Label free electrochemical DNA hybridization discrimination effects at the binary and ternary mixed monolayers of single stranded DNA/diluent/s in presence of cationic intercalators

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Abstract

Electrochemical label free DNA hybridization discrimination of the brain tumor sequence CK20 has been made at the gold–thiol and thiol diluent binary and ternary mixed monolayer interfaces in presence of the [Fe(CN)6]3− and double stranded DNA (dsDNA) specific cationic intercalators, proflavine (PF) and methylene blue (MB), respectively. Thiol hexane labeled single stranded DNA (HS-ssDNA) and thiol diluents such as 6-mercapto-1-hexanol (MCH) and 3-mercaptopropionic acid (MPA) are used to construct the mixed monolayers. Change in the peak-to-peak separation (∆Ep) for the [Fe(CN)6]3− redox reaction indicates the efficiency of the diluents in removing the randomly oriented HS-ssDNA. Smaller ∆Ep 248 mV noticed for the HS-ssDNA/MPA compared to the HS-ssDNA/MCH mixed monolayers (812 mV) indicates the less influence of the MCH diluent on the arrangement of HS-ssDNA layer. However, the hybridization discrimination effect negotiated in presence of both the [Fe(CN)6]3− and PF intercalator showed zero effect for the HS-ssDNA/MPA interface, and ~20–30% effect for the HS-ssDNA/MCH interface. The discrimination effect at the HS-ssDNA/MPA interface further increased to 80% by inserting the MCH at the local defects to form a multicomponent ternary HS-ssDNA/MPA/MCH layer interface. These differential discrimination effects are attributed to the formation of compact and/or defective layer structures, evidenced from their reductive desorption voltammetry in 0.5 M KOH. The presence of single base (C–A) mismatch in the hybrid is diagnosed by a decrease in coulometric charge compared to the perfect dsDNA. The target concentration of 10 pM is detected selectively and sensitively.

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1. Introduction

Low-density electrochemical DNA arrays based on the thiol–gold self-assembled monolayer (SAM) attracts paramount interest by its simple adsorption chemistry and compatibility for micro array construction through photolithography (Drummond et al., 2003; Albers et al., 2003; Dharuman et al., 2005, 2006). Most problems in developing the selective DNA arrays using the gold surface are; (i) tethering orientation controlled recognition layer for high hybridization efficiency with decreased non-specific adsorption of ssDNA and (ii) ability to discriminate the signal arises from the target DNA hybridization (Storhoff et al., 2002; Suda et al., 2003). Conventionally, mixed monolayers of HS-ssDNA/MCH (Herne and Tarlov, 1997; Steel et al., 2000; Petrovykh et al., 2003; Suda et al., 2003) are used. The MCH repels the non-specific DNA–Au binding by its negative –OH head group. Several other studies on the DNA hybridization (Boon et al., 2000; Satjapipat et al., 2001; Huang et al., 2001; Peterson et al., 2001; Storhoff et al., 2002; Drummond et al., 2003; Wackerbarth et al., 2004; Lao et al., 2005; Wong and Gooding, 2006) and DNA–protein interactions (Rajski et al., 1999; Boon et al., 2002, 2003) have followed this. Similarly, the binary mixed monolayer of HS-ssDNA with oligo-(ethylene glycol) terminated thiols is used to control the HS-ssDNA orientation (Boozer et al., 2006). In addition, the indirect DNA hybridization discrimination in presence of the electro active redox probes suffers from the direct diffusion of redox probes through monolayer defects as well as by their non-specific inter-
actions. For example, cationic metal complexes (Steel et al., 2000; Petrovykh et al., 2003; Lao et al., 2005) and/or intercalators (Park and Hahn, 2004; Yau et al., 2002) through electrostatic force. In the context of preventing the non-specific interactions of the cationic intercalators, use of an anionic intercalators (Wong and Gooding, 2006), anionic surfactant micelle sequestrate in presence of the cationic intercalator (Park and Hahn, 2004) and covalent attachment of the MB intercalator during oligonucleotides synthesis (Lai et al., 2006) have been suggested. The studies necessitate the construction of the orientation controlled and compact HS-ssDNA layer to prevent the direct access of the redox active ions, and remains a challenge for developing selective sensors.

The interfacial properties (electronic/compactness) of the mixed monolayer can be tuned by changing the hydrophilicity and/or hydrophobicity, head group functionality, ordering and packing of the alkyl chains (Ma and Lennox, 2000; Shon et al., 2000; Badia et al., 2000; Finklea, 2001; Liu et al., 2004; Love et al., 2005). Here, we constructed the binary HS-ssDNA/MCH and HS-ssDNA/MPA and multi-component ternary HS-ssDNA/MPA/MCH layers with the aim of understanding the influences of the MCH and MPA diluents on the arrangement of the HS-ssDNA strands and hybridization discrimination efficiency. The characterization of these interfaces and hybridization discrimination studies are made in the presence and absence of cationic intercalators in presence of [Fe(CN)₆]³⁻. The MPA provides sufficient charge transfer resistance to the [Fe(CN)₆]³⁻ at the neutral pH 7.4 (–COOH head group with pKa of 5.2 (Sato and Mizutani, 1998; Zhao et al., 1999)) and being used in the design and control of the mixed monolayer properties. The extent of orienting the HS-ssDNA by the these diluents is correlated by the change in the peak-to-peak separation (ΔEₚ) for the [Fe(CN)₆]³⁻ redox reaction, which follows the order HS-ssDNA/MCH > HS-ssDNA/MPA/MCH > HS-ssDNA/MPA. However, the hybridization discrimination negotiated in presence of the [Fe(CN)₆]³⁻ and DNA intercalators showed the order HS-ssDNA/MPA/MCH > HS-ssDNA/MPA/MCH > HS-ssDNA/MPA. That is, while the ternary interface showed 80% discrimination effect, the binary HS-ssDNA/MCH and HS-ssDNA/MPA interfaces showed 20 and 0% effects, respectively. These are presented in Scheme 1. The differential discrimination effects are related to the nature of the diluent interactions with the HS-ssDNA while forming either compact or defective mixed monolayers. Hence, the thiol reductive voltammetry is used in parallel to understand the uniformity and/or compactness of the binary and ternary layers in 0.5 M KOH medium. Cyclic voltammetric (CV) and chronocoulometric (CC) techniques are used to characterize the behavior of these layers. Brain tumor sequence CK20 is used as a model to demonstrate the discriminations of the hybridized, the unhybridized and C–A single base mismatched hybrid surfaces at the ternary mixed monolayer. The target concentration of 10 pM is detected selectively and sensitively. Here, we presented the first preliminary results observed systematically.

2. Experimental

2.1. Materials

K₃Fe(CN)₆, H₂SO₄, H₂O₂, NaCl, NaH₂PO₄, NaOH, KOH, KCl, sodium dodecyl sulfate (SDS), 3,6-diaminoacridine
hydrochloride (proflavine), methylene blue were all obtained from Sigma–Aldrich (St. Louis, MO). Deionized water (DI) was prepared from Milli-Q purifying system (Millipore, Milford, MA). Gold wire of 99.99% purity grade and 1 mm diameter was purchased from Sigma–Aldrich. 0.01 M phosphate buffer of pH 7.4 containing 120 mM NaCl and 2.7 mM KCl was used for all electrochemical measurements. Sodium saline citrate (SSC) buffer at pH 8.0 was also obtained from Sigma–Aldrich. Thiolated short chain 27 mer synthetic oligonucleotides were all synthesized by MWG biotech, Ebersberg, Germany, with HPLC purification. The used sequences are as follows.

- capture probe: 5′-HS-(CH₂)₆-CGA T CTG TTT TAT GTA GGG TTA GGT CA-3′ (I),
- complementary target to I: 5′-TG ACC TAA CCC TAC ATA AAA CAG-3′ (II),
- non-complementary target to II: 5′-TAC CAT TCT CAT CTC TGA AAA CTT CCG-3′ (III),
- single base mismatch target to I: 5′-TGA CCT AAC CCC ACA TAA AAC AG-3′ (IV).

2.2. Electrochemical characterization

2.2.1. Capture DNA immobilization, mixed monolayer formation and hybridization discrimination

The Au wire electrode surface was pretreated as stated in our previous reports (Park and Hahn, 2004; Dharian and Hahn, 2007). The target recognition layer was constructed as follows. Initially, 7 μl of 5 μM HS-ssDNA (I) was immobilized in presence of 1 M NaCl for 2.30 h. Longer immobilization does not have significant effect on the monolayer quality in presence of 1 M NaCl (Peterson et al., 2001). After the extensive rinsing with the blank buffer, the absorptions of MCH and/or MPA were made sequentially by immersing the HS-ssDNA modified surface in 500 μl of 5 mM solutions of the individual diluents in PBS buffer for 1 h. The surface is now allowed to hybridize with 7 μl of 5 μM (used in most of the experiments except in target concentration variation) either complementary (II) or non-complementary (III) targets in the hybridization buffer (4× SSC) for 2 h. At each stage, the Au surface state was assessed by cyclic voltammetric and chronocoulometric techniques intermittently in presence of 25 mM [Fe(CN)₆]³⁻ in PBS buffer at pH 7.4. Hybridization discrimination was done following the charge compensation method reported from our lab (Park and Hahn, 2004) using SDS surfactant and intercalators. The data were acquired using a VMP multichannel potentiostat (PerkinElmer Instruments Boston, MA). CVs were recorded between the potential windows 700 to −600 mV at a scan rate 50 mV s⁻¹. The potentials were referenced with respect to Ag/AgCl reference electrode.

2.2.2. Surface charge density

The redox kinetics of [Fe(CN)₆]³⁻ at the SAM modified surface is greatly influenced by the insulating ability and monolayer defects, e.g., pinholes or grain boundaries, (Finklea et al., 1993; Janek and Fawcett, 1998; Campuzano et al., 2006). CV is being used for qualitative understanding of the mechanism of monolayer formation process (Chaki et al., 2001), phase separation and complexity in the formation of the binary and ternary mixed monolayers (Nishizawa et al., 1997; Imabayashi et al., 1997; Hohara et al., 1999; Satjapipat et al., 2001; Lee et al., 2006). The high dense DNA layers provide lack of signal from electrostatic repulsion, and the partially covered surface lead to the characteristic responses for the [Fe(CN)₆]³⁻ reaction at the surface (Kelley et al., 1997, 1999; Boon et al., 2000; Liu and Barton, 2005). Hence, the monolayer surface coverage determination in presence of the [Fe(CN)₆]³⁻—based on the peak area, indicates the total number of Au sites unmodified, Γₐu and can be calculated using the relation q = nFΓₐu. Here, q is the charge obtained by the CV peak area integration at a given scan rate, n the number of electrons involved in the charge transfer process, F, Faraday constant, 96,484 and A the working electrode’s geometric area (0.078 cm²). Coulometry was made at −350 mV and the current-time (i−t) curves are recorded for 10 s to compare the passage of total charge between the electro inactive and active intercalators in presence of the [Fe(CN)₆]³⁻ following the previous literatures (Boon et al., 2000; Drummond et al., 2003).

3. Results and discussion

3.1. Redox behavior of [Fe(CN)₆]³⁻ at the binary HS-ssDNA/MCH, HS-ssDNA/MPA and ternary HS-ssDNA/MPA/MCH layers

Prior to the formation of mixed monolayers, the integrity of the HS-ssDNA monolayer is monitored in presence of the [Fe(CN)₆]³⁻ and depicted in Figs. 1A, 2A and 2A, respectively. Upon the HS-ssDNA immobilization, the anodic peak current (Iₚₐ) of the unmodified Au surface (Figs. 1 and 2A, curve a) is decreased to 3.7 μA from 15 μA and ΔEₚ is increased to 855 mV (Figs. 1 and 2A, curve b) from 100 mV. This is attributed to the electrostatic repulsion between the negatively charged HS-ssDNAs and [Fe(CN)₆]³⁻ indicating the presence of high impedance to the charge transfer. But the appearance of weak [Fe(CN)₆]³⁻ redox currents in presence of the HS-ssDNA layer (Peterson et al., 2001) suggests the existence of several free Au active sites (Γₐu) at the layer defects on the surface. The Γₐu values are evaluated from the CV peak areas of the unmodified and ssDNA modified surfaces. The observed Γₐu values for the bare and HS-ssDNA monolayer covered surfaces are 13 × 10⁻⁹ and 2 × 10⁻⁹ mol cm⁻², respectively. Hence, the number of DNA molecules immobilized on the Au surface is 1.1 ± 0.03 × 10⁻¹⁰ mol cm⁻² (4.92 × 10¹³ molecules cm⁻²), for the three repeated measurements. This is higher than those reported from different methods, Supplementary data S1. The higher HS-ssDNA density in our study is owing to the fact that the measurement is based on the electrostatic repulsive force rather than the mass change and the adsorption of cationic [Ru(NH₂)₆]³⁺ species onto the negative phosphate backbone of ssDNA. Further, the presence of Faradic current indicates the presence of randomly oriented ssDNA, Scheme 1.

The influence of the conventional MCH diluent on the pre-immobilized HS-ssDNA is presented in Fig. 1A (curve c). Slightly increased reversibility (increased Iₚₐ 6.3 from 3.7 μA
and decreased $\Delta E_p$ (712 mV) for the $[\text{Fe(CN)}_6]^{3-}$ is observed for the HS-ssDNA/MCH layer compared to the lone HS-ssDNA. But the near constant voltammetric profiles of both the HS-ssDNA and HS-ssDNA/MCH monolayer surfaces confirm the presence of negligible amount of the randomly oriented ssDNA strands (Fig. 1A, curves b and c, Scheme 1). In contrast, the mixed monolayer formation in presence of MPA diluent (HS-ssDNA/MPA) (Fig. 2A, curves b and c) results in higher reversibility (increased peak currents ($I_{pa}$ and $I_{pc}$, 12 and 17 $\mu$A, respectively) and decreased $\Delta E_p$ (248 mV) and $\Gamma_{Au}$ ($1 \times 10^{-8}$ mol cm$^{-2}$)) compared to the HS-ssDNA/MCH layer. The activity difference between these two diluents is attributed to their differential hydrophobic and/or hydrophilic interactions with the immobilized HS-ssDNA as well as the different chain lengths and head group functionalities. Compared to the MCH diluent, the high hydrophilicity of the MPA ($–\text{COO}^-$ head group with $pK_a \approx 5.2$ at pH 7.4) could effectively orient the HS-ssDNA molecules in a perpendicular configuration. This is supported from the increased reversibility and $\Gamma_{Au}$ than the

Table 1
Voltammetric peak parameters for different monolayer modified surfaces

<table>
<thead>
<tr>
<th>Surface</th>
<th>$I_{pa}$ ($\mu$A)</th>
<th>$I_{pc}$ ($\mu$A)</th>
<th>$\Delta E_p$ (mV)</th>
<th>$\Gamma_{Au}$ (mol cm$^{-2}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Au</td>
<td>15</td>
<td>26</td>
<td>100</td>
<td>$13 \times 10^{-9}$</td>
</tr>
<tr>
<td>Au–S-ssDNA</td>
<td>3.7</td>
<td>@</td>
<td>855</td>
<td>$2 \times 10^{-9}$</td>
</tr>
<tr>
<td>Au–S-ssDNA–MCH</td>
<td>6.3</td>
<td>@</td>
<td>712</td>
<td>@</td>
</tr>
<tr>
<td>Au–S-ssDNA–MCH–complementary-PF</td>
<td>10</td>
<td>19</td>
<td>292</td>
<td>@</td>
</tr>
<tr>
<td>Au–S-ssDNA–MCH–non-complementary-PF</td>
<td>@</td>
<td>@</td>
<td>800</td>
<td>@</td>
</tr>
<tr>
<td>Au–S-ssDNA–MPA</td>
<td>12</td>
<td>17</td>
<td>248</td>
<td>$1 \times 10^{-8}$</td>
</tr>
<tr>
<td>Au–S-ssDNA–MPA–complementary-PF</td>
<td>11</td>
<td>20</td>
<td>166</td>
<td>@</td>
</tr>
<tr>
<td>Au–S-ssDNA–MPA–non-complementary-PF</td>
<td>13</td>
<td>21</td>
<td>141</td>
<td>@</td>
</tr>
<tr>
<td>Au–S-ssDNA–MPA–MCH</td>
<td>8</td>
<td>@</td>
<td>450</td>
<td>$6 \times 10^{-9}$</td>
</tr>
<tr>
<td>Au–S-ssDNA–MPA–MCH–complementary-PF</td>
<td>11</td>
<td>@</td>
<td>248</td>
<td>@</td>
</tr>
<tr>
<td>Au–S-ssDNA–MPA–MCH–non-complementary-PF</td>
<td>@</td>
<td>@</td>
<td>@</td>
<td>@</td>
</tr>
</tbody>
</table>

@: Not measurable accurately; PF: proflavine intercalator.
Fig. 3. (A) Behavior of the ternary HS-ssDNA/MPA/MCH interface on gold in PBS buffer of pH 7.4 + 25 mM [Fe(CN)₆]³⁻ at a scan rate 50 mV s⁻¹. Curve a: bare Au; curve b: HS-ssDNA; curve c: HS-ssDNA + MPA; curve d: HS-ssDNA + MPA + MCH. (B) DNA hybridization discrimination at the ternary HS-ssDNA/MPA/MCH interface after the interaction of PF intercalator in presence of [Fe(CN)₆]³⁻. Curve a: hybridized surface; curve b: unhybridized surface.

The wide defects in the HS-ssDNA/MPA layer may arise due to the phase separation domains of the individual MPA and HS-ssDNA layers in the range of nanometer scale by their difference in hydrophilicity and/or hydrophobicity, chain lengths and head group functionalities (Chidsey and Loiacono, 1990; Smalley et al., 2004). The presence of phase separated domains is experimentally confirmed by the observation of two successive reductive desorption waves (−0.99 and −0.66 V) in the voltammogram of HS-ssDNA/MPA in 0.5 M KOH, according to RS−Au + e⁻ ↔ RS⁻ + Au (Nishizawa et al., 1997; Imabayashi et al., 1997; Hobara et al., 1999), Supplementary data S3B. On the other hand, the compact layer of HS-ssDNA/MCH showed only a single and diffused thiol reductive peak at −1.2 V, indicating the presence of single-phase domain, Supplementary data S3A. In order to reduce the defect size and the Faradic charge transfer current from the direct diffusion of [Fe(CN)₆]³⁻ at the HS-ssDNA/MPA binary interface, it is treated with the MCH diluent to form the multicomponent HS-ssDNA/MPA/MCH ternary interface. The results are presented in Fig. 3A. Insertion of the hydrophobic MCH (pKₐ ~ 12) at the local defects of HS-ssDNA/MPA is revealed by the increased quasi-reversibility of the [Fe(CN)₆]³⁻ (decreased Iₚa(8/9262A from...
At the unhybridized surface are made free to interact with the extensive washing with the blank buffer, the negative charges by the hydrophobic micelle complex formation between the surfaces are allowed to intercalate with the cationic PF intercalator the formation of dsDNA–SDS complex. In the second step, the hydrophilic phosphate backbones are pointing outward repelling while the DNA bases are pointing inward structurally, the formation of the ternary HS-ssDNA/MCH, HS-ssDNA/MPA and HS-ssDNA/MPA/MCH in presence of [Fe(CN)₆]³⁻ and DNA intercalator

These monolayer interfaces are hybridized with the complementary target (II) (5 µM in 4 x SSC) to form dsDNA. A control experiment using the non-complementary target (III) is also made in parallel with another surface of similar characteristics. Since the PF intercalator is electro inactive within the potential range used, the resulting Faradic current is only from the direct access of [Fe(CN)₆]³⁻ by the bare Au through the layer defects. The observed discrimination effects at the HS-ssDNA/MCH, HS-ssDNA/MPA and HS-ssDNA/MPA/MCH layers are presented in Figs. 1B, 2B and 3B, respectively. The mechanism of this charge compensation discrimination has been reported recently from our lab (Park and Hahn, 2004). Briefly, the mechanism is essentially for reducing the non-specific interactions of the cationic intercalators at the DNA surfaces involving the following three steps. First, both the hybridized and unhybridized surfaces are treated with the SDS surfactant and washed with the blank buffer. It is to remember that the SDS forms a hydrophobic complex only with the hydrophobic ssDNA bases and not with the dsDNA (Bercu et al., 1997). This is because, in the dsDNA, while the DNA bases are pointing inward structurally, the hydrophilic phosphate backbones are pointing outward repelling the formation of dsDNA–SDS complex. In the second step, the surfaces are allowed to intercalate with the cationic PF intercalator. While the unhybridized surface absorbs the PF intercalators by the hydrophobic micelle complex formation between the SDS and intercalator, the hybridized dsDNA surface interacts intercalatively and neutralizes its negative charges. After the extensive washing with the blank buffer, the negative charges at the unhybridized surface are made free to interact with the [Fe(CN)₆]³⁻, but experiences electrostatic repulsion. Hence, the hybridized surface gives intensive redox signal due to the free diffusion of [Fe(CN)₆]³⁻ and the unhybridized surface show a decreased redox signal from the electrostatic repulsion between them. Comparison of the discrimination effects at these three surfaces, Fig. 1B, 2B and 3B, suggests the following. (i) In presence of the cationic PF intercalator, the negative charges are charge compensated effectively at all the hybridized HS-dsDNA surfaces. Charge compensation might occur by both electrostatic and intercalative effects (Yau et al., 2002; Park and Hahn, 2004). The charge compensation is evidenced by the lower ΔEp of 292 mV and the increased peak currents (10 and 19 µA for the Ipa and Ip, respectively) for the hybridized HS-dsDNA/MCH surface (Fig. 1B, curve a) and a nearly constant voltammetric profile for the unhybridized surface (Fig. 1B, curve b) with nearly constant ΔEp (800 mV) and Ip, 7 µA. However, the smaller discrimination effect (~20–30%) indicates the availability of enough layer defects for the direct diffusion of [Fe(CN)₆]³⁻, which leads difficulty to reproduce in a batch experiments (data not shown). This results also confirms the absence of non-specific target hybridization and the existence of free negative charges at the unhybridized surface (Bercu et al., 1997; Park and Hahn, 2004) that are repelling the [Fe(CN)₆]³⁻ diffusion electrostatically. (ii) Similar to the HS-ssDNA/MCH layer, the ternary HS-ssDNA/MPA/MCH layer, Fig. 3B, showed 80% increased discrimination effect. The efficient charge compensation at the hybridized ternary layer witnessed by the decreased ΔEp ~ 248 mV compared to ΔEp ~ 292 mV observed for the HS-ssDNA/MCH binary interface. Nevertheless, the peaks current remains the same for both the surfaces (either Ipa or Ip, ~11 µA, Fig. 3B, curve a). Interestingly, the unhybridized ternary HS-ssDNA/MPA/MCH surface showed lack of signal, probably due to high electrostatic repulsion. (iii) In contrast to these, the HS-ssDNA/MPA layer, Fig. 2B, showed zero discrimination effect, indicated by the constant voltammetric profiles for the [Fe(CN)₆]³⁻ for both the hybridized and unhybridized surfaces, before and after intercalation. The absence of discrimination effect at the HS-ssDNA/MPA interface is attributed to the heterogeneous interactions between the hydrophilic MPA and hydrophobic HS-ssDNA. This would create wider defects allowing the free diffusion of [Fe(CN)₆]³⁻. Even in the presence of the electro active MB intercalator, the hybridization discrimination at the HS-ssDNA/MPA/MCH interface showed similar results, indicting negligible signal contribution from the direct reaction of MB at the bare surface, Supplementary data S4. It may be noted that the intercalators adsorbed onto the negative head groups of the diluents are not electro active and not involved in the electrocatalysis of [Fe(CN)₆]³⁻ (Vericat et al., 2002). Further both the chain length and head group functionality plays an important role in controlling the electron transfer processes and requires further detailed investigation using surface scanning techniques and kept for future works (Chidsey and Loiacono, 1990; Smalley et al., 2004). In this context, our recent report on the comparison of the discrimination effects at the HS-ssDNA–1-mercaptopropane (HS-ssDNA/MP) and HS-ssDNA/MPA suggests that irrespective of the similar alkane chain lengths, the different terminal groups have high impact on the compactness of the layer (Dharuman and Hahn, 2007). Hence, hybridization discrimination effects at all the three surfaces follows the order HS-ssDNA/MCH/MPA > HS-ssDNA/MCH > HS-ssDNA/MPA. The difference in the discrimination effects at the HS-ssDNA/MPA/MCH and HS-ssDNA/MCH is clearly visible in the higher concentration regions >0.01 µM.

The differential discrimination effects between the binary HS-ssDNA/MCH and ternary HS-ssDNA/MPA/MCH layers
could be attributed to different orientation of the HS-ssDNA probes and nature of the layer defects. The ssDNA strands in the ternary layer HS-ssDNA/MPA/MCH may well be separated (for instance in a μm or nm after MCH insertion) from each other. This enhances the freedom for target coiling and lead to the increased hybridization efficiency. Further, the highly negatively charged molecular environment surrounding the HS-ssDNA, created by the MPA and MCH would control this layer property and effectively repels the [Fe(CN)₆]³⁻. This facility is somewhat reduced in the HS-ssDNA/MPA binary layer due to less negative charges (MCH, pKₐ ≈ 12) and not effectively repels the [Fe(CN)₆]³⁻. It has been demonstrated literally that even at the HS-ssDNA/MPA binary interface, 100% discrimination efficiency has been observed, when the HS-ssDNA strand is in perpendicular orientation (Satjapipat et al., 2001). But the complete suppression of Faradic current for [Fe(CN)₆]³⁻ at the hybridized ternary HS-ssDNA/MPA/MCH ternary layer (Fig. 3B) compared to the unhybridized binary HS-ssDNA/MPA and HS-ssDNA/MCH interfaces (Fig. 2B) confirms the dual role of the MCH diluent. These are the defects backfilling to reduce defect size in the HS-ssDNA/MPA layer and enhancing the electrostatic repulsion force on the incoming [Fe(CN)₆]³⁻ by the negative –OH head group. The mechanism of this ternary layer formation is supported from the reductive voltammetric study of these layers in 0.5 M KOH. Fig. 4. Table 2 indicates reductive desorption peak potentials of various thiol alkane chains present in different monolayer. The desorption peak potentials for the HS-ssDNA and MCH diluent occur nearly at the same potential, viz., ∼−1.2 (±0.1) V for all the three HS-ssDNA/MPA/MCH, Fig. 4A and B, HS-ssDNA/MCH (S3A) and MPA/MCH (Fig. 4C) layers, respectively. In contrast, the positive shift in the peak potential for the MPA diluent in presence of the HS-ssDNA (S3B) than in presence of the MCH (Fig. 4C) indicates the high segregation of the hydrophilic MPA domain from the hydrophobic HS-ssDNA domain (Labinis et al., 1992). Therefore, it is possible that the MCH molecules occupied near the HS-ssDNA domain to reduce the defect size when forming the HS-ssDNA/MCH and HS-ssDNA/MPA/MCH interfaces. It is literally supported from the phase separation of long chain hydrophobic 1-dodecanethiol (DDeT) domain and hydrophilic domains composed of 2-aminoethanethiol (AET) and 2-mercaptoethanesulfonic acid (MES) on Au (1 1 1) in a ternary layer those possess defects of 15 nm in size (Phong et al., 2005, 2007). The phase separated domain formation involves the orientation of the initially absorbed flat lying hydrophilic long chain DDeT by the hydrophilic AET or MET (Phong et al., 2007). This followed by the creeping of the post absorbing alkane thiols onto the surface to occupy the defect sites adjacent to the first absorbed hydrophobic alkane thiol. Similar to this, the observation of two reductive desorption peaks for the hybridized and unhybridized surface suggest the following mechanism of ternary layer formation in the present study. Initially, the highly hydrophobic HS-ssDNA is absorbed at the surface by immersion of the cleaned Au electrode in 5 × 10⁻⁶ mol dm⁻³ of the HS-ssDNA in presence of 1 M NaCl solution. This is evidenced by the observation of the highest monolayer coverage, 1.1 × 10⁻¹⁰ mol cm⁻². But the observation of [Fe(CN)₆]³⁻ Faradic current indicates the presence of layer defects and randomly oriented layer DNA layers (Figs. 1 and 2A, curves a and b, Scheme 1). Therefore, the hydrophilic MPA interacts with surface to orient the flat oriented HS-ssDNA to the perpendicular orientation (Fig. 2 curves b and c), creating μm-sized pinhole defects (Wackerbarth et al., 2004; Phong et al., 2005, 2007) indicated by the increased reversibility of [Fe(CN)₆]³⁻ and Iₚ/Au. The insertion of hydrophobic MCH into the local defects of the binary layer of HS-ssDNA/MPA results in reducing the μm pinhole size defects to nm range, confirmed by a decrease in Iₚ/Au from 13 × 10⁻⁹ to 6 × 10⁻⁹ mol cm⁻². Appearance of a re-oxidation wave at −0.67 V in the positive scan is due to re-adsorption of the desorbed HS-ssDNA presents near the electrode surface, similar to the 1-hexadecane thiol and MPA system reported elsewhere (Hobara et al., 1998). This supports the proposed Scheme 1 summarizing the mechanism of layer construction and transduction method adopted in the present study.

### Table 2

<table>
<thead>
<tr>
<th>Surfaces</th>
<th>MCH (V)</th>
<th>MPA (V)</th>
<th>HS-ssDNA (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Au–MCH</td>
<td>−1.1</td>
<td>−0.8</td>
<td>−1.1</td>
</tr>
<tr>
<td>Au–MPA</td>
<td>−1.19</td>
<td>−0.95</td>
<td>−1.2</td>
</tr>
<tr>
<td>Au–S-ssDNA–MPA</td>
<td>−1.10</td>
<td>−0.6</td>
<td>−1.15</td>
</tr>
<tr>
<td>Au–S-ssDNA–MCH–MCH</td>
<td>−1.15</td>
<td>−0.78</td>
<td>−1.15</td>
</tr>
</tbody>
</table>

Potentials given are in volts.

3.3. Comparison of hybridization discrimination effects at the ternary HS-ssDNA/MPA/MCH interface in presence of the electro inactive PF and electro active MB and [Fe(CN)₆]³⁻ and C–A mismatch detection

Use of the electro active MB instead of PF would have the advantage of increasing the detection signal due to its assistance in the long range charge transport through dsDNA (Kelley et al., 1997, 1999; Boon et al., 2000; Drummond et al., 2003). For this purpose, the sensor surfaces after experiments with PF intercalator are incubated first in 5% SDS to remove the cationic intercalators present at the dsDNA (Yau et al., 2002) and at the negatively charged diluents (Vericat et al., 2002; Benitez et al., 2004; Westerlund et al., 2003) by the micelle sequestration. The surface is now de-hybridized in 0.5 M KOH solution for 20 min. The constancy of [Fe(CN)₆]³⁻ signal for the de-hybridized ssDNA and unhybridized surface (data not shown) confirms the effective denaturation of the dsDNA molecules and the high integrity of the HS-ssDNA/MPA/MCH layer on gold, blocking the [Fe(CN)₆]³⁻ redox reaction. The surfaces are now hybridized, MB intercalated and interrogated the [Fe(CN)₆]³⁻ reaction by the CC technique. Fig. 5A compares the discrimination effects in presence of the PF and MB intercalators.

In presence of the MB (Kelley et al., 1997, 1999; Boon et al., 2000), the sensor signal intensity increases by 40% than in...
presence of the PF for the reasons stated above. The direct reaction of MB at the bare surface is neglected as the unhybridized surface HS-ssDNA/MPA/MCH shows lack of signal similar to PF intercalated surface, Supplementary data S3. Therefore, the enhanced signal could be from the intercalative assisted charge transport mediated electrocatalysis of \([\text{Fe(CN)}_6]^{3-}\). This is further evaluated by the detection of the C–A mismatch present in the middle of the duplex in presence of the MB and \([\text{Fe(CN)}_6]^{3-}\), Supplementary data S3. The decreased charge transport is observed for the C–A mismatched hybrid than for the perfect dsDNA (Kelley et al., 1997, 1999; Boon et al., 2000). Both the CV and CC techniques showed a similar decrease in the current and/or charge for the C–A mismatches. However, still the redox signal from the direct diffusion of \([\text{Fe(CN)}_6]^{3-}\) is not completely prevented as observed for the non-complementary control experiment, Fig. 3B. This reveals the fact that the pre-dominant signal contribution is from the \([\text{Fe(CN)}_6]^{3-}\) diffusion through the layer defects and the charge compensation by MB might occur equally and efficiently at both the complementary hybrid and the single base mismatched hybrid surfaces. This necessitates further research in constructing the more compact monolayer interface with the reduced defect size and HS-ssDNA orientation controlled interface to prevent the direct diffusion of \([\text{Fe(CN)}_6]^{3-}\) (Dharuman and Hahn, 2007). Fig. 5B compares the observed discrimination efficiencies at the conventional HS-ssDNA/MCH binary interface with the ternary HS-ssDNA/MPA/MCH interface. Figure clearly demonstrates its higher efficiency in discriminating the hybridized and unhybridized surfaces at all the concentrations studied.

3.4. Calibration curve for HS-ssDNA/MPA/MCH

Analytical performance of the sensor surface is evaluated by constructing a calibration curve using different target concentrations ranging from \(1 \times 10^{-11}\) to \(5 \times 10^{-6}\) M, Fig. 5A. The behavior of these curves suggests the good specificity of the constructed binary and ternary layers in discriminating the complementary hybrid selectively at all concentrations studied. The linearity following the Langmuir isotherm is clearly visible in the nM range and the saturation limit of this sensor falls in \(\mu\)M. The lowest concentration detected is 10 pM. Discrimination is effectively noted at higher target concentrations >0.01 \(\mu\)M. It is inefficient at low target concentrations probably due to the insignificant number of dsDNA formation and nearly equal amount of electrostatic repulsions exerted from the hybridized and unhybridized on the incoming \([\text{Fe(CN)}_6]^{3-}\) leading to the poor discrimination and decreased signal-to-noise ratio.

4. Conclusions

We made a ternary monolayer consists of the HS-ssDNA and short chain MPA and MCH diluents to enhance the hybridization effect than the conventional HS-ssDNA/MCH binary interfaces for the first time. From the comparison of the discrimination effects between the binary HS-ssDNA/MCH and ternary HS-ssDNA/MPA/MCH layers, especially at the high concentration region, it is apparent that the discrimination ability of the transducer is greatly limited by the direct diffusion of the electro active ions and intercalators as well as the random orientation of the HS-ssDNA. That is, the layer defects in the binary layers of the HS-ssDNA/diluent are probably created by the phase separation of thiol alkanes of same chain lengths with different head group functionalities. Although the MCH seems to form a compact layer, still the defects could allow the diffusion of electro active ions affecting the hybridization discrimination efficiency. We also showed that the inter change of hydrophilic and hydrophobic diluents of the short chains diluents with different head groups could be efficiently used to achieve the goal while retaining the molecular environment surrounding HS-ssDNA intact. Therefore, in continuing search for the simultaneous control of both the non-specific adsorption of DNA and layer defects, our future works will focus on the control of defect type and density in the mixed monolayers of binary and multi-component SAMs of HS-ssDNA/diluents. The knowledge can then be used to increase the detection sensitivity and selectiv-
Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2007.11.015.

References