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Improving the Stability and Sensing of Electrochemical Biosensors by Employing Trithiol-Anchoring Groups in a Six-carbon Self-assembled Monolayer

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Abstract

Alkane thiol self-assembled monolayers (SAMs) have seen widespread utility in the fabrication of electrochemical biosensors. Their utility, however, reflects a potentially significant compromise. While shorter SAMs support efficient electron transfer, they pack poorly and are thus relatively unstable. Longer SAMs are more stable, but suffer from less efficient electron transfer, thus degrading sensor performance. Here we use the electrochemical DNA (E-DNA) sensor platform to compare the signaling and stability of biosensors fabricated using a short, six-carbon monothiol with those employing either of two commercially available, trihexylthiol anchors (a flexible Letsinger-type and a rigid adamantane type). We find that all three anchors support efficient electron transfer and E-DNA signaling, with the gain, specificity and selectivity of all three being effectively indistinguishable. The stabilities of the three anchors, however, vary significantly. Sensors anchored with the flexible trithiol exhibit enhanced stability, retaining 75% of their original signal and maintaining excellent signaling properties after 50 days storage in buffer. Likewise these sensors exhibit excellent temperature stability and robustness to electrochemical interrogation. The stability of sensors fabricated using the rigid trithiol anchor, by comparison, are similar to those of the monothiol. Both exhibit significant (>60%) loss of signal upon wet storage or thermocycling. Employing a flexible trithiol anchor in the fabrication of SAM-based electrochemical biosensors may provide a means of improving sensor robustness without sacrificing electron transfer efficiency or otherwise impeding sensor performance.

Keywords

electrochemistry; DNA detection; stability; serum; trithiols

Introduction

Since the mainstream development of gold-thiol self-assembled monolayers (SAMs) in the early 1980s,^{1,2} they have found widespread utility in the fabrication of chemical and biological sensors [reviewed in refs 3 and 4]. While SAMs have been employed in the fabrication of a wide range of optical,^{5,6} mechano-acoustical^{7,8} and electronic sensors,^{9,10} perhaps their most valuable application has been in fabrication of electrochemical sensors [e.g., see refs 11 and 12], where they provide a convenient route for the site-specific attachment of biomolecular sensing elements to an electrode surface. Examples of such

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electrochemical sensors range from traditional sandwich assays¹³ to reagentless sensing architectures¹⁴ such as the electrochemical DNA sensor (E-DNA).¹⁵⁻¹⁹

While the ease of fabricating alkane thiol SAMs has ensured their widespread use (for reviews see, e.g., refs 20, 21, and 22), a significant limitation of this chemistry is that many SAMs exhibit relatively poor stability.²³ For example, the long term storage of gold SAMs in media such as phosphate buffered saline results in significant monolayer loss after only several days.²⁴ It has also been shown that DNA-modified SAMs on gold nanoparticles are unstable at elevated temperatures and high salt concentrations.²⁵ One approach to improve the stability of sensors based on DNA-modified SAMs has been the use longer-chain alkane thiols, which produce SAMs with improved stability (as a result of improved packing interactions²³ and the reduced accessibility of oxygen²⁶). These, however, lead to sluggish electron transfer, which can reduce electrochemical signaling.¹⁷ Alternative approaches to improved SAM stability have also been explored that may avoid this potential pitfall. Examples include trihexylthiol anchors, which have been employed to immobilize DNA on gold nanocrystals²⁵ and gold thin-films²⁷ for optical sensing applications. To date, however, the use of multi-thiol SAMs in *electrochemical* biosensor applications has seen little investigation. In response we compare here the stability and signaling characteristics of electrochemical biosensors fabricated using either a commonly employed, six-carbon monothiol (6-mercapto-hexane), a rigid, adamantane-based trihexylthiol, or the flexible Letsinger trihexylthiol²⁵ as the means of anchoring the probe DNA to an interrogating electrode (all kindly provided by Fidelity Systems, Inc., Gaithersburg, MD) (Fig. 1).

Experimental Methods

Linear probe E-DNA sensors were fabricated using standard procedures,²⁸ which we describe in brief here. The structures of the modified probe DNAs, a generous gift from Fidelity Systems, Inc. (Gaithersburg, MD), were as follows:

where “thiol” represents one of the three thiols denoted (Fig. 1) and MB indicates a monocarboxymethylene blue-succinimidyl ester (methylene blue) attached to a six-carbon amino modifying group appended to the 3' phosphate. These constructs, used as delivered, were reduced for 1 hr at room temperature in the dark in 10 mM tris(2-carboxyethyl)phosphine hydrochloride (Molecular Probes, Carlsbad, CA) and then diluted to 1 μM in 6X SSC buffer (Sigma-Aldrich, St. Louis, MO; 90 mM sodium citrate, 0.9 M NaCl, pH 7.0). Gold rod electrodes (2 mm diameter, CH Instruments, Austin, TX) were cleaned as previously described²⁷ then incubated in this solution for 30 min at room temperature in the dark, rinsed with distilled, deionized (DI) water and then incubated in 3 mM 6-mercapto-1-hexanol in DI water for 1 h. Following this the electrodes were rinsed in DI water and stored in 6X SSC until use.

Fabricated sensors were interrogated using standard AC voltammetric methods²⁸ (25 mV amplitude, 50 Hz frequency, -0.1 V to -0.5 V vs. Ag/AgCl) in the absence and presence (200 nM) of either a 17-base, fully complementary target or the equivalent 3-base mismatch targets (both obtained from Integrated DNA Technologies, Coralville, IA and used as received) with the following sequences:

Target Sequence:

3 Base-Pair Mismatch Sequence:

For tests run with target the electrodes were incubated for ~5 min with the target in 6X SSC buffer (or 50% fetal calf serum; Sigma-Aldrich)); replicate measurements indicate that sensor equilibration is complete in this timeframe.²⁹ Values with reported error bars (Fig. 3-6) represent the average and standard deviations of triplicate measurements performed on independently fabricated electrodes.

Apparent electron transfer rates were determined using square wave voltammetry. By measuring the peak current at various frequencies and following the Lovric formalism for an adsorbed species,³⁰⁻³² we obtain a parabolic plot of $1/f$ vs. I_p/f (where f is the interrogation frequency and I_p is the peak current – Fig. 2). As such, this plot reaches a maximum at a critical frequency which is directly related to the electron transfer rate constant.³⁰

Testing conditions were as follows. For solution storage, sensors were stored in the dark, at room temperature under 6X SSC buffer in parafilm sealed, but air-filled containers. Background currents were measured using ACV every 3 days for 50 days (Fig. 4a). On days 0, 25 and 50, the sensors were challenged with 200 nM target to determine signal suppression, and then regenerated using the standard 30s distilled water wash²⁹ before returning the electrodes to storage buffer. For dry storage, sensors were fabricated, tested, and regenerated as above, before being air dried and stored in sealed, dry test tubes at room temperature in the dark under air. On days 1, 15, and 30, these sensors were returned to buffer for approximately 20 min before being tested, regenerated, and re-dried (Fig. 5). To simulate polymerase chain reaction cycling, sensors were placed in 95°C 6X SSC buffer for 25s, 55°C for 30s, and 75°C for 55s for each cycle. Cycles were repeated 9 times with the current being measured every other cycle. In order to determine the robustness of each sensor to repeated electrochemical interrogation we subjected freshly fabricated electrodes in 6X SSC to repeated rounds of AC voltammetry using the parameters described above.

Results

As a test bed for our comparative studies we have employed an E-DNA sensor¹⁸ comprised of a linear, 17-base DNA probe attached at the 5' terminus to the relevant thiol and modified with a redox active methylene blue tag at the opposite terminus (Fig. 1). The modified probe is deposited from aqueous solution onto a gold electrode followed by “backfilling” with excess 6-mercapto-1-hexanol as a co-adsorbate, which forms a continuous, mixed SAM and consequentially removes any nonspecifically adsorbed DNA.³³ In the absence of target, the flexible single-stranded probe supports rapid collision between the redox tag and the electrode, ensuring a large faradaic current. Upon hybridization, the formation of a rigid probe-target duplex reduces the collisional frequency and thus leads to a large change in measured signal (Fig 1).

All three sensor architectures support efficient electron transfer between the redox tag and the electrode surface. For example, immediately after fabrication, sensors exhibit similar high signaling currents regardless of the thiol type (1 to 2 $\mu\text{A}/\text{mm}^2$ – data not shown). This suggests that electron transfer through all three SAMs remains relatively efficient. Likewise, all three sensor architectures exhibit apparent electron-transfer rates of ~40 to 70 s^{-1} (Fig. 2), which are an order of magnitude more rapid than the electron transfer observed, for example, in E-DNA sensors fabricated using a (relatively stable) eleven-carbon alkane-thiol SAM.¹⁷

Consistent with similar electron transfer properties, all three anchors support efficient E-DNA sensing. Specifically, sensors fabricated with the various anchoring chemistries exhibit similar binding trends with respect to target DNA concentration (Fig. 3, top). Likewise, all three architectures exhibit similar specificity (ability to distinguish mismatched from perfect

target). For example, all three sensors exhibit similarly reduced signal changes when challenged with a 3-base mismatch target (Fig. 3, middle). The selectivity (*i.e.*, ability to perform in complex matrices) of each sensor also remains constant regardless of the anchoring chemistry: all three sensors perform equally well when challenged in buffer or in 50% blood serum (Fig. 3, bottom). Finally, all three anchors produce readily regenerable sensors; after a 30 s wash in room temperature, deionized water we recover in excess of 91% of the original signal for all three architectures.

Sensors employing the flexible trithiol anchor exhibit significantly enhanced solution-storage stability. When stored for 50 days in aqueous buffer at room temperature, such sensors exhibit only a 30% loss in signaling current (current observed in the absence of target). In contrast, sensors fabricated using the rigid trithiol or monothiol anchors exhibit losses of 60% and 75% respectively (Fig. 4, left). Given that the absolute current measured in buffer is related to the number of signaling probe DNA immobilized on the electrode surface, these results suggest that the flexible trithiol anchor significantly reduces the loss of probe DNA during storage. (Loss of the co-adsorbate, in contrast, would likely result in an increase in faradaic current as loss of the insulating alkane thiol would give direct access to the electrode surface for efficient electron transfer).³⁴ Sensors fabricated with the flexible trithiol anchor likewise maintain their ability to signal the presence of target: after 50 days storage in buffer, these sensors, similarly to those fabricated using the rigid trithiol, exhibit ~50% signal suppression in the presence of saturating concentrations of target, which is close to the 65% suppression observed when freshly fabricated sensors are challenged with saturating target (Fig. 4, right). Conversely, while freshly fabricated monothiol anchored sensors also exhibit 65% signal suppression, this falls to only 14% suppression by day 50.

In contrast to the improved solution stability produced by the flexible trithiol, all three anchors perform rather poorly under dry storage conditions. After 30 days of dry storage at room temperature under air, sensors fabricated from each of the three anchors exhibit 50% loss of the original buffer current (no target) when they are reintroduced to buffer in order to perform the relevant electrochemical measurements (Fig. 5, left). Likewise, all three sensors behave similarly, exhibiting slightly reduced signal suppression when challenged with saturating concentration of target DNA after dry storage (Fig. 5, right).

The flexible trithiol anchor provides enhanced thermo-stability and enhanced robustness to repeated electrochemical interrogations. For example, when subjected to 9 simulated polymerase chain reaction cycles (95°C for 25s, 55°C for 30s, and 75°C for 55s), sensors fabricated using the flexible trithiol anchor retain 80% of their original signaling current (Fig. 6). Sensors fabricated using either rigid trithiol or monothiol anchors, in contrast, exhibit losses of up to 55% under these same conditions. Likewise, while the flexible trithiol anchor exhibits only a 7% loss in signaling current after 50 AC voltammetric scans sensor fabricated using either rigid trithiol or monothiol anchors lose 25% of their original signaling current (Fig. 7).

Discussion

Here we have shown that a flexible, trihexylthiol anchor significantly improves the solution-phase storage stability, thermo-stability and interrogation robustness of an electrochemical biosensor, and, critically, achieves these improvements without sacrificing electron transfer efficiency or otherwise degrading sensor performance. The use of trithiol anchors to improve the stability of electrochemical biosensors represents an advance over earlier efforts to improve sensor stability via increasing SAM thickness,¹⁷ an approach that suffers from a trade-off between improved stability and reduced electron transfer efficiency. Taken

together this suggests that the flexible trithiol linkage chemistry may be of significant, general utility in the fabrication of electrochemical biosensors.

While little work appears to have been reported on the use of trihexylthiols and other oligothiol in electrochemical biosensors, similar improvements in sensor stability have been reported for the use of such anchors in optical sensor platforms. These include the pioneering work of Letsinger and co-workers, who first developed the flexible trithiol employed here and demonstrated that it leads to improved thermostability in an optical biosensor based on DNA-modified gold nanoparticles.²⁵ Likewise, Sakata *et al.* have shown that a rigid, “tripodal” trithiol (distinct from the rigid trithiol employed here) leads to improved thermostability in an optical biosensor comprised of a fluorescently-tagged DNA probe attached to a thin gold film.²⁷ Finally, Mirkin and co-workers have shown that a short chain hexaethylthiol (triple-disulfide) anchor termed DSP (cyclic disulfide-containing phosphate derivative) exhibit enhanced stability when used to fabricate DNA-silver nanocrystal materials for optical sensing applications.³⁵

In the above-mentioned references, as well as in our work presented within, it is presumed that the trihexylthiol anchor enhances stability via the mechanism of enhancing the affinity of the thiol-modified DNA probe for the gold surface. As a result of the three thiol-gold bonds than can form, the adhesion strength of each DNA probe is increased when compared to the mono-thiol equivalent.²⁷ If one of these bonds is compromised, the remaining bonds would retain the DNA probe on the surface, thus maintaining sensor integrity. Given that each of the individual thiols in the trithiols used within are attached to the probe DNA via six-carbon chains identical to the monothiol anchor it appears unlikely that improved van der Waals interactions are playing a role in the enhanced stability we have observed, as is thought to be true for the C11 linkers previously reported.¹⁷ We likewise assume, however, that the relatively poorer performance of the rigid trithiol in this application arises because its rigidity precludes optimal packing. Irrespective, however, of the precise mechanism (or mechanisms), the improved stability and efficient electron-transfer properties of the flexible trithiol anchor will likely prove of utility in the fabrication of a wide range of electrochemical sensors.

Acknowledgments

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References

1. Nuzzo RG, Allara DL. *J Am Chem Soc.* 1983; 105:4481–4483.
2. Ulman A. *Chem Rev.* 1996; 96:1533–1554. [PubMed: 11848802]
3. Chaki NK, Vijayamohan K. *Biosens Bioelectron.* 2002; 17:1–12. [PubMed: 11742729]
4. Wink T, vanZuilen SJ, Bult A, vanBennekom WP. *Analyst.* 1997; 122:R43–R50.
5. Yu Q, Chen S, Taylor AD, Homola J, Hock B, Jiang S. *Sens Actuators B.* 2005; 107:193–201.
6. Hong S, Kang T, Moon J, Oh S, Yi J. *Colloids Surf A.* 2007; 292:264–270.
7. Gronewold TMA, Glass S, Quandt E, Famulok M. *Biosensors and Bioelectronics.* 2005; 20:2044–2052. [PubMed: 15741074]
8. Länge K, Rapp B, Rapp M. *Anal Bioanal Chem.* 2008; 391:1509–1519. [PubMed: 18265962]
9. Lucarelli F, Marrazza G, Turner APF, Mascini M. *Biosens Bioelectron.* 2004; 19:515–530. [PubMed: 14683635]
10. Arora K, Chand S, Malhotra BD. *Anal Chim Acta.* 2006; 568:259–274. [PubMed: 17761267]

11. Rubinstein I, Sabatani E, Maoz R, Sagiv J. Organized monolayers on gold electrodes, in *Electrochemical Sensors for Biomedical Applications*. 1986
12. Privett BJ, Shin JH, Schoenfisch MH. *Anal Chem*. 2008; 80:4499–4517. [PubMed: 18491869]
13. Umek RM, Lin SW, Vielmetter J, Terbrugge RH, Irvine B, Yu CJ, Kayyem JF, Yowanto H, Blackburn GF, Farkas DH, Chen YP. *J Molec Diag*. 2001; 3:74–84.
14. Palecek E. *Trends Biotechnol*. 2004; 22:55–58. [PubMed: 14757035]
15. Fan C, Plaxco KW, Heeger AJ. *Proc Natl Acad Sci USA*. 2003; 100:9134–9137. [PubMed: 12867594]
16. Fan C, Plaxco KW, Heeger AJ. *Trends Biotech*. 2005; 23:186–192.
17. Lai RY, Seferos DS, Heeger AJ, Bazan GC, Plaxco KW. *Langmuir*. 2006; 22:10796–10800. [PubMed: 17129062]
18. Ricci F, Lai RY, Heeger AJ, Plaxco KW, Sumner JJ. *Langmuir*. 2007; 23:6827–6834. [PubMed: 17488132]
19. Ricci F, Lai RY, Plaxco KW. *Chem Commun*. 2007; 36:3768–3770.
20. Tarlov, MJ.; Steel, AB. DNA-Based Sensors. In: Rusling, JF., editor. *Biomolecular Films – Design, Function, and Applications*. Marcell Dekker; New York: 2003. p. 545-608. *Surfactant Science Series* 111
21. Drummond TG, Hill MG, Barton JK. *Nature Biotech*. 2003; 21:1192–1199.
22. Lucarelli F, Marrazza G, Turner APF, Mascini M. *Biosens Bioelectron*. 2004; 19:515–530. [PubMed: 14683635]
23. Poirier GE, Tarlov MJ, Rushmeier HE. *Langmuir*. 1994; 10:3383–3386.
24. Flynn NT, Tran TNT, Cima MJ, Langer R. *Langmuir*. 2003; 19:10909–10915.
25. Li Z, Jin R, Mirkin CA, Letsinger RL. *Nucl Acids Res*. 2002; 30:1558–1562. [PubMed: 11917016]
26. Everett WR, Fritsch-Faules I. *Anal Chim Acta*. 1995; 307:253–268.
27. Sakata T, Maruyama S, Ueda A, Otsuka H, Miyahara Y. *Langmuir*. 2007; 23:2269–2272. [PubMed: 17269799]
28. Xiao Y, Lai RY, Plaxco KW. *Nat Protocols*. 2007; 2:2875–2880.
29. Lubin AA, Lai RY, Baker BR, Heeger AJ, Plaxco KW. *Anal Chem*. 2006; 78:5671–5677. [PubMed: 16906710]
30. Komorsky-Lovric S, Lovric M. *Anal Chim Acta*. 1995; 305:248–255.
31. Lovric M, Komorsky-Lovric S. *J Electroanal Chem*. 1988; 248:239–253.
32. Lovric M, Komorsky-Lovric S, Murray RW. *Electrochim Acta*. 1988; 33:739–744.
33. Herne TM, Tarlov MJ. *J Am Chem Soc*. 1997; 119:8916–8920.
34. Porter MD, Bright TB, Allara DL, Chidsey CED. *J Am Chem Soc*. 1987; 109:3559–3568.
35. Lee JS, Lytton-Jean AKR, Hurst SJ, Mirkin CA. *Nano Lett*. 2007; 7:2112–2115. [PubMed: 17571909]

Figure 1.

(Top) We have compared the signaling properties and stabilities of E-DNA sensors fabricated using both the commonly employed monothiol 6-mercapto-1-hexane to anchor the probe DNA or either of two trihexylthiol anchors: a highly flexible Letsinger type trithiol (flexible trithiol), and a rigid adamantane-based trithiol. In all three architectures the thiol-anchored probe was co-deposited with 6-mercapto-1-hexanol to form a mixed SAM.

(Bottom) The E-DNA sensor we have employed is a “signal-off” sensor in which the addition of a complementary target strand results in a decrease in measured faradaic current. Because all of the sensing components are strongly adsorbed to the sensor surface, the

sensor is readily regenerated with a 30 s distilled water rinse, as shown. The representative data presented here were collected using a sensor fabricated with a monothiol anchor.

Figure 2.

All three of the thiol anchors we have characterized support efficient electron transfer. For example, all three sensor architectures exhibit electron transfer rates of ~ 40 to 70 s^{-1} , an order of magnitude more rapid than, for example, the electron transfer rate observed for E-DNA sensors fabricated using an eleven-carbon alkane-thiol SAM ($\sim 7 \text{ s}^{-1}$).¹⁷ The plots shown reach a maximum frequency (f_{max}) which is related to the standard rate constant (k_s) by the expression $k_s = k_{\text{max}} f_{\text{max}}$, where k_{max} is the theoretically calculated critical kinetic parameter (1.18 for values of the transfer coefficient, α , between 0.25 and 0.85).³²

Figure 3.

All three of the thiol anchors we have characterized support efficient E-DNA signaling. **(Top)** Each construct responds similarly to the addition of a 17-base pair perfect match target, saturating at target concentration (200 nM) and **(Middle)** all three architectures exhibit similar sequence specificity; as shown, significantly poorer signal suppression is observed when the sensors are challenged with a 3-base mismatched target (3bpmm). **(Bottom)** Likewise the nature of the anchoring thiol does not affect E-DNA selectivity; all three sensors perform equally well when challenged in 50% blood serum, with the two trithiols producing signal suppressions within error of those observed in pure buffer. The data on this and the following figures represent the average of measurements conducted on

at least three independent sensors. The error bars, which are indicated on only one data point for clarity, represent the standard deviation of these measurements.

Figure 4.

The wet storage stability of sensors fabricated using the flexible trithiol anchor is significantly greater than that of sensors fabricated using either the monothiol or rigid trithiol anchors. **(Left)** After 50 days, rigid trithiol and monothiol anchored sensors exhibit losses of 60% in peak current (no target), presumably due to loss of probe from surface. Conversely, sensors fabricated using the flexible trithiol anchor exhibit only a ~20% loss of peak current. **(Right)** In contrast, sensors fabricated using either trithiol maintain their ability to signal under wet storage conditions: whereas the signal suppression (in the presence of saturating target) observed for monothiol-based sensors drops significantly after storage, the signaling of trithiol-based sensors is largely retained.

Figure 5.

All three of the thiol anchors we have characterized here fail upon dry storage. **(Left)** After 30 days of dry storage, under ambient air conditions, all three sensor architectures lose ~50 % of the original current in buffer (no target). **(Right)** Likewise, dry storage significantly inhibits signaling in all three sensor architectures.

Figure 6.

The thermostability of sensors anchored with the flexible trithiol is significantly greater than that of either rigid trithiol- or monothiol-based sensors. As shown, for example, the flexible trithiol anchor maintains 80% of its original signal after nine simulated polymerase chain reaction (PCR) cycles. Under the same conditions, in contrast, the monothiol- and rigid trithiol-based sensors lose 60% of their original signaling current.

Figure 7.

The flexible trithiol readily withstands repeated electrochemical interrogations. As demonstrated, after 50 AC voltammetric (ACV) scans (from -0.1 V to -0.5 V, 50 Hz, 25 mV amplitude) sensors fabricated using the flexible trithiol maintain 94% of their original peak current. In contrast the signaling current of monothiol and rigid trithiol-based sensors is significantly degraded, presumably due to loss of the probe DNA from the electrode surface.