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Modular Synthesis of Bifunctional Linkers for Materials Science

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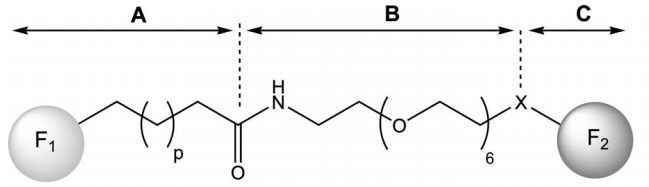
A practical synthesis of α,ω-bifunctional linkers is described that is based on three building blocks, namely, thioctic acid, a spacer-arm of seven ethylene glycol units, and a functional motif dedicated to the selective immobilization of purposely tagged proteins. Such representative motifs are biotin, haloacetamide, maleimide, and nitrilotriacetic acid derivatives. The building blocks are connected through alkyl, amide, and/or carbamate linkages.

Introduction

The stable immobilization of biomacromolecules onto materials or nanoparticles has received growing interest over the last few decades.[1a] A challenging goal in biotechnology, molecular electronics, and biosensors is the grafting of proteins that retain their full activity on the surfaces of the devices.[1b] The synthesis of appropriate linkers capable of connecting surfaces to specific proteins is still a difficult task; most often, detailed protocols are not available or the reactions suffer from poor reproducibility, poor yields, and unspecified purity of the prepared bifunctional molecules. Several protocols were recently proposed by using either high molecular weight molecules as spacer arms, usually polyethylene glycols (PEGs),[2a–2c] or on the contrary very short ones.[2d] Heterotelechelic oligoethylene glycols (OEGs), in particular thiol end-functionalized OEGs, are also commercially available and used for gold surface modification.[2e,2f] Nevertheless, α,ω-bifunctional linkers based precisely on seven ethylene glycol units [i.e., OEG of 14 CH_2 groups named $(EG)_7$] are not marketed, and their synthesis is rarely mentioned in the literature.[2g]

We describe here a modular and practical route to synthesize a series of $(EG)_7$-containing bifunctional linkers designed to immobilize different tagged proteins and mutants onto metal surfaces in a regularly oriented manner (Figure 1). In the course of a program dedicated to the label-free detection of analytes through the use of biosensors based on sum-frequency generation (SFG) spectroscopy,[3a] we needed to graft proteins as dedicated receptors onto the surface of silver- or gold-coated optical elements.[3b] Various chemical functionalities able to bind selectively to metals, metal oxides, semiconductors, or glasses have been already described for forming dense self-assembled monolayers (SAMs).[4] However, the most commonly encountered class of SAMs is derived from the chemisorption of alkanethiols or disulfides onto gold due to strong S–Au interactions.[5]



F_1 = metal surface anchoring group
F_2 = reactive group for oriented immobilization of proteins
X = alkyl, amide or carbamate linkage
A, **B**, **C** = building blocks for the modular synthesis (see Figure 2)

Figure 1. Bifunctional linkers.

In our study, the disulfide function (F_1 in Figure 1) of thioctic acid (TA) was chosen to interact with a gold or silver surface, as it was already shown that this "masked" dithiol motif allows the corresponding linkers to be handled without risk of any side reactions, leading to stable double anchorage on the device.[1a]

The functions selected for protein immobilization (F_2 in Figure 1) were (i) a biotin molecule giving stable complexes with avidin-tagged proteins;[6] (ii) a halogenoacetyl residue or (iii) a maleimide moiety, both susceptible to fix cysteine-tagged proteins by nucleophilic substitution or Michael addition respectively;[7] and (iv) a nitrilotriacetic acid molecule (NTA) able to complex histidine-tagged proteins with the aid of a Ni^{II} cation.[8]

The design of the spacer arm linking the F_1 and F_2 motifs was inspired from our previous work dedicated to germanium-ATR devices.[9] The alkyl chain of TA connected to a few ethylene glycol units through a peptide bond is expected to favor the formation of SAMs. In order to get accurate spectroscopic detection at the interface, we considered an organic layer as thin as possible. Hence, a short

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OEG chain of seven units was chosen, as this length is known to be the minimum length for inducing nonfouling behavior.[10] Thus, the bifunctional linkers (Figure 1) were constructed by combining building blocks **A**, **B**, and **C**, as shown in Figure 2. It is worth noting that ester linkages between the blocks are excluded from our synthetic plan for stability reasons in biological fluids (X in Figure 1).

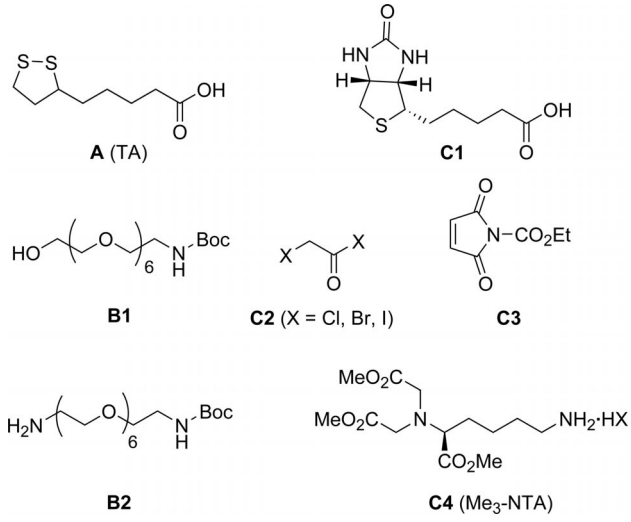
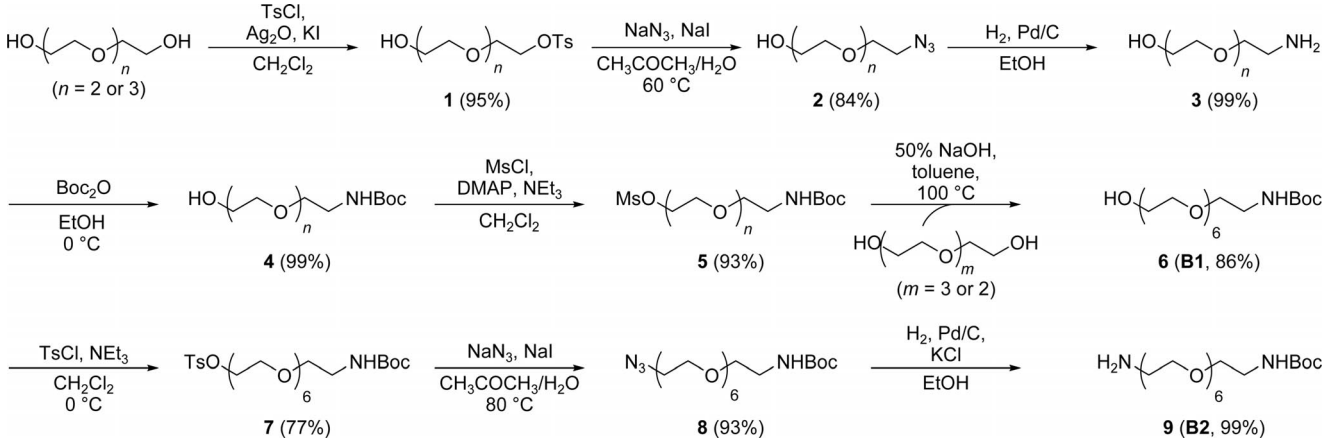


Figure 2. Selected building bocks. TA = thioctic acid; NTA = nitrilotriacetic acid.

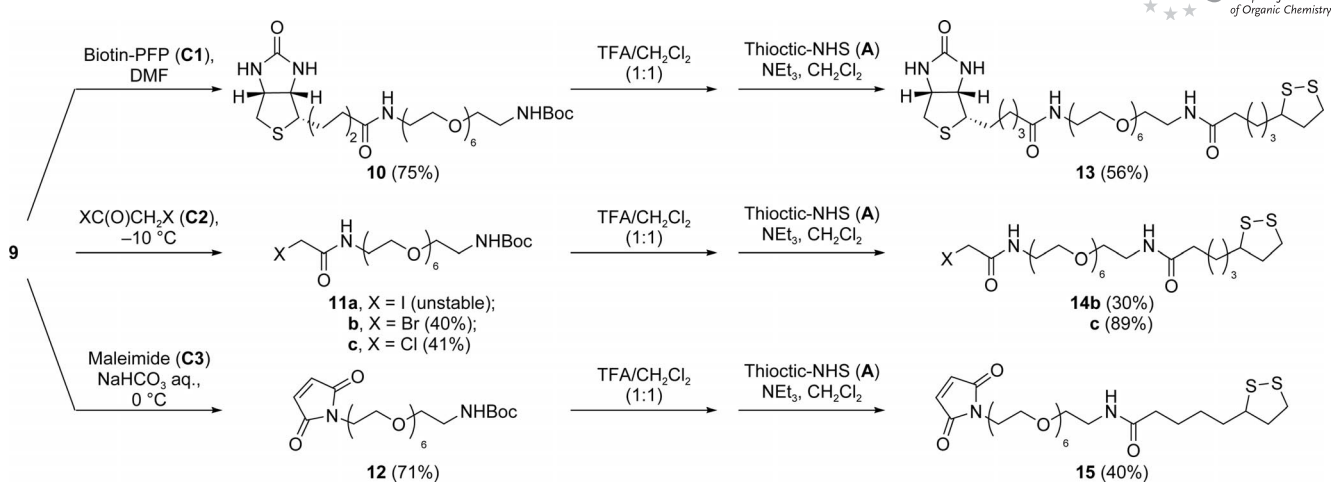
Results and Discussion

Seven-unit monoprotected amino-OEG (block **B1**) was first synthesized in six steps from commercially available triethylene and tetraethylene glycols (Scheme 1). The $(EG)_7$ motif could be constructed by nucleophilic substitution (ether bond-forming reaction), either from activated $(EG)_4$ (i.e., **5** with $n = 3$) coupled to $(EG)_3$ or from activated $(EG)_3$ (i.e., **5** with $n = 2$) coupled to $(EG)_4$. Both synthetic schemes have been tested, but taking into account the yields of individual steps and the easiness of intermediates purification, we recommend the latter strategy, (i.e., $n = 2$ and $m = 3$ in Scheme 1). Monotosylation of triethylene glycol on a 10-gram scale was first realized in the presence of silver oxide[11] with an optimized yield of 95% of **1** after chromatography. Formation of corresponding azide **2** followed by catalytic hydrogenation afforded amine **3**, which after *tert*-butyloxycarbonyl protection provided intermediate **4** in 82% yield (overall yield for four steps). Activation of the hydroxy function by mesylation to afford **5** allowed the $(EG)_3$ chain to be lengthened by S_N2 reaction with tetraethylene glycol in the presence of concentrated aqueous NaOH in hot toluene (100 °C); spacer arm **6** was recovered by liquid–liquid extraction in 86% yield (overall yield for six steps: 62.5%). Amino alcohol **6** was then converted in three conventional steps into corresponding monoprotected diamine block **B2**. Tosylation to afford **7**, substitution with azide to afford **8**, and reduction by hydrogen in the presence of KCl led to spacer arm **9** in 64.4% yield over three steps (overall yield for nine steps: 40.2%). The other building blocks are commercially available (**A**, **C1**, and **C2**; Figure 2) or easily prepared. *N*-(Ethoxycarbonyl)maleimide (**C3**) was obtained according to Keller.[12] The trimethyl ester of NTA (**C4**) was synthesized from ε-*N*-(benzyloxycarbonyl)-(L)-lysine in three steps following the protocol of McGeary.[13]

In principle, two modes for the assembly of the building blocks could be considered, that is, the formation of **A** and **B** followed by the coupling to **C**, or the formation of **C** and **B** followed by the coupling to **A**. Practically, we found that the latter mode (although it is the less convergent one) is the most efficient, because it requires only one chromatographic purification step on the disulfide-containing molecules. Biotinylated derivative **10** was obtained by treating **9** with biotin (**C1**, Scheme 2). Different activation methods of **C1** were evaluated, that is, the in situ activation with dicyclohexylcarbodiimide and an additive (DMAP, NHS, HOBT, etc.), preformation of the *N*-hydroxysuccinimidyl ester or preformation of the pentafluorophenyl (PFP) ester.[14a] The highest yields of **10** were recorded by using the PFP ester method, whose performance was already mentioned in the field of polymer chemistry[14b] and bioorganic chemistry.[14c] Iodoacetamide derivative **11a** could not be



Scheme 1. Synthesis of α,ω-functionalized EG heptamers. TsCl = tosyl chloride; Boc = *tert*-butyloxycarbonyl; MsCl = mesyl chloride; DMAP = dimethylaminopyridine.

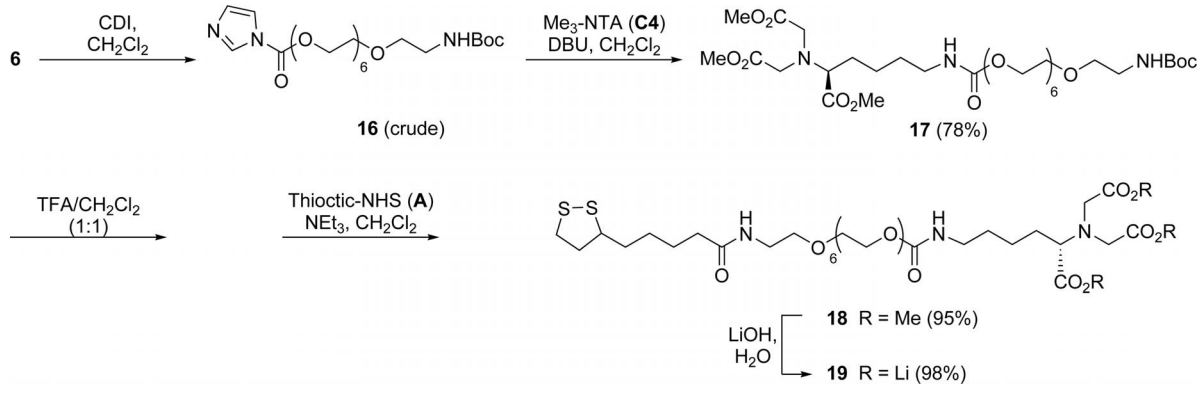


Scheme 2. Synthesis of linkers assembled with **9**. PFP = pentafluorophenyl; DMF = *N*,*N*′-dimethylformamide; TFA = trifluoroacetic acid; NHS = *N*-hydroxysuccinimidyl.

isolated (highly unstable compound). Bromoacetamide **11b** was prepared by adding bromoacetyl bromide to **9** in dichloromethane. A low temperature and a short reaction time were required to avoid *N*-Boc deprotection. Rapid neutralization with aqueous $NaHCO_3$ allowed the product to be recovered in 40% yield after purification. Compound **11b** is light and air sensitive. At last, chloroacetamide derivative **11c** was readily obtained from **9** and chloroacetyl chloride; it could be purified and stored without significant degradation (Scheme 2). Maleimide derivative **12** was synthesized from **9** and **C3** in equimolar amounts, in ice-cold aqueous sodium hydrogen carbonate,[15] in 71% yield after chromatography (Scheme 2). Boc cleavage from precursors **10**, **11b**, **11c**, and **12** was realized in the presence of trifluoroacetic acid (TFA), and the TFA salts of the corresponding primary amines were neutralized by addition of a tertiary amine before coupling with TA (block **A**). The NHS-ester of **A** previously prepared and isolated[16] was used as a pure reagent to obtain better yields of final targets **13**, **14b**, **14c**, and **15**, respectively, than by applying in situ activation with carbodiimide. The PFP-ester of **A** gave similar results.

The NTA-containing linker was constructed by using a different strategy because two nucleophilic partners, namely, blocks **B1**/**B2** (OEG spacer) and **C4** (derived from L-lysine with free ε-NH_2), have to be connected. To shorten the total synthesis, spacer **6** (instead of **9**) was considered for activation by a bis-electrophilic reagent containing one carbon (phosgene-type reagent). In this case, the created link between the building blocks is a carbamate function (Scheme 3). Thus, imidazole carboxylate derivative **16** was synthesized from alcohol **6** and carbonyldiimidiazole (CDI) in dichloromethane and was directly engaged with amine **C4** in the presence of DBU (10 equiv.). Carbamate **17** was obtained after purification in 78% yield over two steps. TA coupling was then realized as described previously to obtain triester **18** in 95% yield. Saponification with lithium hydroxide[17] in water furnished quantitatively final target **19**.

Bifunctional linkers **13**, **14b**, **14c**, **15**, and **19** were fully characterized by IR, ^1H NMR, and ^{13}C NMR spectroscopy and HRMS (^{13}C spectra are given in the Supporting Information). Their stability in solution (≈10^{-2} M, CD_3OD, 25 °C) was monitored by NMR spectroscopy over several months: **13** and **19** are stable and **15** and **14c** very slowly



Scheme 3. Synthesis of linker assembled with **6**. CDI = carbonyldiimidazole; DBU = diazabicycloundecane.

degrade (half-life ≈ 4–6 months). In the pure state, the linkers can be preserved in the freezer without damage.

The immobilization of proteins on SAMs constructed from the previous molecules is currently under investigation. For instance, partially biotinylated SAMs were formed on gold substrates by using mixtures of linker **13** (0.1 to 10 mol-%) and passivation molecules [i.e., similar molecules where the reactive terminus X-F_2 (Figure 1) is replaced with a methoxy group] in ethanol solution (1 mM, 18 h, 20 °C).[18] XPS analyses (X-ray photoelectron spectroscopy) giving N/C atomic ratios consistent with the considered molar compositions, and cyclic voltammetry measurements showing the decrease till the suppression of the Au surface electroactivity depending on the organic layers compactness, confirmed the successful synthesis of SAMs. Using the QCM-D method (quartz crystal microbalance with dissipation measurement), we could monitor the avidin binding process onto the biotinylated surfaces.[18] The maximal protein immobilization reached (≈500 ng/cm^2) corresponds to the data usually reported in the literature for a protein monolayer.[19]

Conclusions

We have disclosed optimized protocols for the synthesis of a series of TA-based linkers featuring a seven-unit oligoethylene glycol spacer and a terminal function dedicated to the immobilization of tagged proteins. Our syntheses make use of easily available building blocks and efficient coupling methods. The connecting links are alkyl, amide, or carbamate functions, more stable than ester functions versus hydrolytic degradation. The sequence in which the blocks are connected (i.e., **C** + **B** and then **C**–**B** + **A**) was chosen to allow chromatographic purification without excessive material loss. The overall yields of linkers **13** (biotin), **14b** (bromoacetamide), **14c** (chloroacetamide), **15** (maleimide), and **19** (nitrolotriacetic acid) are 16.9 (12 steps), 7.2 (12 steps), 14.7 (12 steps), 11.4 (12 steps), and 46.3% (10 steps), respectively. Individual blocks **6** and **9** and diblock intermediates **10**, **11b**, **11c**, **12**, and **17**, and the final constructs can be stored in the freezer without damage when they are fully purified.

Supporting Information (see footnote on the first page of this article): Experimental procedures and spectroscopic characterization of **1**–**19** and ^{13}C NMR spectra of major compounds.

Acknowledgments

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