Optical Control of Aptamer-Based Sensors Using a Photocleavable Linker

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Abstract

Aptamer-based sensors rely on the ability of nucleic acids to bind to target molecules with high affinity and specificity via molecular recognition. Due to their ease of production and the high thermal stability of nucleic acids, aptamer-based sensors are emerging as attractive candidates for detecting a wide range of chemical and biological targets. A current limitation of aptamer-based sensors, however, is the inability to control their activity in a time-resolved manner, limiting their utility in applications that require precise temporal control over function. Here, we demonstrate that temporal control over structure-switching (SS) aptamer sensors can be achieved using an equilibrium-shifting photocleavable linker that is placed between the aptamer and complementary strand. Installation of this linker significantly increases the effective molarity of the complementary strand, temporarily shifting the equilibrium of the sensor to disfavor target binding, and thus rendering the sensor functionally inert to the target molecule. To restore activity, the linker can be cleaved by UV irradiation, which returns the sensor into its functional equilibrium state, and allows for a dose-dependent response in the presence of target. To demonstrate the generalizability of our photocleavable linker design, we have shown that the linker can be grafted onto a different SS biosensor and still provide time-resolved control over activity. This represents a key benefit of our approach, as it allows the caging strategy to be easily adapted for use with aptamer sensors for a wide range of target molecules. Together, this research demonstrates that precise temporal control over aptamer sensors can be achieved without modifying the actual nucleotide sequence of the sensor, creating a generalizable method for control of sensor function. We anticipate that this will be particularly useful when deploying aptamer sensors in complex biological environments.

1. Introduction

Biosensors are analytical devices that are capable of binding to a specific target molecule and converting this molecular recognition event into a detectable signal. Natural receptors such as antibodies and enzymes have commonly been employed as the recognition element in

biosensors, but nucleic acid aptamers have emerged over the past two decades as a promising alternative.(Liu et al, 2009) Aptamers are short oligonucleotides that adopt folded structures, which allow them to bind to a broad range of small molecule or protein targets. Unlike antibodies, where production involves host animals or hybridoma cell lines, aptamers are generated through a combinatorial selection process termed Systematic Evolution of Ligands via EXponential enrichment (SELEX) (Tuerk et al, 1990; Ellington et al, 1990). This *in vitro* selection process does not require animals or cell lines, and can yield aptamers having high binding affinity and specificity for the desired target. And, as an additional key benefit relative to antibodies, aptamers can be chemically synthesized, reducing batch-to-batch variation and allowing for the introduction of functional group modifications. Additionally, the robust nature of nucleic acids offers higher resistance to thermal or chemical denaturation compared to protein-based affinity reagents, enabling aptamer sensors to function under a diverse range of assay conditions (Peterson et al, 2015).

While the utility of aptamer biosensors has been demonstrated in a variety of bioanalytical applications, the ability to achieve temporal control over their sensing activity remained relatively unexplored. We recognized that the availability of generalizable methods for achieving time-resolved control of biosensor function would expand the repertoire of potential applications for aptamer sensors. To meet this need, we envisioned utilizing a covalent self-caging strategy similar to that employed by naturally occurring enzymes and protein receptors, which allows for stimuli-responsive control of their ligand-binding activity. In nature, self-caged proteins, such as zymogens (Nelson et al, 2000) and protease-activated receptors (Saito et al, 2005), are temporarily inactivated by a covalently-bound inhibitor. However, these proteins can be converted into their active form in a stimuli-responsive manner through cleavage of the peptide chain at a site between the active protein and the inhibitor.

We hypothesized that this approach would be well-suited to SS biosensors, as these are comprised of an aptamer receptor bound to a short complementary strand via Watson-Crick base pairing. The aptamer and complementary strand are often labeled with a fluorophore and quencher, respectively, providing a dose-dependent fluorescent signal as target binding displaces the complementary strand (Nutiu et al, 2003). Importantly, binding of the aptamer to the complementary strand and the target is mutually exclusive, and thus the complementary strand can be viewed as a reversible inhibitor. By analogy to the covalent self-caging strategy used by proteins, we anticipated that covalently linking the aptamer to the complementary strand would shift the equilibrium of this interaction to favor binding of the complementary strand

even in the presence of high concentrations of the target molecule. However, stimuli-responsive cleavage of the linker would be expected to restore the SS biosensor to its functional state, allowing for target-dependent displacement of the complementary strand to generate a dose-dependent fluorescence signal (**Figure 1**) (Tan et al, 2016).

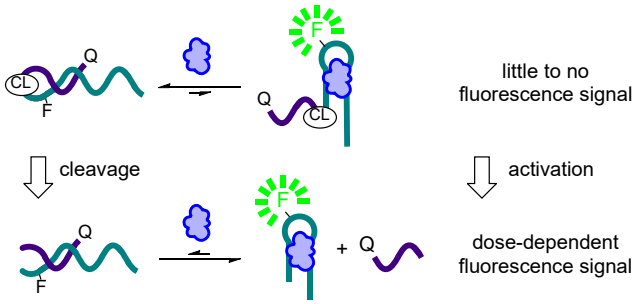


Figure 1. The equilibrium of a covalently caged biosensor is temporarily shifted to disfavor target binding. This equilibrium shift can be reversed by cleaving the stimuli-responsive linker, restoring the ability of the biosensor to bind to the target molecule and generate a dose-dependent fluorescent signal. F = fluorophore, Q = quencher, CL = cleavable linker. (Reproduced from ref. 8 with permission from American Chemical Society).

To generate the stimuli-responsive linker between the aptamer and complementary strand, we chose a photolabile group that can be cleaved by UV irradiation (Tang et al, 2006). Light represents an ideal choice as an external stimulus for the activation of caged biomolecules due to its bioorthogonality and noninvasiveness (Riggsbee et al, 2010; Mayer et al, 2006). Photocaging methods have been employed to achieve photoresponsive control over the activity of functional nucleic acids (Heckel et al, 2005; Lusic et al, 2007; Hwang et al, 2014; Ting et al, 2004). However, these methods rely on sequence-specific modification of nucleobases, which limits their generality. In comparison, our approach requires no modifications to the nucleotide sequence of the biosensor, imparting significantly higher generalizability and ease of use. Additionally, the shift in equilibrium observed in covalent self-caging is largely independent of the chemical structure of the cleavable linker, and thus we envision future expansion of our approach to encompass additional linker-stimuli combinations.

We first developed this method using the DNA biosensor for L-tyrosinamide (L-Tym), and then validated the versatility by grafting the same photocleavable linker onto the DNA biosensor for ochratoxin A (OTA) (Chen et al, 2012). We found that with a small amount of optimization (e.g. changing the concentration of the biosensor), we were able to achieve an analogous level of photoresponsive control over biosensor activity (Tan et al., 2016). One potential hurdle to

broadly adapting this approach would be instances of biosensors where the termini of the aptamer and complementary strand are separated by a large amount of sequence distance and cannot be truncated, as we did with the L-Tym biosensor. In these cases, the intervening nucleotides could act as part of the linker to still allow photoresponsive control of the biosensor. However, additional optimization would be required, and there is likely a maximum number of nucleotides that can be accommodated. Fortuitously, many SS biosensors do have close alignment of the aptamer and complementary strand termini, and thus based upon our initial experience with the L-Tym and OTA biosensor architectures, we anticipate that the photocleavable linker can be easily transferred to other sensors to impart a similar level of stimuli-responsive control.

2. Materials

2.1. DNA Synthesis

1. 6-Fluorescein phosphoramidite (Glen Research, cat. No. 10-1964)
2. 5'-BHQ-1 phosphoramidite (Glen Research, cat. No. 10-5931)
3. PC spacer phosphoramidite (Glen Research, cat. No. 10-4913)
4. Spacer phosphoramidite 9 (Glen Research, cat. No. 10-1909)
5. All DNA was synthesized by the University of Utah DNA/Peptide Synthesis Core Facility using an ABI 394 synthesizer

Table 1. DNA sequences

Name	Sequence (5'-3')
TA-0S	BHQ1-CACATCAAT/PC spacer/FAM/ATTGATGTGGTGTGTGAGTGCGGTGCCC
TA-1S	BHQ1-CACATCAAT/PC spacer/PEG_3/FAM/ATTGATGTGGTGTGTGAGTGCGGTGCCC
TA-2S	BHQ1-CACATCAAT/PC spacer/PEG_3/PEG_3/FAM/ATTGATGTGGTGTGTGAGTGCGGTGCCC
TA-3S	BHQ1-CACATCAAT/PC spacer/PEG_3/PEG_3/PEG_3/FAM/ATTGATGTGGTGTGTGAGTGCGGTGCCC
OA-1S	GATCGGGTGTGGGTGGCGTAAAGGGAGCATCGGACA/FAM/ PEG_3/PC spacer/TGTCCGAT/BHQ1
OA-CS	TGTCCGAT/BHQ1

2.2. Preparation of Stock Solutions

1. Binding buffer: 10 mM Tris-HCl, 100 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.5
2. 7.5 mM L-tyrosinamide (L-Tym, Sigma-Aldrich) freshly prepared in binding buffer
3. 5 μM purified DNA in binding buffer

2.3. Denaturing PAGE for Gel Separation and Purification

1. Multiple Mini-Vertical Gel Casting Chamber GCC-204 (C.B.S. Scientific)
2. Vertical Mini-Gel System MGV-102 (C.B.S. Scientific)
3. VWR power supplies (300V)
4. Tris/Borate/EDTA (TBE) buffer (1x): 90 mM Tris, 90 mM boric acid and 2.5 mM EDTA in Milli-Q purified water
5. Polyacrylamide gel buffer: 8% acrylamide/bisacrylamide (29:1) and 8 M urea prepared in TBE buffer
6. *N,N,N′,N′*-Tetramethylethylenediamine (TEMED)
7. Ammonium persulfate (APS)
8. RNA loading dye, 2x (New England BioLabs)
9. Crush and soak buffer: 0.5 M ammonium acetate, 1 mM EDTA, pH 8
10. Amicon Ultra 0.5 mL centrifugal filters 10 kDa membrane (EMD Millipore)
11. Corning Costar Spin-X centrifuge tube filters, pore size 0.22 μm
12. Fisher Scientific accuSpin Micro 17 microcentrifuge
13. Maestrogen UltraBright UV transilluminator
14. Typhoon FLA-7000 laser scanner
15. ImageQuant TL 8.1 software

2.4. Photocleavage and T_m Measurements

1. Corning 96-well flat bottom clear polystyrene plate (wrap the lid with aluminum foil)
2. Cleavage box - a 14 cm tall white card box wrapped in aluminum foil (**Figure 2**)
3. Feit Eelctric 60 W Equivalent Red Spiral SFL light bulb
4. Maestrogen UltraBright UV transilluminator

5. Shimadzu UV-1800 UV-VIS spectrophotometer with an MMC-1600 8 multi-cell (cell path length = 1 cm)
6. Origin 9.1 software

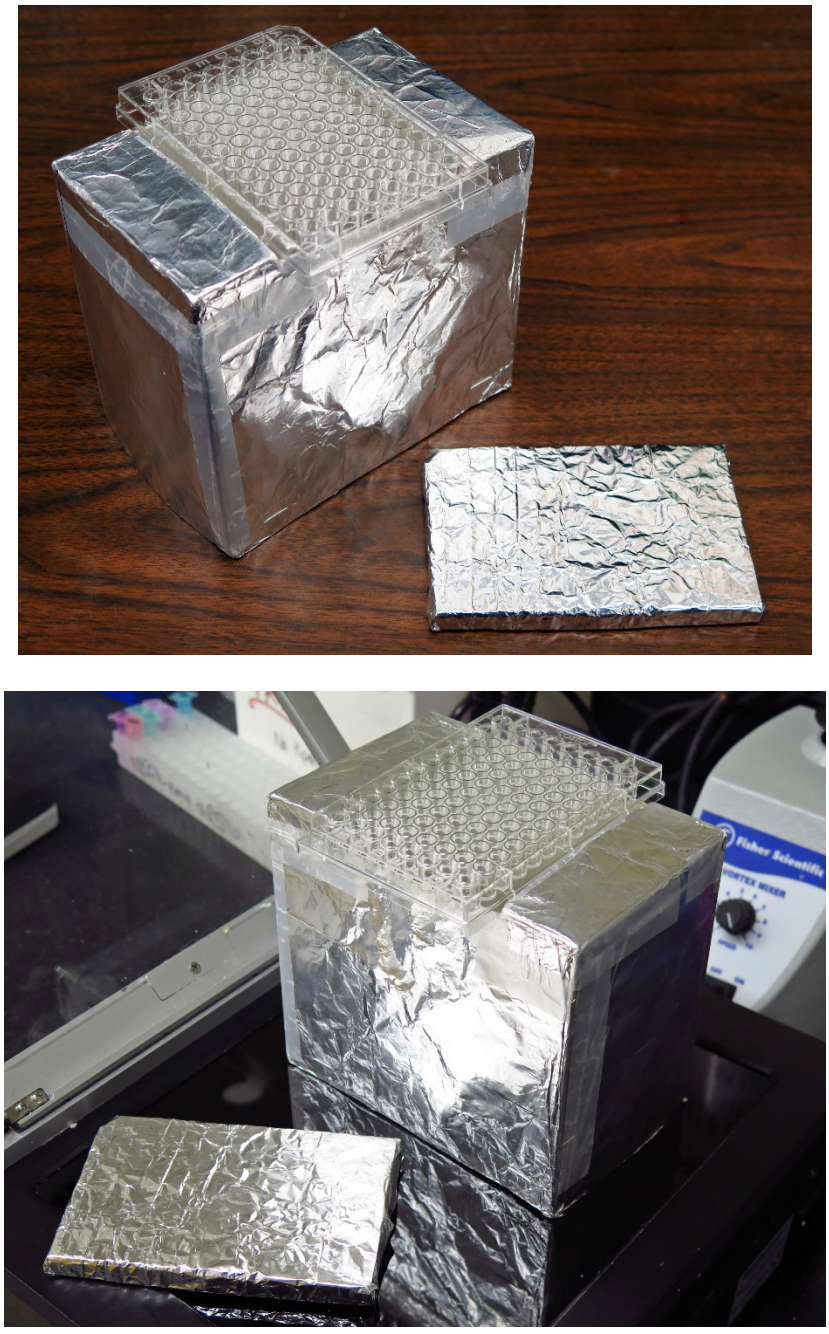


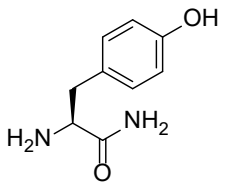
Figure 2. Experimental setup for the UV irradiation process, which includes the cleavage box, a clear bottom 96-well plate, and a lid wrapped in aluminum foil.

2.5. Fluorescence Measurements

1. Corning 384-well flat bottom black polystyrene plate
2. Corning 96-well flat bottom clear polystyrene plate
3. Biotek Synergy Mx Microplate Reader
4. Fisher Scientific Isotemp Standard Lab incubator

3. Methods

To demonstrate our covalent self-caging approach, we chose the SS biosensor for L-tyrosinamde (L-Tym) (**Figure 3**). The DNA aptamer for L-Tym was initially reported by the Gatto lab in 2001,(Vianini et al, 2001) and was developed into a fluorescence polarization sensor by the Peyrin lab in 2011.(Zhu et al, 2011) Our laboratory further adapted these sequences to generate a fluorescence-based SS biosensor by truncating the aptamer strand and labeling the aptamer and complementary strand with fluorescein (FAM) and black hole quencher 1 (BHQ1), respectively.(Feagin et al, 2015)



L-Tym

Figure 3. Chemical structure of L-Tym

The aptamer and complementary strand are joined by a hairpin loop that consists of a photocleavable (PC) spacer and triethylene glycol (PEG_3) spacer units (**Figure 4**). The PC spacer we chose for our biosensor contains an *ortho*-nitrobenzyl group, which can be cleaved by irradiation with 365 nm light. We later discovered that the PC spacer is sensitive to ambient light and can be cleaved to some extent by the standard overhead lighting in our laboratory. Thus, the crude biosensors are purified using polyacrylamide gel electrophoresis (PAGE) to remove any cleaved products and all experiments are performed in a dark room with illumination from a red light, as this wavelength does not result in linker cleavage. Because the magnitude of equilibrium shifting is dependent on the gain in duplex stability from the installation of the hairpin loop, the

effect of linker length on the duplex stability of the biosensors was explored. Biosensors containing a varying number of spacer units were synthesized and the thermal melting temperature of each structure was determined by monitoring the temperature-dependent hyperchromicity at 260 nm. In addition to optimizing the linker length, the biosensor concentration was also tuned to provide optimal signal-to-background, further improving the performance of our biosensor.

Figure 4. Structure of L-Tym biosensor. FAM = fluorescein, BHQ1 = Black Hole Quencher 1. (Reproduced from ref. 8 with permission from American Chemical Society).

Once the linker length and biosensor concentration were optimized, we tested our hypothesis for equilibrium shifting of the biosensor. The caged biosensor was irradiated at 365 nm for varying lengths of time prior to the addition of L-Tym. After incubation for 20 min at 25 °C in the presence of varying concentrations of L-Tym, fluorescence intensity was measured using a microplate reader, which enabled calculation of the percentage of complementary strand that was displaced in response to the target molecule. An additional experiment was conducted to demonstrate the ability of our biosensor to respond to L-Tym in real time as the uncaging occurs. In this experiment, the caged biosensor was combined with varying concentrations of L-Tym, and fluorescence intensity was measured at varying times during the irradiation of the sample.

This protocol describes the application of our photocaging method using the L-Tym biosensor. However, with only a small amount of re-optimization, this approach can be applied to a SS biosensor for a different small molecule target.

3.1. DNA Purification Using Denaturing PAGE

1. Prepare 8% denaturing polyacrylamide gels in a gel casting chamber (GCC-204). Inject a mixture of 50 mL of polyacrylamide gel buffer, 50 μL of TEMED, and 200 μL of 10% w/v APS into a gel casting chamber pre-loaded with glass plates and spacers.
2. Insert 1-well gel combs into each gel cassette immediately and let it sit for 30 min, or until the gel is solidified.
3. Pre-run the gel for 20 min (260 V). While waiting, prepare a 60 μM crude biosensor solution in binding buffer.
4. After 20 min, turn off the power supply and remove the gel comb. Load a mixture of 150 μL of 60 μM biosensor solution with 150 μL of 2x RNA loading dye onto the 8% polyacrylamide gel.
5. Turn on the power supply (260 V) and let the gel run for 30 min.
6. When gel separation is complete, carefully remove the polyacrylamide gel from the gel cassette.
7. To visualize the bands, excise a small vertical section from the gel and place it on the Maestrogen UltraBright UV transilluminator (high intensity, 365 nm). The remainder of the gel is kept away from any UV exposure to avoid unwanted cleavage of the PC spacer.
8. Locate the upper band (uncleaved biosensor) on the excised gel and align it to the remainder of the gel. Then, excise the indicated area on the remainder of the gel to collect the purified biosensor.
9. Cut the gel section containing the purified biosensor into small pieces and soak the gel pieces in 2 mL of crush and soak buffer.
10. Cool the mixture in a freezer until the buffer is frozen, then heat on a heat block at 90 ^{o}C for 2 hours (or 65 ^{o}C overnight).
11. Let the mixture cool to room temperature and transfer it to a 0.22 μm Corning Costar Spin-X centrifuge tube filter (try to minimize transfer of any gel pieces). Spin the filter tube at 2,000 X G for 2 minutes in a Fisher Scientific accuSpin Micro 17 microcentrifuge to remove any remaining gel pieces.
12. Transfer the eluted fraction to an Amicon Ultra 0.5 mL centrifugal filter (10 kDa) and spin at 15,000 X G for 15 minutes in a Fisher Scientific accuSpin Micro 17 microcentrifuge to remove the crush and soak buffer. Repeat this step twice by diluting the purified biosensor with binding buffer.

13. To recover the purified biosensor, place the Amicon Ultra filter device upside down in a clean collection tube. Spin the tube at 1,000 X G for 2 minutes to transfer the purified biosensor from the filter device to the collection tube.

3.2. Optimization of Biosensor Concentration

1. Prepare biosensor solutions having varying DNA concentrations (0.1, 0.3, 1, and 3 μM) in 10 mM Tris-HCl, 100 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.5.
2. Incubate the samples at 90 °C for 5 min followed by rapid cooling in ice to ensure proper annealing. Allow the solutions to warm to room temperature prior to use.
3. Transfer 100 μL of each solution into different wells on a 96-well flat bottom clear polystyrene plate.
4. Place the sample plate on top of the cleavage box and irradiate the sample with UV light (high intensity, 365 nm) for 40 min on a Maestrogen UltraBright UV transilluminator. Incubate the sample solutions at 25 °C for 15 min. The microplate containing sample is covered with a lid at all times.
5. After cleavage, transfer 50 μL of each solution into different wells on a 384-well flat bottom black polystyrene plate.
6. Measure the fluorescence intensity using excitation/emission wavelengths of 490/520 nm on a Biotek Synergy Mx Microplate Reader. All fluorescence values are standardized using a control solution containing only fluorophore-labeled aptamer in the same concentration.
7. Plot the percent of biosensors dehybrized versus biosensor concentration.
8. Using the result of this analysis, we choose the biosensor concentration (1 μM) that provides ~95% of hybridization between the aptamer and complementary strand after cleavage. We have found that this approximate level of hybridization provides optimal signal-to-background while still allowing strand displacement to take place in the event of target binding.
9. Calculate the average K_D value for the duplex using equation 1, in which [Apt] is aptamer concentration, [CS] is complementary strand concentration, and [Apt-CS] is the concentration of the hybridized duplex.

$$K_D = \frac{[Apt][CS]}{[Apt-CS]} \quad (1)$$

3.3. T_m Measurements

1. Prepare 2 μM of each of the biosensor in **Table 1** and transfer 100 μL of each solution into different wells on a 96-well flat bottom clear polystyrene plate.
2. Perform cleavage following **Step 4** in **Section 3.2**.
3. After cleavage, dilute the cleaved and uncleaved biosensor with binding buffer into a final concentration of 1 μM.
4. Anneal the cleaved and uncleaved biosensors following **Step 4** in **Section 3.3**.
5. Transfer the sample solutions to an MMC-1600 8-cell cuvette for UV measurements using a Shimadzu UV-1800 UV-VIS spectrophotometer.
6. Record the UV absorbance at 260 nm, with a ramp rate of 0.5 ºC/min in 0.5 ºC intervals from 5 ºC to 90 ºC.
7. Determine the T_m values by taking the first derivative of the melting curves using Origin 9.1 software.

3.4. Quantification of Cleavage Kinetics on Denaturing PAGE

1. Prepare 2 μM biosensor solution in 10 mM Tris-HCl, 100 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.5, and transfer 100 μL of the solution into each well on a 96-well flat bottom clear polystyrene plate.
2. Perform cleavage following **Step 4** in **Section 3.2**.
3. Dilute cleaved and uncleaved biosensors to a final concentration of 1 μM using an equal volume of 2x RNA loading dye.
4. Perform gel separation on denaturing PAGE using 10-well gel combs following **Step 1-6** in **Section 3.1**.
5. Stain the gel with SYBR Green II solution for 10 min followed by fluorescence imaging on a Typhoon FLA-7000 laser scanner using a SYBR Green II filter set.
6. Quantify the cleavage yield using ImageQuant TL 8.1 software and fit the data to a first order kinetic model using Origin 9.1 software.

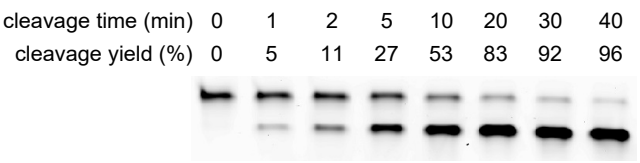


Figure 5. Monitoring the photocleavage of L-Tym biosensor using denaturing PAGE. (Reproduced from ref. 8 with permission from American Chemical Society).

3.5. Dose-Responsive Fluorescence Measurements

1. Prepare 2 μM biosensor solution in10 mM Tris-HCl, 100 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.5, and anneal by following **Step 4** in **Section 3.3**.
2. Prepare cleaved biosensor following **Steps 1-2** in **Section 3.2** with varying lengths of time, ranging from 0 to 20 min.
3. Prepare L-Tym sample solutions in varying concentrations from the L-Tym stock in **Section 2.2**.
4. Mix cleaved and uncleaved biosensor (final concentration = 1 μM) with L-Tym sample solutions in a 384-well flat bottom black polystyrene plate to give a final volume of 50 μL.
5. Cover the sample plates with a lid and incubate the samples at 25 ºC for 20 min.
6. Measure the fluorescence intensity using excitation/emission wavelengths of 490/520 nm on a Biotek Synergy Mx Microplate Reader. All fluorescence values are standardized using a control solution containing 1 μM of fluorophore-labeled aptamer.
7. Calculate the percent displacement (*%D*) using the standardized fluorescence values and equation 2:

$$\%D = \left(\frac{F-F_0}{F_m-F_0}\right) \times 100 \quad (2)$$

in which F is the measured fluorescence, F_o is the fluorescence of the uncleaved biosensor in the absence of ligand, and F_m is the fluorescence of the fluorophore-labeled aptamer strand.

3.6. Real-Time Monitoring of Biosensor Response upon UV Irradiation

1. Prepare 2 μM biosensor solution and anneal by following **Step 4** in **Section 3.3**.
2. Mix uncleaved biosensor (final concentration = 1 μM) with L-Tym sample solutions in a 96-well flat bottom clear polystyrene plate to give a final volume of 100 μL. A 96-well plate is used in this assay, as it has a higher surface area exposed to the UV light, and thus provides more efficient photocleavage.
3. Place the sample plate on top of the cleavage box. Irradiate the sample with UV light (high intensity, 365 nm) on a Maestrogen UltraBright UV transilluminator while measuring the fluorescence intensity after each UV irradiation. All fluorescence values

are standardized using a control solution containing only 1 μM fluorophore-labeled aptamer.

4. Calculate the percent displacement (*%D*) using the standardized fluorescence values and equation 2.

Notes

1. The PC modifier used in our biosensor design is PC Spacer Phosphoramidite (Glen Research, cat. No. 10-4913), not to be confused with PC Linker Phosphoramidite (Glen Research, cat. No. 10-4920).
2. As previously mentioned, the PC spacer utilized in our biosensor is sensitive to ambient light. To prevent any unwanted cleavage, all biosensor solutions were kept away from ambient light. All experiments were performed in a dark room with illumination from a red light, and samples were stored in tubes wrapped in aluminum foil for transporting outside of the dark room.
3. It is important to note that small molecule degradation may occur under red light. The red light used in these experiments was compatible with L-Tym, but we found that OTA was degraded when exposed at a close distance to this light source.
4. The conditions for DNA gel purification (voltage and separation time) were optimized for TA-1S. A different biosensor with different DNA sequence may require re-optimization to achieve good separation.
5. The choice of centrifugal filter used in **Section 3.1** was based on the molecular weight of the biosensor. Uncleaved TA-1S has a molecular weight of ~13,000 g/mol and therefore a 10kDa filter was used to retain the purified TA-1S.
6. The cleavage box (**Figure 4**) used in this method was constructed with a white card box with dimensions of 14 cm (h) x 15 cm (l) x 10 cm (d). The exterior wall of the box is wrapped with aluminum foil to prevent any ambient light from entering the box, which may interfere with the cleavage of the PC spacer.
7. The UV transilluminator generates heat when the light source is on, which can build up inside the cleavage box and contribute to sample evaporation during UV irradiation. To minimize the sample evaporation, release the heat by lifting the cleavage box from the UV transilluminator from time to time throughout the cleavage process. We suggest carrying out this step at 10 minute intervals, we have found this to be effective in keeping sample evaporation to a minimum.

Acknowledgements

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