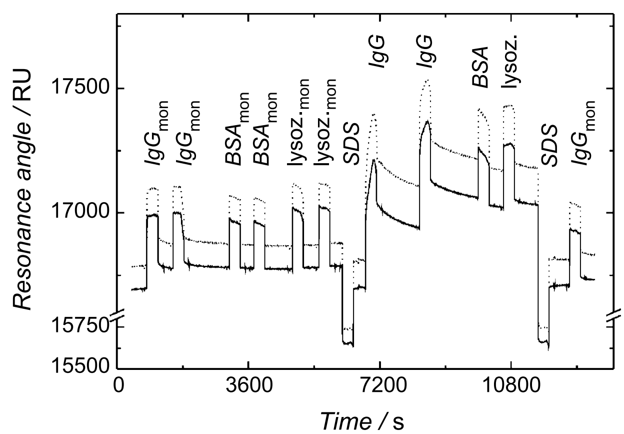


Fig. 2. SPR measurement of nonspecific protein binding to a *SAM* prepared in $20\,\mu M$ HS–C_{12}–EG_3 (**2**); goat *IgG*, *BSA*, and hen egg lysozyme were each applied twice at $1\,mg\ cm^{-3}$ concentration in *PBS*; in the first sequence, monomeric proteins were applied which had been isolated by gel filtration on a Superdex® 200 column; after washing with 0.5% *SDS*, two injections of unpurified goat *IgG* and single injections of *BSA* and lysozyme were applied; after a second wash with 0.5% *SDS*, purified *IgG* was injected again; the solid and the dotted line reflect the SPR signal from two different spots on the same chip which are serially passed by the same solution with ~1 s time difference

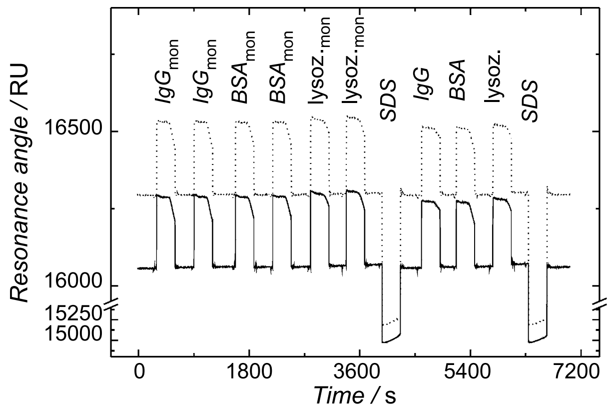


Fig. 3. SPR measurement of nonspecific protein binding to a *SAM* prepared in 20 μM HS–C_{12}–EG_4 (**3**); the sequence of injections was closely similar as in Fig. 2 (see legend within the figure)

The SPR signals from the two separate flow cells were simultaneously recorded (solid and dotted lines in Figs. 2 and 3), thereby monitoring protein adsorption on two different regions of the same chip. The two flow cells were serially perfused with the same solution, the time delay being in the order of 1 s. After insertion of the chip, the baselines in the two flow cells showed a minor drift but became constant within 30–60 min. Then, 100 mm^3 volumes of protein (1 mg cm^{-3}) were injected at the same flow (see Figs. 2 and 3). In a first series, monomeric proteins (goat *IgG*, *BSA*, lysozyme) were applied all of which had been purified by HPLC gel filtration to eliminate aggregates (<5% dimers and traces of oligomers, data not shown). After a washing step with 0.5% *SDS*, goat *IgG*, *BSA*, and lysozyme were injected which had not been purified by gel filtration. Finally, another washing step with 0.5% *SDS* was performed in order to check for reversibility of protein adsorption.

Figures 2 and 3 show the nonspecific binding of proteins to *SAM*s of HS–C_{12}–EG_3 (**2**) and HS–C_{12}–EG_4 (**3**), which differ by a single ethylene glycol unit only. Surprisingly, the nonspecific adsorption of protein was much more pronounced on the EG_3-terminated *SAM* (Fig. 2), as compared to the EG_4-terminated *SAM* (Fig. 3). On the EG_3-terminated *SAM*, the first injection of monomeric *IgG* caused a signal increase of +83 and +89 resonance units (RU) in flow cell 1 and 2 (solid and dotted line in Fig. 2), albeit further injections of monomeric proteins (*IgG*, *BSA*, and lysozyme) caused little additional binding. An injection of 0.5% *SDS* removed most of the adsorbed protein, with residual binding corresponding to 5 and 23 RU in flow cell 1 and 2, as compared to the initial baseline. Subsequent injection of unpurified commercial proteins (see previous paragraph) led to dramatic nonspecific adsorption, corresponding to +240 and +300 RU after the first injection and another +120 and +110 RU after the second injection of goat *IgG* in flow cell 1 and 2 (solid and dotted line in Fig. 2). The following injections of *BSA* and lysozyme had minor effects on top of slow continuous *IgG* desorption. The second injection of 0.5% *SDS* caused a perfect return of both traces to the level obtained after the first *SDS* injection. In separate experiments it was shown that the enhanced adsorption of *IgG* (in comparison to *BSA* and lysozyme), as well as the enhanced adsorption of commercial proteins over gel filtered proteins were general observations and not caused by the order of injections shown in Figs. 2 and 3 (data not shown).

The pronounced nonspecific adsorption of proteins to the EG_3-terminated *SAM* in Fig. 2 is in contrast with the minimal protein adsorption on the EG_4-terminated *SAM* in Fig. 3. Together, all six injections of monomeric proteins caused a signal increase of +11 and +7 RU in flow cell 1 and 2 (solid and dotted line in Fig. 3), which was also exactly reversed in the subsequent washing step with 0.5% *SDS*. Even more surprising was the lack of adsorption with unpurified *IgG*, *BSA*, and lysozyme in the next cycle, with the total signal increases amounting to +6 and +9 RU in the two flow cells. When compared with the signal for a dense layer of *IgG* (~2500 RU) [9], the resonance angle shift of <10 RU for the EG_4-terminated *SAM* corresponds to <0.4% monolayer coverage by *IgG* and is almost negligible.

The protocol in Fig. 3 was applied to *SAM*s prepared from thiols **1**–**6** at 500 μM or at 20 μM thiol concentration. The most characteristic parameter was the SPR angle shift observed after the first injection of monomeric *IgG* (compare Fig. 2), thus this criterion was used for a comparison of different *SAM* types (see Table 1). The striking contrast between high adsorption on thiol **2** (exemplified in Fig. 2) and low adsorption on thiol **3** (depicted in Fig. 3) was confirmed in triplicate experiments (see Table 1). Since no comparable literature data were available for thiols **2** (HS–C_{12}–EG_3) and **3** (HS–C_{12}–EG_4), it was critical to also test the widely used thiol **1** (HS–C_{11}–EG_3) for protein adsorption after storage of the *SAM* under water (see Table 1). Under the testing conditions, thiol **1** showed the same increased

Table 1. Nonspecific adsorption of monomeric goat *IgG* at 1 mg cm^{-3} towards *SAM*s prepared from the *OEG*-terminated alkanethiols **1–6** shown in Fig. 1; the thiol concentration was either 500 μM or 20 μM during *SAM* formation; the extent of adsorption is given in RU, one RU corresponding to 0.0001° SPR angle shift; for comparison, 25 RU corresponds to ~1% of maximal coverage by *IgG* [9]; the data have been averaged from 3 to 4 chips for each *SAM* type, each chip yielding two SPR traces from two different spots on the chip (solid and dotted line in Figs. 2 and 3)

Thiol	Adsorption/RU	
	500 μM	20 μM
1, HS–C_{11}–EG_3	115 ± 21	69 ± 41
2, HS–C_{12}–EG_3	85 ± 28	77 ± 26
3, HS–C_{12}–EG_4	16 ± 12	4 ± 2
4, HS–C_{15}–COO–EG_3	–[a]	36 ± 8
5, HS–C_{15}–CONH–EG_8	12 ± 3	8 ± 4
6, HS–C_{11}–CONH–EG_6	–[a]	25 ± 14

[a] Not determined

level of protein adsorption as thiol **2**, from which follows that the startling difference between thiol **2** (HS–C_{12}–EG_3) and thiol **3** (HS–C_{12}–EG_4) was solely due to the single additional ethylene glycol unit in the latter. The reduced protein repellency of EG_3-terminated *SAM*s after storage is also reflected in the data for thiol **4** (HS–C_{15}–COO–EG_3), taking into account that the shortness of EG_3 was partially compensated for by the long hydrophobic anchor which may confer higher stability of the *SAM* over time. Unexpectedly, thiol **3** (HS–C_{12}–EG_4) was not topped by thiols **5** (HS–C_{15}–CONH–EG_8) and **6** (HS–C_{11}–CONH–EG_6), in spite of their longer *OEG* termini. Thiol **5** was equal to thiol **3** within experimental error, while thiol **6** appeared slightly more adsorptive.

A pleasant side effect of the study was the finding that *SAM*s formed in 20 μM thiol were always at least as good as those formed at 500 μM (see Table 1), allowing for minimization of thiol consumption. In addition, it was found that the 1 m*M* stock solutions of the thiols maintained full activity over several months when stored at −25°C.

Role of the Gold Precleaning Method

In the beginning of this study it was found that an essential precondition for a protein resistant *SAM* was precleaning of gold with boiling *SC1*, *i.e.* 2 × 20 min incubation at ~70°C in a mixture of water, 25% ammonia, and 30% hydrogen peroxide (5/1/1, $v/v/v$) [5]. When commercial BIAcore® chips (SIA kit) were used that had been precleaned by sonication in ethanol according to the manufacturer's instructions, then even thiol **4** (HS–C_{15}–CONH–EG_8) gave severe adsorption of *IgG*, equivalent to full monolayer coverage (data not shown). Precleaning with piranha and/or ozone afforded only moderate improvements, whereas boiling in *SC1* gave the satisfactory results reported above. Unfortunately, *SC1* is extremely irritating and potentially toxic. Even the small amount of fumes released before heating can cause severe irritation when working for several minutes in front of a half way closed hood, especially with pre-sentitized personnel. In case of an accident, repeated inhalation of cortison for a 24 h period in a hospital is indispensable to exclude the development of a potentially fatal lung edema.

Conclusions

The main intention of this study was to select types of protein-resistant *SAM*s on gold that can be prepared in larger batches and then consumed in the course of 1–2 weeks. Since no relevant data were available in the literature, a representative variety of *OEG-AT*s was examined by this criterion. The results in Table 1 show that a clear distinction was found between EG_3-terminated *SAM*s which were rather unsatisfactory, and *SAM*s with EG_4 or longer *OEG* chains which showed high protein resistance. Interestingly, the most dramatic difference was found in that pair of thiols which differed by a single ethylene glycol unit only, *i.e.* thiol **2** and thiol **3**. Although this finding is similar to the trends previously reported for mixed *SAM*s of *OEG*-terminated and *OEG*-lacking alkanethiols [4], the dramatic superiority of EG_4- over EG_3-terminated *SAM*s after 1–2 weeks of storage could not have been anticipated without explicit testing.

Other findings of practical value were that (i) longer hydrophobic tails also helped to enhance long term stability of protein resistance, up to the point that thiol **3** proved satisfactory, in spite of its short EG_3 chain, (ii) gel-filtered proteins showed much less adsorption than unpurified commercial proteins, (iii) repeated challenge of a *SAM* with protein followed by washing with *SDS* significantly improved protein resistance, (iv) even at 20 μM thiol concentration *SAM*s with long term protein resistance could be formed, and (v) the choice of method for gold precleaning was most essential. Together with the new synthetic routes in Schemes 1 and 2, the above findings should help to reliably prepare *OEG*-terminated *SAM*s for applications which require long term stability of protein resistance.

Experimental

Water was always taken directly from a Milli-Q50 system. Analytical grade solvents and materials were used, as long as they were commercially available. NaH (60%, in mineral oil) was obtained from Acros Organics. 1,11-Dibromoundecane, 1,12-dibromododecane, potassium *t*-butoxide, CH_3COSK, *t*-butyl acrylate, thiourea, tri(ethylene glyol) (for reaction with 1,11-dibromododecane), and trityl mercaptan were purchased from Aldrich. Sephadex LH-20 was obtained from Amersham. CH_3COOH, CH_3CN, $CHCl_3$, CH_2Cl_2, HCl (37%), and toluene were purchased from J.T. Baker. Tri(ethylene glycol) (for reaction with 1,12-dibromododecane) and tetra(ethylene glycol) were obtained from Fluka. Aqueous NH_3 (25%), anhydrous KH_2PO_4, H_2O_2 (30%, aqueous solution), I_2, KCl, CH_3OH, ninhydrine, H_3PO_4, NaCl, NaOH, Na_2SO_4, TLC plates (silica 60, without fluorescent indicator), and triphenylphosphine were purchased from Merck (Germany). Azido-EG_8 was obtained from Polypure (Norway). Glass substrates (D263 T Dünnglas, 12 mm × 12 mm × 0.3 mm) for SPR were purchased from Präzisions Glas & Optik GmbH, Iserlohn, Germany. Ethanol (analytical grade) for *SAM* formation was obtained from Roth (Germany). *BSA* (no. 775 827, Fraction V, fatty acid free) was purchased from Roche (Austria). Goat *IgG* (I-5256), 16-hydroxyhexadecanoic acid, lysozyme (L-6876), and Na_2HPO_4 were obtained from Sigma. *HS–*C_{15}*–COO–*EG_3 (thiol **4**) was available from a previous study [11]. HS–C_{11}–CONH–EG_6 (thiol **6**) and HS–C_{15}–CONH–EG_8 (thiol **5**) were synthesized as described before [10], except that commercial N_3–EG_8 (Polypure, Norway) was used for the synthesis of thiol **5**.

NMR spectra were recorded on a Bruker WM300 spectrometer or on a Bruker DPX200 spectrometer at 300 MHz or 200 MHz (as specified) in 5 mm dual $^1H7/^{13}C$ probes. Mass spectra were measured on a Kratos MS50T spectrometer.

20-Bromo-3,6,9-trioxa-1-icosanol (**7**, $C_{17}H_{35}BrO_4$)
Both 4.7 g tri(ethylene glycol) (31 mmol) and 0.41 g NaH (17 mmol) were dissolved in dry *DMF* and stirred for 30 min. The resulting solution was treated with 20 g 1,11-dibromoundecane (6.3 mmol) and subsequently stirred for 17 h. The reaction was quenched with CH_3OH and the solvent was evaporated. The resulting oil was dissolved in 250 cm^3 CH_2Cl_2, washed four times with water, and dried ($MgSO_4$). Subsequently, the solvent was evaporated and the residue was purified by column chromatography (silica 60, ethyl acetate) yielding 2.3 g **7** (60%), as verified by ^1H NMR and mass spectrometry [22].

20-Sulfanyl-3,6,9-trioxa-1-icosanol (HS–C_{11}–EG_3, **1**, $C_{17}H_{36}O_4S$)
Both 2.3 g **7** (6 mmol) and 2.36 g thiourea (31 mmol) were dissolved in ethanol and refluxed for 14 h under N_2 atmosphere. The resulting solution was treated with 1.7 g NaOH (42.5 mmol) in a few cm^3 water and refluxed for an additional 5 h under N_2 atmosphere. The mixture was then treated with HCl in ice-cold water, 300 cm^3 CH_2Cl_2 were added, and the organic layer was washed three times with water. The solvent was evaporated and the oil-like residue was recrystallized from ethanol giving rise to 1.41 g **1** (70%). The composition and purity were verified using ^1H NMR, TLC, and mass spectrometry. ^1H NMR (300 MHz, $CDCl_3$): $\delta = 3.75$–3.5 (m, 12H), 3.4 (t, $J = 7$ Hz, 2H), 2.5 (q, $J = 7$ Hz, 2H), 1.5–1.1 (m, 19H) ppm; MS (CI): $m/z = 337$ (MH^+).

S-Trityl S-(12-bromododecyl) sulfide (**8**, $C_{31}H_{39}BrS$)
Both 14.35 g 1,12-dibromododecane (44 mmol) and 2.76 g trityl mercaptan (10 mmol) were dissolved in 300 cm^3 CH_3CN under Ar atmosphere and 9.12 g K_2CO_3 (66 mmol) were added. The mixture was refluxed for 23 h during which time the color turned to light yellow. The solvent was removed by rotary evaporation, the residue was dissolved in CH_2Cl_2 (100 cm^3), and washed with 1 *M* HCl (50 cm^3), 1 *M* NaOH (50 cm^3), and brine (100 cm^3). The organic layer was dried (Na_2SO_4) and filtered. The filtrate was subjected to rotary evaporation and purified by repeated recrystallization from *n*-hexane, yielding 3.12 g **8** (6.0 mmol). ^1H NMR (200 MHz, $CDCl_3$): $\delta = 1.15$–1.50 (m, 18H, –$(CH_2)_9$–), 1.87 (tt, $J_{AB} = J_{BC} = 6.9$ Hz, 2H, CH_2(A)–CH_2(B)–CH_2(C)–Br), 2.15 (t, $J = 7.2$ Hz, 2H, CH_2–S), 3.42 (t, $J = 6.9$ Hz, 2H, CH_2–Br), 7.21–7.33 (m, 9H, trityl: H3, H4, H5), 7.43 (d, $J = 6.9$ Hz, 6H, trityl: H2, H6) ppm.

21-Tritylsulfanyl-3,6,9-trioxa-1-heneicosanol (**9**, $C_{37}H_{52}O_4S$) and *24-Tritylsulfanyl-3,6,9,12-tetraoxa-1-tetracosanol* (**10**, $C_{39}H_{56}O_5S$)
Potassium *t*-butoxide (217 mg, 1.93 mmol) was suspended in 10 cm^3 tri(ethylene glycol) or tetra(ethylene glycol) and vigorously stirred under Ar for 15 min. After addition of 1012 mg **8** (1.93 mmol) the mixture was gently heated to 90°C and reacted at this temperature for 5 h. The mixture was allowed to cool to r.t., diluted with $CHCl_3$ (30 cm^3), washed with water (2 × 40 cm^3), dried (Na_2SO_4), and filtered. After evaporation of the filtrate the residue was subjected to chromatography on silica 60 (80 g, 3.5 cm ID column) in $CHCl_3$ yielding 672 mg **9** (1.06 mmol) or 721 mg **10** (1.22 mmol), as colorless oils that gradually crystallized. ^1H NMR (**9**, 200 MHz, $CDCl_3$): $\delta = 1.10$–1.50 (m, 18H, –$(CH_2)_9$–), 1.59 (m, 2H, CH_2–CH_2–CH_2–O), 2.14 (t, $J = 7.4$ Hz, 2H, CH_2–S), 3.46 (t, $J = 6.7$ Hz, 2H, CH_2–CH_2–CH_2–O), 3.61–3.75 (m, 12H, O–CH_2–CH_2–O), 7.21–7.32 (m, 9H, trityl: H3, H4, H5), 7.43 (d, $J = 6.9$ Hz, 6H, trityl: H2, H6) ppm; ^1H NMR (**10**, 200 MHz, $CDCl_3$): $\delta = 1.10$–1.50 (m, 18H, –$(CH_2)_9$–), 1.55 (m, 2H, CH_2–CH_2–CH_2–O), 2.15 (t, $J = 7.2$ Hz, 2H, CH_2–S), 3.45 (t, $J = 6.7$ Hz, 2H, CH_2–CH_2–CH_2–O), 3.55–3.75 (m, 16H, O–CH_2–CH_2–O), 7.15–7.35 (m, 9H, trityl: H3, H4, H5), 7.45 (d, $J = 6.9$ Hz, 6H, trityl: H2, H6) ppm.

21,21′-Dithiobis(3,6,9-trioxa-1-heneicosanol) (**11**, $C_{36}H_{74}O_8S_2$) and *24,24′-Dithiobis(3,6,9,12-tetraoxa-1-tetracosanol)* (**12**, $C_{40}H_{82}O_{10}S_2$)
The corresponding trityl derivative (**9** or **10**, 0.5 mmol) was dissolved in 8 cm^3 CH_3OH under Ar atmosphere and I_2 crys-

tals (1 mmol) were added. The reaction mixture was stirred at r.t. and the reaction was monitored by TLC (chloroform/methanol, 90/15, v/v). When no trityl derivative could be detected anymore, the solution was diluted with 10 cm^3 $CHCl_3$ and subjected to rotary evaporation. The residue was redissolved in CH_3OH (2.5 cm^3) and chromatographed in the same solvent on Sephadex LH-20 (1.5 cm × 95 cm, at a flow of 0.4 cm^3 min^{-1}), yielding 0.2 mmol disulfide as colorless crystals. The product was pure by TLC in ethyl acetate. The R_f of the product ($R_f = 0.06$) was very distinct from that of the byproduct trityl iodide ($R_f = 0.40$), thus flash chromatography (*e.g.* in ethylacetate or $CHCl_3$ with a low percentage of CH_3OH) should be a good alternative to chromatography on Sephadex LH-20. ^{1}H NMR (**11**, 200 MHz, $CDCl_3$): $\delta = 1.23$–1.40 (m, 32H, –$(CH_2)_8$–), 1.50–1.75 (m, 8H, CH_2–CH_2–CH_2–O and CH_2–CH_2–CH_2–S), 2.49 (t, $J = 6.1$ Hz, 2H, –OH), 2.69 (t, $J = 7.1$ Hz, 4H, CH_2–S), 3.47 (t, $J = 6.9$ Hz, 4H, CH_2–CH_2–CH_2–O), 3.55–3.80 (m, 24H, O–CH_2–CH_2–O); ^{1}H NMR (**12**, 200 MHz, $CDCl_3$): $\delta = 1.20$–1.40 (m, 32H, –$(CH_2)_8$–), 1.50–1.75 (m, 8H, CH_2–CH_2–CH_2–O and CH_2–CH_2–CH_2–S), 2.31 (s, 2H, –OH), 2.69 (t, $J = 7.2$ Hz, 4H, CH_2–S), 3.45 (t, $J = 6.7$ Hz, 4H, CH_2–CH_2–CH_2–O), 3.55–3.80 (m, 32H, O–CH_2–CH_2–O) ppm.

21-Sulfanyl-3,6,9-trioxa-1-heneicosanol (HS–C_{12}–EG_3, **2**, $C_{18}H_{38}O_4S$) and *24-Sulfanyl-3,6,9,12-tetraoxa-1-tetracosanol* (HS–C_{12}–EG_4, **3**, $C_{20}H_{42}O_5S$)

CH_2Cl_2 (3 cm^3) and CH_3COOH (1.5 cm^3) were added to the symmetric disulfide (0.1 mmol **11** or **12**) and the solution was stirred under Ar atmosphere. Zinc powder (163 mg, 2.5 mmol) was added against the Ar flow and the mixture was stirred under Ar until all disulfide had been converted into free thiol according to TLC in $CHCl_3/CH_3OH$ (90/15, v/v). The suspension was filtered through cotton wool, diluted with 20 cm^3 $CHCl_3$, washed with 0.5% aqueous HCl (2 × 20 cm^3), dried (Na_2SO_4), and filtered. The filtrate was taken to dryness and dried at 1–10 Pa, yielding 0.18 mmol **2** or **3** as a colorless oil that was found to be pure by TLC in the above eluent, except for traces of disulfide. ^{1}H NMR (**2**, 200 MHz, $CDCl_3$): $\delta = 1.25$–1.50 (m, 16H, –$(CH_2)_8$–), 1.50–1.70 (m, 4H, CH_2–CH_2–CH_2–O and CH_2–CH_2–CH_2–S), 2.54 (quartettoid, $J = 7.3$ Hz, 2H, CH_2–SH), 3.47 (t, $J = 6.7$ Hz, 2H, CH_2–CH_2–CH_2–O), 3.50–3.80 (m, 12H, O–CH_2–CH_2–O); ^{1}H NMR (**3**, 200 MHz, $CDCl_3$): $\delta = 1.20$–1.50 (m, 16H, –$(CH_2)_8$–), 1.50–1.70 (m, 4H, CH_2–CH_2–CH_2–O and CH_2–CH_2–CH_2–S), 2.53 (quartettoid, $J = 7.2$ Hz, 2H, CH_2–SH), 3.45 (t, $J = 6.7$ Hz, 2H, CH_2–CH_2–CH_2–O), 3.50–3.80 (m, 16H, O–CH_2–CH_2–O) ppm.

Preparation of Gold Chips for Surface Plasmon Resonance

All glassware and tweezers were precleaned, in a closed and well ventilated hood (!), by submerging them in or filling them with *SC1* (a 5:1:1 mixture of water, 25% NH_3, and 30% H_2O_2, $v/v/v$, see Results section for the high risks of this reagent!), heating to 70–80°C for 20 min, and rinsing with water (5×). This standard cleaning cycle (boiling *SC1* + 5× rinsing with water) was repeated [11]. Subsequently, the glass substrates (12 mm × 12 mm × 0.3 mm) were cleaned in the precleaned glass dishes by another two standard cleaning cycles. The cleaned slides were washed in ethanol (3×), blown dry with N_2 gas, and coated by thermal evaporation at 10^{-4} Pa pressure with chromium (2.8–3.0 nm at 0.05 nm/sec) and gold (41 nm at 0.6 nm/sec) in a Bal-Tec Med020 system (Baltech AG, Liechtenstein).

SAM Formation on the Gold Surfaces

Thiol stock solutions (1 m*M* in ethanol) were prepared in glass vials with screw-thread open top caps and separate *PTFE* septa which had been precleaned in boiling *SC1* and rinsed in water. The stock solutions were stored at −25°C for up to several months and thawed in desiccators over blue gel before use. The gold chips were cleaned by two standard cleaning cycles (boiling *SC1* + 5× rinsing, boiling *SC1* + 10× rinsing), just as described above for precleaning of the bare glass slides. The gold chips were rinsed in ethanol (3×) and immersed for at least 1 min in the same solvent (CH_3CN) or solvent mixture (CH_3CN/ethanol, 1/1, v/v) which was subsequently used for *SAM* formation. After this equilibration, the chips were immersed in 500 μM or 20 μM thiol solutions which had been prepared in weighing dishes by diluting an ethanolic 1 m*M* stock solution with CH_3CN. The glass dishes were tightly closed and kept under ambient conditions for 36 h. The coated chips were rinsed in the same solvent as previously used during *SAM* formation (3×) and then bath-sonicated in the same solvent in the weighing dish for no longer than 3 min. The chips were again rinsed in the same solvent (2×), followed by 3 rinses in ethanol, and one rinse in water. Each chip was individually transferred into a 14 cm^3 Falcon tube that had been filled with water before. The water was carefully decanted and the tube was slowly filled with water again. All Falcon tubes of one batch were screw capped and placed in a water-filled beaker which was placed in a bath sonicator for no longer than 3 min. Subsequently, the water was decanted and the Falcon tube was carefully refilled. This step was repeated, the tubes were completely filled with water and capped. They were kept in the dark at 4°C for 1–3 weeks.

Measumement of Protein Adsorption by Surface Plasmon Resonance

SPR measurements were performed in a BIAcore® X system (BIAcore® AB, Uppsala, Sweden) at 25°C with a flow rate of 10 mm^3 min^{-1}. The stored chips were washed with water (3×), blown dry with N_2 gas and mounted on the chip holder with double sided adhesive tape. The newly inserted chip was equilibrated with *PBS* (140 m*M* NaCl, 2.7 m*M* KCl, 10 m*M* Na_2HPO_4, and 1.8 m*M* KH_2PO_4, $pH = 7.3$ was usually obtained without adjustment, otherwise minute amounts of HCl or NaOH were used to adjust the *pH* to 7.3) for about 1 h before measurements of protein adsorption were performed.

All injections had a volume of 100 mm^3. The *SDS* solution (0.5% in water) was the original "BIAdesorb solution 1". *PBS* was used as running buffer, as well as to dissolve the commercially obtained proteins (goat *IgG*, *BSA*, and hen egg lysozyme, each at 1 mg cm^{-3} protein concentration). A fraction of these proteins had been purified by HPLC gel filtration in *PBS* on a HR 10/30 column (10 mm ID, 30 cm column length) of

Superdex® 200 (Amersham) at a flow rate of $0.5\,cm^3\,min^{-1}$ in order to remove dimers and higher aggregates. The monomers of *IgG*, *BSA*, and lysozyme eluted as expected for M_r of 150000, 66000, and 14600, as compared to BioRad gel filtration standard. The monomer fractions from many column runs were pooled and the protein concentration was determined from UV-Vis spectrum ($\varepsilon_{280} = 210000\,M^{-1}\,cm^{-1}$ for *IgG* according to the manufacturer's declaration, $\varepsilon_{280} = 44300$ and $39300\,M^{-1}\,cm^{-1}$ for *BSA* and lysozyme [27]) and diluted to $1\,mg\,cm^{-3}$ with *PBS*. The typical injection sequence is shown in Fig. 2 (see legend to Fig. 2). In each injection, $140\,mm^3$ protein solution was pulled into the yellow tip of a $200\,mm^3$ digital pipette, then $10\,mm^3$ air were also pulled into the yellow tip by turning the pipette to the $150\,mm^3$ setpoint. This little air bubble served to clear the $100\,mm^3$ sample loop from buffer and thus to ensure an instantaneous rise from 0 to $1.0\,mg\,cm^{-3}$ protein concentration in each individual injection of protein.

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