Short communication

Reusable biosensors via in situ electrochemical surface regeneration in microfluidic applications

Seokheun Choi*, Junseok Chae

Department of Electrical Engineering, Arizona State University, Tempe, AZ, USA

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Biosensors support biological and clinical diagnostic applications, offering high accuracy, precision, and functionality. Viable reusable biosensors for enclosed microfluidics are challenging because regeneration methods must ensure precision and sensitivity after recycle. Self-Assembled Monolayers (SAMs) and electrochemical processes show promise; intermediate and long chain SAMs are commonly used, but during desorption, re-adsorption and non-specific adsorption degrades biosensors, limiting reusability. Overcoming these limits is the focus of this work. We tested a prototype and regeneration method using densely packed, short-chain SAMs. Used in conjunction with controlled surface roughness, they reduced re-adsorption and non-specific molecule adsorption. Results confirmed short-chain SAM formation in enclosed microfluidic devices, and desorption promoted “clean” surface recycling for up to 50 cycles. Sensor reusability was evaluated in situ and in real time by Surface Plasmon Resonance (SPR), with a Relative Standard Deviation (RSD) of less than 0.82%, suggesting reusable microfluidics biosensors are finally within reach.

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1. Introduction

Integrating biosensors in microfluidics tools that support biological and clinical diagnostics is a popular practice; it promises advantages such as improved portability, low sample/reagent volume requirements, rapid analysis time, and high throughput analysis (Lee et al., 2007; Beebe et al., 2002; Barry and Ivanov, 2004). Several types of biosensors are commercially available or under development, and their disposability takes advantage of low cost materials and batch fabrication (Ahn et al., 2004; Yu and Chen, 2002). Growing demand for high accuracy, high precision (Asanov et al., 1998), and increased functionality in these sensors has created a new platform for exploring reusability in microfluidic applications.

Biosensors consist of a biological recognition element (bio-receptor) and a transducer (Mohanty and Kougianos, 2006). Researchers have approached reusability by removing bio-receptor target complexes from the transducer’s solid surface, and immobilizing fresh bio-receptors on the surface (Diaz-Gonzalez et al., 2005; Choi and Chae, in press). Covalent coupling with Self-Assembled Monolayers (SAMs) is a widely used immobilization technique that offers a convenient, flexible way to generate thin, well-ordered monolayers of biological molecules (Shankaran and Miura, 2007). A popular approach for removing the bio-receptor target complex is to desorb the SAM linker molecules along with all bound biomolecules (Yuan et al., 2008; Raiber et al., 2005; Kim et al., 2008). Integrated microfluidics make SAM desorption difficult to manipulate, however, because the sensing surface is completely enclosed by polymer layers and many micro-components and micro-channels (Canaria et al., 2006). Electrochemical reductive desorption of SAMs can be controlled by applying a small voltage, and individual electrodes can be flexibly implemented within array formats (Canaria et al., 2006). This is why the electrochemical desorption method is a very attractive option for regenerating biosensors in microfluidic environments. Previous studies show that incomplete SAMs desorption impacts sensing surface regeneration, limiting sensor reusability (Choi and Chae, in press; Kim et al., 2008). There are two ancillary concerns: re-adsorption of detached molecules, and non-specific adsorption of target biomolecules, such as proteins. Overcoming those issues in microfluidic applications is the focus of this work.

SAMs with intermediate (8 < n < 16) or long chain lengths (n ≥ 16) are commonly used as linker molecules in existing biosensor applications to immobilize biomolecules. They offer a high degree of surface coverage with little defect (Anandan et al., 2009), but their detached SAMs have significant re-adsorption rates, posing a practical obstacle to reuse the sensing surface (Shepherd et al., 2004; Pesika et al., 2006). Accumulation is significant because re-adsorption prevents complete regeneration of the biosensor surface, interferes with subsequent immobilization cycles, and degrades sensor sensitivity. These result in the use of short-chain SAMs as linker molecules to immobilize molecular
probes. They promote higher detection sensitivity than long chain SAMs (Anandan et al., 2009), and have lower steric hindrance, leading to the ordering of the structures by the terminal groups (Jang and Keng, 2008). In addition, they require shorter incubation time (≏6 h) for their full formation than that of the long chain SAMs (≏24 h) (Anandan et al., 2009).

Non-specific adsorption occurs as a result of imperfect SAM formation on the sensing surface. Non-specific adsorption may cause false positive/negative errors (Ladd et al., 2008). Target biomolecules bond on the empty spaces, causing a hysteresis response throughout multiple electrochemical desorption/reconstruction cycles. Very few studies have been reported to elucidate the effect of the SAM formation on non-specific adsorption, and our work explores the hysteresis effect by forming a high quality, highly packed layer of short-chained SAMs to reduce non-specific adsorption rates, and by changing the roughness of the sensing surface to facilitate appropriate SAM adsorption and desorption behaviors (Du et al., 2000; Tkac and Davis, 2008; More et al., 2002; Kropman and Blank, 1998; Schwartz, 2001).

This paper reports reusable biosensors intended for integration in microfluidics applications. The in situ electrochemical surface regeneration method using short-chained SAMs holds promise for promoting viability in reusable microfluidics-based sensor technology.

2. Materials and methods

2.1. Gold surface preparation

Glass substrates (BK7, n = 1.517) were first cleaned in piranha solution (a 3:1 ration of H2SO4 and H2O2) for 10 min. The cleaned glass was then coated with Cr/Au (2 nm/47 nm) by thermal evaporation. Gold roughness was controlled by the deposition rate (0.01 nm/s for 0.8 nm roughness, and 0.5 nm/s for 9.8 nm roughness). The substrates were annealed by hydrogen flame for several seconds, then immersed in an ethanol solution of alkanethiols [COOH(CH2)n-SH] with an EDC/NHS mixture before fibrinogen immobilization. Anti-fibrinogen was covalently immobilized on COOH-terminated SAMs [COOH(CH2)2-SH] with an EDC/NHS mixture before fibrinogen injection was injected.

2.2. Microfluidic device fabrication

Top and bottom glass substrates had Cr/Au electrodes and the spacer between the two substrates was made of PDMS (Polydimethylsiloxane). The PDMS layer was 1 mm thick, with two mechanically cut channels: one reference and one sample channel. The PDMS, glass substrate, and electrode layers were bonded together using oxygen plasma (Harrick Plasma Inc.) at 18 W for 1 min. The Cr/Au electrode on the bottom layer (2 nm/47 nm) was evaporated and patterned on a 150 μm-thick glass substrate for SPR measurement. The bottom Cr/Au layer was additionally patterned for an electrical contact. The top glass (1 mm thick) was mechanically drilled with six holes: two inlets, two outlets, and two electrical contacts. Electrical contact holes were filled with a conductive silver paste (Ted Pella Inc.) for feedthroughs. The contacts were used for applying voltage to the top Au layer; current was applied between the top and bottom electrodes, with the negative potential with respect to the bottom electrode.

2.3. Chemicals

All chemicals were used as purchased from suppliers, without additional purification. We used Anti-fibrinogen and fibrinogen from Calbiochem as target proteins. The ω-carboxylic acid alkanethiols [COOH(CH2)n-SH] with n = 2 (3-mercaptopropionic acid) and n = 10 (11-mercaptopoundecanoic acid) and ethanolamine came from Sigma Aldrich. Mediatech, Inc. supplied the phosphate buffer solution (PBS) 1× (1.15 g/L-Na2HPO4, 0.20 g/L-KCl, 0.20 g/L-KH2PO4, 8.0 g/L-NaCl, pH 7.4), sodium acetate (NaAc), and absolute ethanol. We also used Fisher Scientific’s 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS).

2.4. Linear sweep voltammetry

Linear sweep voltammetry was used to obtain the operating potential of SAM desorption in the microfluidic device. A computer-controlled potentiostat (PGSTAT302N, Eco Chemie) performed the ex situ electrochemical voltammetric measurements with custom-made electrodes placed at the bottom hole of the cell with a silicone rubber O-ring. The top hole was tightly fitted with a silicon rubber stopper with a Pt wire counter and Ag/AgCl reference electrodes.

2.5. SPR measurement of surface modification

The fabricated microfluidic prototype was mounted on a SPR analytical system (Biosensing Instrument Inc.) to monitor SAM desorption in situ and in real time. Angle shift was recorded as each solution inducing surface modification was delivered through the microfluidic channels by an external syringe pump.

All surface modification steps were monitored upon electrochemical desorption on the metal electrode. SPR angle shift was determined by differential measurement using the two channels (reference and sensing) of the microfluidic device. Initially, PBS was circulated for 20 min until the angle shift stabilized. Once the angle shift stabilized, the Au electrode in the sample channel was modified with COOH-terminated SAM from 2 mM 3-mercaptopropionic acid (MPA) solution in ethanol for four different periods of time: 10 min, 30 min, 1 h, and 12 h. Electrode modification was always followed by a thorough PBS rinse.

The first target molecule, fibrinogen, was captured by anti-fibrinogen. The antibody was immobilized on the SAM by activating the MPA carboxylic acid groups with freshly prepared 400 mM EDC/100 mM NHS in water for 15 min. The produced NHS-esters reacted with amine functions present in the 1 μM anti-fibrinogen in 10 mM NaAc pH 5.3 (sodium acetate) solution. Once the protein immobilization completed, PBS was used to wash the electrode and remove weakly bound excess proteins. After that, 1 μM fibrinogen was injected.

To desorb the antibody-antigen complexes along with the SAMs on the electrode, a reductive current was applied for the next cycle. DC potential was applied twice to the top electrode (anode) from the bottom Au electrode (cathode) for 30 s. Reductive desorption of immobilized complexes was performed by applying −0.9 V to the working electrode (twice for 30 s).

3. Results and discussion

Each task was studied in a fully enclosed microfluidic device. The microfluidic device was comprised of two channels in a PDMS spacer between glass substrates, with Chromium/Gold (Cr/Au) oxidation/reduction electrodes placed on the top and bottom (Fig. 1). Molecular binding/detachment occurs at the bottom gold electrode, being monitored by SPR in real time. SAM formation solution [3-Mercaptopropionic acid (3-MPA) in ethanol] was introduced from the inlet port, and the COOH-terminated SAM [COOH(CH2)2-SH] was formed on the bottom electrode. Antibodies were then covalently immobilized on the SAM to capture subsequently injected antigens. When reductive potential was applied at the bottom electrode with respect to the top electrode, the short-chain SAMs were dissociated from the surface along with
the antibody–antigen complexes. The electrode was then regenerated and prepared for reuse in the next biomolecular sensing experiment.

First, we conducted ex situ electrochemical measurement using linear sweep voltammetry to obtain the operating potential of SAM desorption in the microfluidic device. Reductive potential, $E_p$, of the desorbing COOH-SAM appeared at $-0.7680$ V for $n = 2$ and $-0.8887$ V for $n = 10$, respectively. Reductive desorption peak potentials were more negatively shifted by 105 mV for $n = 2$ and by 133 mV for $n = 10$ when the surface was modified by antibody–antigen complexes.

### 3.1. Re-adsorption of the desorbed SAM molecules

We observed in situ SPR sensorgrams of two chain lengths ($n = 2, 10$), shown in Fig. 2. Reductive potentials of $-0.9$ and $-1.0$ V were applied to the working electrode for $n = 2$ and 10 SAMs, respectively. Variable parameters were the duration of the voltage application and the flow of the buffer solution. Before applying the reductive potential, SPR angle values of intermediate and short-chain SAMs were established at approximately 200 and 100 mDeg, corresponding to surface coverages of 1.67 and 0.83 ng/cm$^2$, respectively. These numbers indicate tightly packed SAMs formation (alkanethiol monolayer, $7.6 \times 10^{-10}$ mol/cm$^2$) (Balasubramanian et al., 2006). Fig. 2a shows that the intermediate chain SAMs suffer from extensive re-adsorption reaction. Upon $-1.0$ V for 1 s with no flow, most of the detached SAM readily re-adsorbs onto the surface within tens of seconds (Fig. 2a(1)). We increased the duration of the voltage application to 30 s, yet the SPR angle value returned to 169 mDeg, which means that 83% of the desorbed SAM species were re-adsorbed on the same surface (Supplementary Fig. 1a). A flow of 10 μL/min did not help reducing the re-adsorption. Fig. 2a(2) shows that two consecutive potential applications with the flow at 10 μL/min have the re-adsorption of 58.5%. On the other hand, only 18% of the detached short-chain SAMs ($-0.9$ V for 1 s) re-adsorbs with no flow and the re-adsorption reduces to 10% as the duration of the voltage application increases (Fig. 2b(1) and Supplementary Fig. 1b). When $-0.9$ V was applied for twice for 30 s with the flow of 10 μL/min, re-adsorption of the detached SAM was almost negligible (Fig. 2b(2)). Pesika et al. explained the reductive desorption by a simple model that takes into account diffusion of the thiolate into the bulk solution, stating that the solubility of the thiolate increases as the chain length decreases (Pesika et al., 2006), which is in good agreement with our results. Beside, Fig. 2 shows that the flow of the buffer solution does not seem to increase the solubility for both intermediate and short-chain SAMs. Therefore, the re-adsorption decreases significantly as the chain length decreases and the duration of the potential application increases.

We studied the surface stability by repeating the formation and removal cycles of the short-chain COOH-SAM up to 50 times. SAMs were repeatedly formed for 1 h and removed by applying $-0.9$ V for 30-s twice on the working electrode. Fig. 2c shows the surface reproducibility data generated by SPR. The repeated angle shift of the recycled SAM formations was 2.43% of RSD (Relative Standard Deviation) during 50 cycles. No sign of re-adsorption occurs throughout the repeated formation and removal of the COOH-SAM. After the 50th SAM removal, the SPR baseline shifted by merely 3 mDeg.

### 3.2. Non-specific adsorption

To study the effect of the non-specific adsorption on the regeneration of the surface, we activated the COOH-SAM using EDC/NHS, then quenched the activated carboxyl groups using 1M ethanolamine to block any biomolecule adsorption on the SAM (Supplementary Fig. 2). This process allows us to distinguish non-specific adsorption from biomolecules being adsorbed on the empty space of the SAM. Fig. 3a shows that the roughness of the gold surface affects non-specific adsorption and that non-specific adsorption of fibrinogen is a function of the surface roughness and the incubation time of the SAM. The non-specific adsorption decreases as the SAM incubation time increases. However, incubation duration is not effective above 10 min. In this study, non-specific biomolecule adsorption is closely related to SAM formation time and surface roughness where the SAM is formed. This result aligns with other studies which suggest SAM formation completes over 90% within approximately 10 min. SAMs formed for 12 h on the gold surface had non-specific adsorption of 29 mDeg ($4.28 \times 10^{10}$ molecules/cm$^2$) whereas the 0.6 nm-rough gold surface showed negligible non-specific adsorption for as short a period of time as 1 h.

### 3.3. Reusability

The microfluidic device has a working electrode with 0.6 nm roughness. SAMs were formed for 1 h to minimize non-specific adsorption. The reusability of the sensing surface was studied by repeating the formation and removal of anti-fibrinogen–fibrinogen complexes via electrochemical desorption of the short-chain

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**Fig. 1.** A schematic of a microfluidic device. (a) Device configuration (PDMS spacer between glass substrates with Cr/Au electrodes); (b) A-A’ cross section; (c) B-B’ cross section (Channel 1: Reference channel, Channel 2: Sample channel).
Fig. 2. Re-adsorption of detached SAMs; SPR angle changes for re-adsorption of the detached SAM molecules upon the reductive electrochemical desorption in the microfluidic device. (Red arrow: reductive potential application, R: re-adsorption.) (a) Re-adsorption of Intermediate chain COOH-SAM \((n=10)\). The DC voltage \((1.0 \, \text{V})\) was applied for \(1 \, \text{s}\) without active flow in the microfluidic channel. (2) \(1.0 \, \text{V}\) application for twice for \(30 \, \text{s}\) with active flow in the channel at \(10 \, \mu\text{L/min}\). Most of the detached molecules were re-adsorbed upon releasing the voltage. (b) Re-adsorption of short-chain COOH-SAM \((n=2)\). The re-adsorption significantly reduces when the short-chain SAM \((n=2)\) was desorbed. The DC voltage \((0.9 \, \text{V})\) was applied for \(1 \, \text{s}\) without active flow in the microfluidic channel. (2) By applying \(0.9 \, \text{V}\) for twice for \(30 \, \text{s}\), the re-adsorption became negligible. (c) Surface reproducibility with short-chain SAM \((n=2)\). The SAM was formed and removed repeatedly up to 50 cycles and re-adsorption issue was not observed throughout 50 cycles. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

COOH-SAM. Fig. 3b shows an SPR profile sequence of surface modification steps. SPR angle shift increased by \(1800 \, \text{mDeg}\) when \(40 \, \mu\text{L}\) of \(2 \, \text{mM} \, 3\text{-MPA} \) (3-Mercaptopropionic acid) in ethanol was introduced at the rate of \(10 \, \mu\text{L/min}\). After the angle shift being stabilized, the SAM was formed for \(1 \, \text{h}\). Then, unformed SAM residues were thoroughly washed by PBS. Once the SAM covered the surface, EDC/NHS mixture activated carboxylic acid groups of the SAM to effectively immobilize anti-fibrinogen, and fibrinogen was bonded to the anti-fibrinogen. Finally, reductive desorption removed the immobilized complexes by applying \(-0.9 \, \text{V}\) to the work electrode twice for \(30 \, \text{s}\). We repeated the cycle 15 times as shown in Fig. 3c. The binding activity of fibrinogen maintained over \(97\%\) after 15 regeneration cycles, and no clear signal degradation caused by non-specific adsorption was observed during the 15 cycles. This result
demonstrated that the microfluidic biosensor could be reused with reproducibility of an RSD less than 0.82%.

In situ desorption of the short-chain SAMs in a microfluidic channel successfully delivered a regenerative surface for reusable biosensors. SPR sensorgrams demonstrated that the re-adsorption was reduced substantially by using short-chain SAM and the surface was completely regenerated by two consecutive 30 s-long desorption process while intermediate-chain SAMs tended to re-adsorb. SPR profiles showed that anti-fibrinogen and fibrinogen complexes bound on the COOH-SAM were completely reconfigured throughout 15 cycles without hysteresis.

4. Conclusion

Reusable biosensors are useful in applications where detailed sensor calibration is required, as in biochemical science and analytical chemistry. In this paper, we reported a prototype sensor and an in situ electrochemical surface regeneration technique, targeting microfluidic-based reusable biosensors. Previously, incomplete surface regeneration caused by re-adsorption of the detached molecules and non-specific adsorption of biomolecules had obscured development of reusable microfluidic-based biosensors. Here, our prototype and technique seem to be effective on overcoming the adsorption issues using short-chain SAMs that have high solubility into bulk solution. We demonstrated that re-adsorption and non-specific adsorption on the empty space could be mitigated by densely packed, short-chain SAMs, and that non-specific adsorption on the empty space due to the imperfect SAM formation could be significantly reduced by controlling sensor surface roughness.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2009.08.003.

References


Choi, S., Chae, J., in press. Microfluidics and Nanofluidics.


Fig. 3. Non-specific adsorption of proteins and real-time SPR profiles of the reusable device. (a) SPR angle changes of fibrinogen adsorption on a gold substrate as a function of the surface roughness of gold layer and the incubation time of the COOH-SAM in the microfluidic device. The COOH-SAM was incubated, activated with EDC/NHS, and quenched the activated carboxyl group to block the fibrinogen adsorption onto the SAM. Then, fibrinogen was flown through the microfluidic channel and the SPR angle shift was monitored. (b) Real-time SPR profiles and schematics of step-by-step procedure of surface modification in microfluidic device. (c) Injection of COOH-SAM solution. (d) COOH-SAM incubation. (e) Activation of surface carboxylates. (f) Immobilization of anti-fibrinogen. (g) Reductive desorption for twice for 30 s at −0.9 V.

(c) Surface reproducibility with protein modifications. First, fibrinogen was selectively bonded to the anti-fibrinogen immobilized on the COOH-SAM and the surface was completely regenerated by electrochemical desorption. We repeated 15 cycles. Non-specific adsorption of fibrinogen does not appear throughout 15 cycles.