Reversible Biosensors

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Towards Maintenance-Free Biosensors for Hundreds of Bind/Release Cycles**

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Abstract: *A single aptamer bioreceptor layer was formed using a common streptavidin–biotin immobilization strategy and employed for 100–365 bind/release cycles. Chemically induced aptamer unfolding and release of its bound target was accomplished using alkaline solutions with high salt concentrations or deionized (DI) water. The use of DI water scavenged from the ambient atmosphere represents a first step towards maintenance-free biosensors that do not require the storage of liquid reagents. The aptamer binding affinity was determined by surface plasmon resonance and found to be almost constant over 100–365 bind/release cycles with a variation of less than 5 % relative standard deviation. This reversible operation of biosensors based on immobilized aptamers without storage of liquid reagents introduces a conceptually new perspective in biosensing. Such new biosensing capability will be important for distributed sensor networks, sensors in resource-limited settings, and wearable sensor applications.*

Biological detection using sensors that operate in an unattended mode, with little or no power consumption, without consumables, and with unobtrusive form factors is an ultimate goal for medical diagnostics, homeland security, and other applications.[1] The selectivity of biosensors is given by using bioreceptors where selective molecular recognition is provided by bioreceptor–target interactions through multivalent and cooperative binding, hydrogen bonding, and charge–charge interactions.[2] Many natural and artificial bioreceptors have been demonstrated, including antibodies, enzymes, phages, carbohydrates, oligosaccharides, peptides, molecularly imprinted polymers, and aptamers.[3] Aptamers, which are nucleic acid molecules engineered to bind non-nucleic acid targets,[3m,n] are among the most widely studied bioreceptors.[4] In sensing applications, their attractive features include the ability for selection to diverse targets without the use of animals or animal cells, binding affinities similar to those of antibodies, and improved selectivity and stability by using non-natural nucleic acid bases.[5]

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The high binding affinity of bioreceptors often leads to an irreversible sensor response.[6] Thus, for repetitive detection, a new bioreceptor layer must be re-deposited before each measurement[7] providing repetitive detection but complicating sensor design by the need for removal of analyte-loaded bioreceptors and deposition of fresh ones. To simplify sensor operation, we have developed a reversible aptamer sensor by chemically induced aptamer unfolding and release of its bound target.[8] Follow-up studies by other groups have shown aptamer reversibility over a limited number of bind/release cycles.[9] Operation of aptamers over several tens of bind/release cycles led to degradation of the aptamer binding efficiency.[10] The regeneration of immobilized antibodies[11] was shown to be less attractive over aptamers in re-usable applications.[9a,12]

Herein, we demonstrate 365 bind/release cycles of reversable operation of immobilized aptamers with the high stability of the binding affinity (K_d) that varied by $< 5\,\%$ of relative standard deviation (RSD) over these cycles. Furthermore, as the first step towards maintenance-free biosensors without the need for storage of liquid reagents, we also tested the reversible operation of the immobilized aptamer bioreceptor layer for 100 bind/release cycles by using deionized (DI) water for aptamer unfolding. We show that water scavenged from the ambient atmosphere can be used for chemically induced aptamer unfolding and target release.

Scenarios where multiple biosensing cycles will be important include wearable sensors, sensors in resource-limited settings, and sensors for unattended operation.[13] For example, in wireless sensor networks for biological surveillance in airports, subways, and other public places, the advantages of conventional disposable biosensors would be cancelled out by application requirements (e.g., unobtrusive form factor of an individual sensor node, unattended operation, no storage of liquid reagents on board). Our 365 and 100 bind/release cycles of reversible operation of immobilized aptamers is the first step towards such ubiquitous unobtrusive biosensors.

To demonstrate the applicability of our approach, we selected a well-studied GGTTGGTGTGGTTGG thrombin aptamer[14] as a model bioreceptor. This aptamer folds into a monomolecular quadruplex with two G-quartets connected by a TGT and two TT loops.[15] Previous studies of this aptamer provided information on its K_d value, which ranged from 1.4 to 450 nM depending on the experimental methods.[16] We selected a common streptavidin–biotin immobilization strategy because of the well-known excellent stability of such surface-immobilized bioreceptors and the uncommonly strong ($K_d \approx 10^{-14}$ M) streptavidin–biotin interaction.[17]

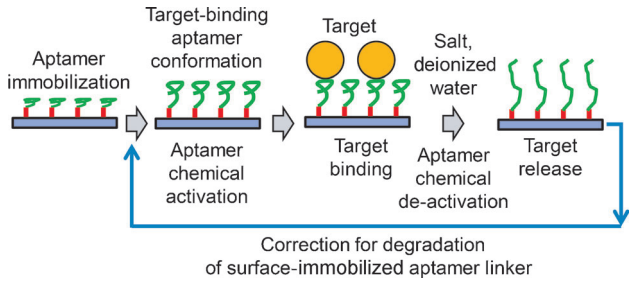

Figure 1. Strategy for increasing the number of cycles for the reversible operation of a maintenance-free biosensor with immobilized bioreceptors. The critical aspect for the reversible operation for an increased number of cycles is the correction for the predictable degradation of surface-immobilized aptamer linkers.

Details of the reversible biosensor operation for hundreds of bind/release cycles are shown in Figure 1. The aptamer molecules were immobilized onto a sensor chip. The chip was further used for reversible biosensing without a replacement of the immobilized aptamer layer by using chemical activation and deactivation of the aptamer bioreceptor layer. Chemical activation was done by exposing the immobilized aptamer layer to a buffer, which permits aptamer folding into its target-binding conformation. Chemical deactivation of the aptamer bioreceptor layer was carried out to unfold the immobilized aptamer and to release the bound target. As chemical deactivation agents for 100–365 bind/release cycles, we used DI water and alkaline solutions with high salt concentration. A critical step for the maintenance-free reversible biosensor operation over hundreds of bind/release cycles was the correction for the predictable loss of surface-immobilized aptamers. This loss was due to the instability of the aptamer attachment, which became noticeable over hundreds of cycles.

As a transduction platform, we selected surface plasmon resonance (SPR)[18] and utilized its most advanced implementation using high-end laboratory SPR instrumentation. We chose this instrumentation with a very high detection sensitivity[18] to fully explore the bioreceptor–target binding characteristics over hundreds of cycles without limitations of the measurement instrumentation and to provide guidelines for further portable in-the-field applications of reversible biosensors that could be based on diverse transduction principles.

The oligonucleotides of the thrombin aptamer GGTTGGTGTGGTTGG and a control sequence of a DNA randomer GGTGGTGGTTGTGGT were extended with twenty T nucleotides, functionalized with biotin at their 5′-end, and further immobilized onto a standard streptavidin-functionalized chip with a loading density of approximately 100 pg mm^{-2} or 3.6×10^{11} molecules cm^{-2} assuming a 15 kDa molecular weight of these DNA molecules. This bioreceptor density provided an SPR chip response of approximately 100 response units (RU, 1 RU = 10^{-6} refractive index units).

Binding of the thrombin target to immobilized aptamers was done using five thrombin concentrations (0.5, 1, 2, 4, 8 nM) in a HEPES thrombin binding buffer (see the Supporting Information). Chemical deactivation of the immobilized aptamer layer after each cycle was done for approximately 0.5 minutes with an aqueous solution of 50 mM NaOH and 1M NaCl. Chemical activation of the immobilized aptamer layer was done for approximately five minutes with HEPES buffer to fold the immobilized aptamers into their target-binding conformation.[8,15,19] The immobilized control DNA randomer showed a lack of response to thrombin (Supporting Information, Figure S1).

A reversible sensor response (Figure 2 A) illustrates five thrombin bind/release cycles. These cycles were presented to the sensor chip in 73 segments, totaling as 365 cycles of a reversible operation of immobilized aptamers on a streptavidin-functionalized chip. Results of the 365 bind/release cycles with five thrombin concentrations are illustrated in Figure 2 B. The total time for the 365 bind/release cycles of biosensor operation was 112 hours.

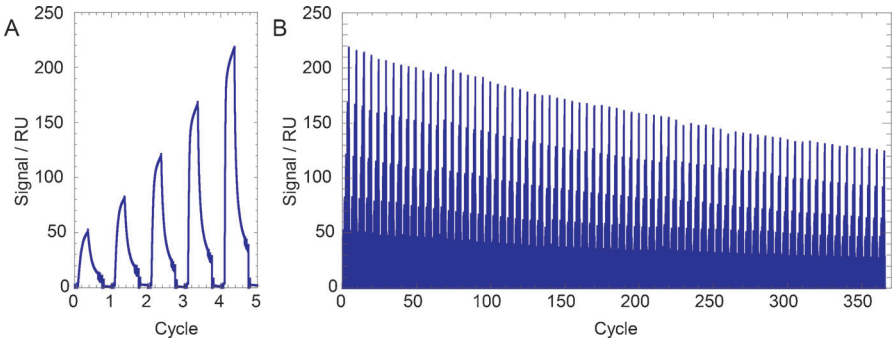

Figure 2. Reversible operation of the immobilized aptamer bioreceptors for 365 bind/release cycles. Thrombin aptamer GGTTGGTGTGGTTGG was chosen as a model. A) Five regeneration cycles of immobilized aptamers upon exposure to five different concentrations of thrombin. B) Sensor response over 365 bind/release cycles. Chemical deactivation solution: 50 mM NaOH/1 M NaCl. Thrombin concentrations: 0.5, 1, 2, 4, and 8 nM. Each cycle was run for 18.3 min.

Although the magnitude of the biosensor response decreased by approximately 40% over these many cycles, we found that the binding affinity K_d of the aptamer for thrombin was very stable. The K_d value of 2.5 ± 0.1 nM (mean $\pm1\,\sigma$) was calculated by using a global fit of the binding interactions at the five different thrombin concentrations. The calculated K_d value versus the number of bind/release cycles of the reversible performance of the immobilized aptamers and a histogram of the K_d values over 365 bind/release cycles are depicted in Figure 3 A and B, respectively.

The K_d value was constant over the 365 cycles of regeneration with a reproducibility of better than 5% RSD. Such an excellent stability of the K_d value over hundreds of bind/release cycles is a significant milestone towards the development of maintenance-free biosensors.

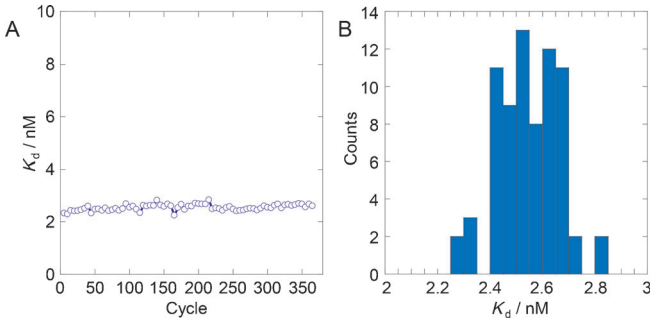

Figure 3. Immobilized aptamers display a highly stable binding affinity constant K_d over 365 bind/release cycles. A) K_d as a function of the number of performed bind/release cycles. B) Histogram of K_d over 365 bind/release cycles, $K_d = 2.5 \pm 0.1$ nM (mean $\pm 1\,\sigma$).

Other metrics of stability of the sensing surface with immobilized aptamers included the association rate constant $k_a = (4.9 \pm 0.3) \times 10^6\,\text{M}^{-1}\,\text{s}^{-1}$, the dissociation rate constant $k_d = (1.3 \pm 0.1) \times 10^{-2}\,\text{s}^{-1}$, and the predicted binding capacity of the SPR sensor surface, R_{max} (Figure S2).

Initially, we postulated that the possible reasons for the signal loss from the layer of immobilized aptamers could include the degradation of the binding ability of the immobilized aptamer owing to poorly selected chemical deactivation conditions and/or the poor stability of the streptavidin–biotin chemistry with immobilized aptamers. However, the high stability of the binding affinity over the 365 cycles (see Figure 3) demonstrated that the immobilized aptamer molecules did not experience degradation upon reuse for 365 cycles. Therefore, the reduction in binding capacity of the aptamer-functionalized streptavidin chip was further analyzed from the standpoint of streptavidin–biotin linker chemistry.

Whereas the streptavidin-biotin interaction is uncommonly strong with $K_d \approx 10^{-14}$ M as reported by several studies,[17] its finite dissociation rate was noticeable over our experiments with hundreds of cycles. The results of a global fit to the change in the maximum signal prior to the thrombin dissociation phase for the five thrombin concentrations are presented in Figure 4 A. This global fit led to an estimated dissociation rate for biotin from streptavidin of $(1.6 \pm 0.1) \times 10^{-6}\,\text{s}^{-1}$. This calculated rate constant was smaller than the rate constants of $2.4 \times 10^{-6}\,\text{s}^{-1}$ and $5.4 \times 10^{-6}\,\text{s}^{-1}$ previously determined by competition experiments with radiolabeled and unlabeled biotin with streptavidin.[17] The 1.5–3.5-fold smaller dissociation rate observed in our experiments could be due to the re-association of the biotinylated aptamer to the streptavidin surface.

We further applied our experimentally determined dissociation rate for biotin from streptavidin ($1.6 \times 10^{-6}\,\text{s}^{-1}$) as a correction factor to the plasmon resonance signal shown in Figure 2 B. This correction successfully normalized the sensor signal for the duration of the 365 regeneration cycles (Figure 4 B). These results supported our initial assumption that the observed change in binding signal during the repeated regeneration of the binding surface was not due to the degradation of the immobilized aptamer molecules but rather to the loss of some of the aptamer molecules from the surface.

The high binding specificity of the aptamer to thrombin over numerous interferents has been reported (Table S1). These earlier studies were performed with interferents at relatively low concentrations. Herein, we selected bovine serum albumin (BSA) as a known model interferent, but at a higher concentration (5000 nM) than in previous studies. Our results (Figure S3) show minimal non-specific binding of BSA at this concentration.

Next, with the goal of using multiple biodetection cycles in biosensors operated without an on-board supply of liquid reagents, we evaluated moisture scavenging from ambient air as DI water. Such a reversible operation process of immobilized aptamers without the storage of liquid reagents introduces a new perspective for biosensing.

The use of scavenged DI water for the chemical deactivation of aptamers over 100 cycles was our next important step towards unobtrusive maintenance-free biosensors without the need for reagent storage. DI water was previously used to remove salts from the bound aptamers.[5b,19]

We designed and built a system for the collection of liquid water from ambient air (Figure S4) and collected water volumes that were adequate for sensor operation. The results of the water-scavenging process are illustrated in Figure S5, demonstrating the ability of the prototype shown in Figure S4 to collect up to approximately 5 $\mu\text{L}\,\text{min}^{-1}$ of liquid water from a small area of $< 1\,\text{cm}^2$ cooled down to 2 °C. The electrical conductivity of the water scavenged from ambient air was measured to be $31 \pm 14\,\mu\text{S}\,\text{cm}^{-1}$ and thus lies within the range of values (5–160 $\mu\text{S}\,\text{cm}^{-1}$) that were reported for rainwater.[20]

The loading density of aptamers immobilized onto a streptavidin-functionalized chip in these experiments was approximately $70\,\text{pg}\,\text{mm}^{-2}$, which corresponds to 2.5×10^{11} molecules cm^{-2}. To ensure complete regeneration of the

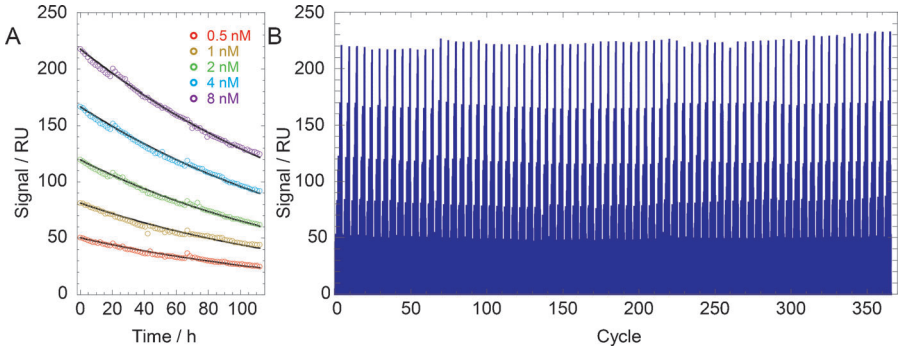

Figure 4. Accurate detection of thrombin over 365 bind/release cycles with immobilized thrombin aptamer bioreceptors. A) Global fit of the signal decay over 365 bind/release cycles for five different thrombin concentrations. Experimental data points from five concentrations: ○; respective single-exponential fits: ——. B) Corrected sensor response. Chemical deactivation solution: 50 mM NaOH/1 M NaCl.

sensor surface, the injection time of DI water for chemical deactivation of the immobilized aptamers was 15 minutes. The total operation time for 100 bind/release cycles using DI water as the chemical deactivator was 46 hours.

Results of the sensor response that were corrected to take the dissociation rate of biotin from streptavidin into account are shown in Figure 5. The calculated dissociation rate for biotin from streptavidin was $(1.6 \pm 0.1) \times 10^{-6}\ s^{-1}$, which is the same as the value calculated for the biosensor that used an alkaline chemical-deactivation solution with a high salt concentration. The uncorrected results (Figure S6) illustrate that the response magnitude of this biosensor decreased by approximately 20% over 100 cycles; however, the binding affinity K_d of the aptamer for thrombin was very stable in this sensor. The K_d value, calculated by using a global fit for the binding interactions at five different thrombin concentrations, was 9.4 ± 0.3 nM (Figure S7), demonstrating a reproducibility of better than 5% RSD. The k_a, k_d, and R_{max} values for this biosensor are summarized in Figure S8.

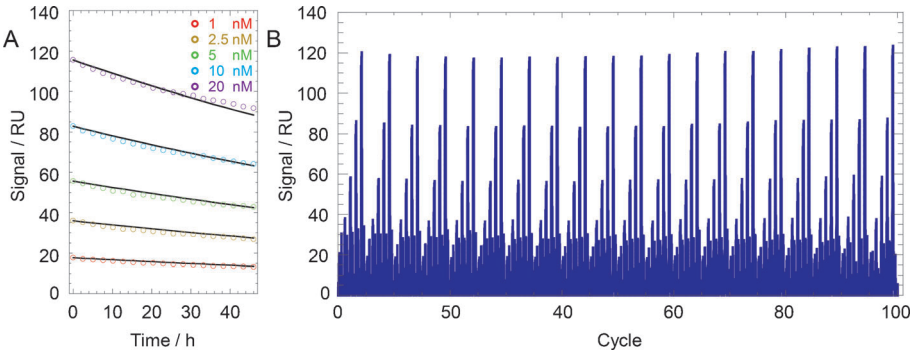

Figure 5. Reversible operation of immobilized aptamer bioreceptors for 100 bind/release cycles using deionized water for chemical deactivation of the layer of immobilized aptamers. A) Global fit of the signal decay over 100 bind/release cycles for five different thrombin concentrations. Experimental data points from five concentrations: ○; respective single-exponential fits: ——. B) Corrected sensor response. Chemical deactivator: DI water. Each cycle was run for 30.7 minutes.

The small differences between the calculated K_d values obtained in our studies for 100–365 bind/release cycles using DI water and alkaline solutions with high salt concentration as the chemical-deactivation agents could be attributed to the slight variability in the surface density of immobilized aptamer molecules and slight variations in the activity of different batches of thrombin.[21]

For the correct folding of the aptamer molecules after their regeneration, different ions are needed.[22] Otherwise, the operation of aptamers in solutions of low ionic strength could result in a significant loss of target-binding selectivity. For the operation of maintenance-free biosensors, different ions can be produced by electrochemical processes.[23] For example, we generated 10 mM of Mg^{2+} ions within approximately three minutes in 500 μL of scavenged water. Using a conservative estimation for a total operational volume of 1000 μL in a microfluidic system, such biosensors could perform unattended measurements at least once a day for hundreds of times.

In conclusion, we have shown the reliable, reversible operation of aptamer bioreceptors that were immobilized onto a sensor surface upon multiple exposures to the regeneration conditions. These biosensors were operated for 365 and 100 bind/release cycles when using 50 mM NaOH/1M NaCl and deionized water as the regeneration agents, respectively. The aptamer binding affinity was almost constant within 5% RSD. A gradual decrease in the sensor signal was determined to be related to the slow loss of immobilized aptamers from the surface. Although we have selected a streptavidin–biotin immobilization strategy because of the excellent known stability of such surface-immobilized bioreceptors, we have found that over hundreds of bind/release cycles, the streptavidin–biotin linker noticeably affects the sensor response magnitude. Of course, covalent attachment approaches are also possible, for example, with widely used thiol, amino, epoxy, or carboxylic acid moieties, and other types of functionalization.[24] Although the covalent attachment could potentially negatively affect the binding affinity of the immobilized bioreceptors through hindering of the binding sites, the opportunities of its applicability should be evaluated. Although in this biosensing study, we employed an optical-transduction method, other transduction principles such as gravimetric, electrical, or calorimetric principles, could be easily employed. The selection of a particular transduction method will be related to the allowed power consumption and system size, its ability to achieve the desired detection limit in the presence of different system instabilities, and other requirements for a particular application. In field applications, sensor stability is one of the key requirements.[25] To improve the environmental stability of aptamers (e.g., against ubiquitous nucleases), several known aptamer protection methods can be applied.[26] Our current work is focused on the implementation of our multivariable resonant-sensor platform[27] for the development of biosensing nodes for distributed sensor networks and wearable sensors.

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