

Selection of DNA Aptamers for Sensing Uric Acid in Simulated Tears

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Abstract

Uric acid is a biomarker for a range of diseases and hyperuricemia is the cause of many diseases including gout. While most biosensors for detecting uric acid relied on enzymatic reactions, in this work a library-immobilization method was used to obtain DNA aptamers for uric acid. After 18 rounds of selection, two representative aptamers were obtained with a K_d around 1.2 μM measured by isothermal titration calorimetry (ITC). Based on their difference in binding to xanthine, which differs from uric acid by only one oxygen atom, these two aptamers have different binding orientations to uric acid. ITC also indicated that the UA-1 aptamer specifically required a high concentration of Na^+ for binding, which cannot be replaced by Li^+ , K^+ or Mg^{2+} . Combined ITC and fluorescence spectroscopy data indicated the need of three Na^+ ions, which explained the requirement of a high Na^+ concentration. The UA-1 aptamer was engineered into a fluorescent biosensor based on the strand-displacement reaction, resulting in a limit of detection of 90 nM uric acid. This sensor was also tested in simulated tears with a limit of detection of 350 nM uric acid.

Introduction

Uric acid (2,6,8-trihydroxypurine, UA) is the primary end product of purine metabolism and a potent antioxidant in human.^[1] UA is produced from purines by xanthine oxidase.^[2] In the majority of mammals, UA is further converted to allantoin by uricase. Because of a lack of uricase in humans, UA exists in high concentrations in various human body fluids from several tens micromolar to several millimolar, for example, 150-450 μM in blood,^[1c, 3] 25-150 μM in tears,^[4] and 1.4-4.4 mM in urine.^[5] UA is a weak acid (pK_a 5.75 and 10.3). At the physiological pH, UA predominantly circulates in the form of a monovalent sodium salt called urate.^[2, 6] An abnormal UA level is a risk factor for many diseases, such as gout, diabetes, high blood pressure, kidney and heart diseases, Parkinson's disease and Alzheimer's disease.^[1a, 2]

An early method for the detection of UA uses the color change of reaction with phosphotungstic acid.^[7] Due to the inherent toxicity of phosphotungstic acid, new methods have been developed for both in vivo detection and the detection of extracted UA based on light absorption, fluorescence, chromatograph and electrochemistry.^[1a, 8] The majority of recent reported methods involved enzymatic oxidation of UA to produce H_2O_2 as a by-product, which could be subsequently quantified electrochemically,^[3a, 5, 9] or by a spectroscopic method in the presence of a chromogenic or fluorogenic probe.^[3b, 10] In such methods, aside from natural uricase, a variety of enzyme mimics were also developed.^[3b, 10a, 11] In these enzyme-based methods, multiple reaction steps involving various enzyme cascade reactions were often performed. Thus, developing biosensors for UA based on simple binding reactions instead of enzyme catalysis is needed.

Aptamers are single-stranded nucleic acids that can selectively bind target molecules.^[12] DNA aptamers are particularly attractive for biosensing applications due to their high stability, programmable structure and versatile signaling methods.^[13] For the detection of small molecules, DNA aptamers have advantages over antibodies,^[14] and many metabolite binding aptamers have been reported.^[15] A class of riboswitch can sense the oxidized purine derivatives (xanthine and UA).^[16] Given the large size and RNA nature of riboswitches, DNA aptamers are preferred for detection outside cells for their low cost, high stability and ease of modification.^[14, 17] However, DNA aptamers for UA have not yet been reported. Based on the structure of UA with a xanthine scaffold, we reason that UA is a viable target for DNA aptamers.^[18]

In this work, we performed library-immobilized aptamer selection experiments and obtained two aptamers for UA. While both bind UA with a similar affinity, one showed lower binding to xanthine, suggesting these two aptamers bind UA with different orientations. Using one of the aptamers, we designed a highly sensitive and selective biosensor for UA and tested its performance in simulated tears.

Results and Discussion

1. Selection of UA-binding DNA aptamers.

We used a library-immobilized aptamer selection strategy as reported in literature (Figure 1a).^[19] UA ($pK_a \sim 5.7$) has a relatively low water solubility of 60 mg/L (0.36 mM) at neutral pH and the solubility can be increased by raising the pH. Thus, we used a buffer of pH 8.3 (20 mM Tris, 1 M NaCl, 10 mM $MgCl_2$ and 5 mM KCl) to elevate its solubility. In addition, UA could precipitate in high salt buffers upon storage (Figure S1). To ensure consistent results, we freshly prepared UA solutions for each round of selection with the assistance of heating in hot water to promote dissolution. A 73-nucleotide library containing a 30-nucleotide random region flanked by two constant regions for hybridization to PCR primers was used (Figure 1a), and the library sequence was designed to form a hairpin. The initial library contained around 10^{14} random DNA sequences and was immobilized on beads via hybridization. The sequences that can bind UA and form a hairpin were expected to be released from the beads, and they were amplified by PCR.

The initial UA concentration was 5 mM. The selection stringency was increased by gradually decreasing the UA concentration to 50 μ M over a total of 18 rounds (Table S2). The round 18 library was sent for sequencing. The resulting 29,961 sequences were assigned to 354 groups by the Geneious software. The first 40 most abundant groups were aligned using Clustal Omega and assigned to different families (Table S3). We pick 9 most abundant sequences and aligned them in Figure 1b. UA-2 and UA-6 are from one family, where three regions of conserved nucleotides can be observed (Figure 1b, in green). The rest sequences were all similar to UA-1. These UA-1 like sequences all contain a hairpin, as shown in the middle red-black-red regions (the two red regions can base pair). The conserved nucleotides are in boldface blue. Interestingly, these conserved nucleotides switched sides (UA-3 and UA-9), indicating the same way of binding but

the target flipped the side. This is a strong indication of a successful selection, and similar observations were also made with the RNA aptamer for theophylline.^[20]

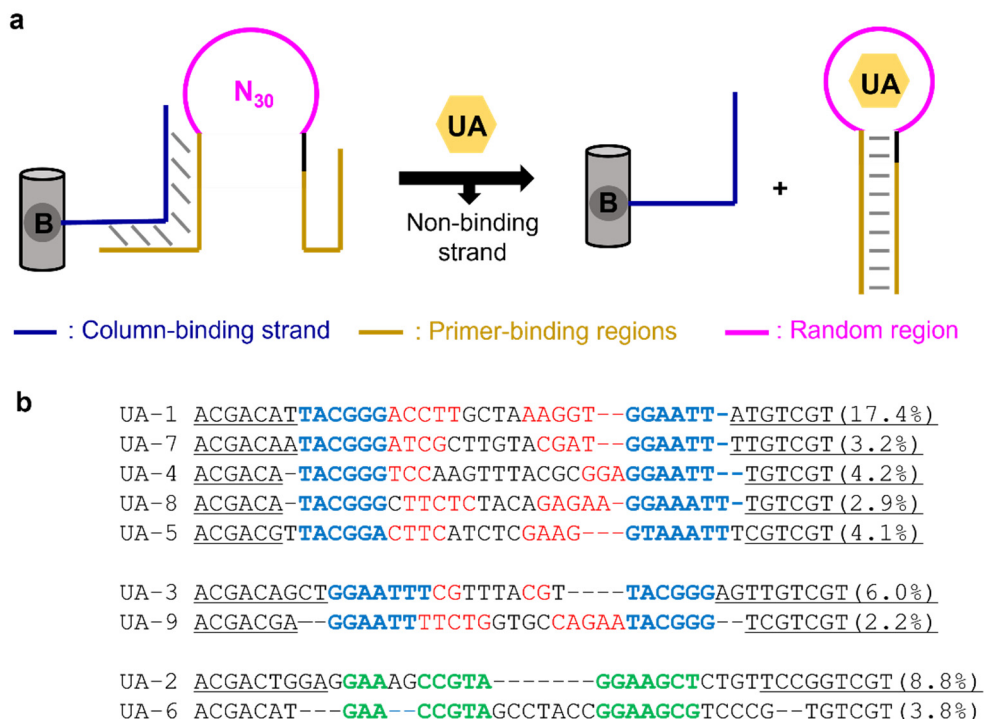


Figure 1. (a) A schematic representation of the library-immobilized aptamer selection for UA. (b) Alignment of the first 9 most abundant sequences, which can be divided into three families, and the top two families have the same way of binding. The underlined regions base pair.

2. ITC for confirmation of binding.

To test whether the sequences can bind UA, we used isothermal titration calorimetry (ITC).^[21] Since all the sequences can fold in an overall hairpin structure, we truncated most of the constant primer binding regions and kept five base pairs to produce 40-nt aptamers. The Mfold predicted secondary structures of the truncated UA-1 and UA-2 aptamers are shown in Figure 2a and 2b.^[22] The ITC traces and integrated heat for UA-1 and UA-2 are shown, and the rest are shown in Figure S2. The fitted K_d values for UA-1 and UA-2 were 1.3 and 1.2 μ M, respectively. All the other tested sequences could also bind, but with slightly higher K_d values (Table S4). In addition, we also titrated UA into a random sequence (Figure S2h), and no heat was generated, indicating the specific binding between the aptamers and UA.

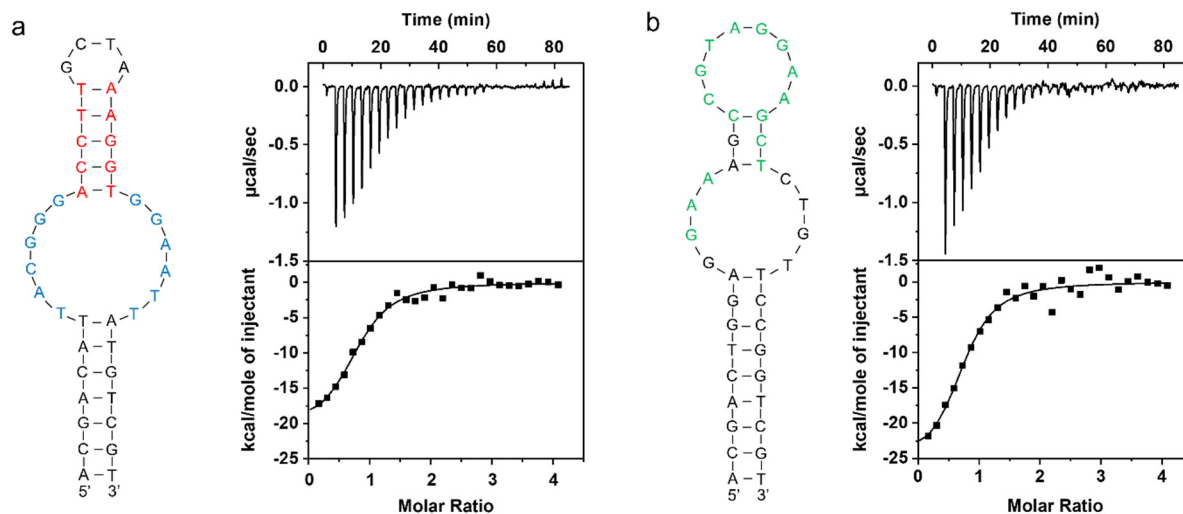


Figure 2. The predicted secondary structures of (a) UA-1 and (b) UA-2 (10 μM) and their ITC traces and integrated heat of titrating 200 μM UA into the aptamers.

UA and xanthine differ only by an oxygen atom (Figure 3a). Considering this similarity, we also tested the binding of xanthine by UA-1 and UA-2 (Figure 3b and 3c). Interestingly, UA-2 showed a similar binding affinity to xanthine, whereas UA-1 had a lower binding (an accurate K_d cannot be fitted). This suggests that these two aptamers bind UA from different orientations. UA-1 might interact with the exocyclic oxygen, whereas UA-2 binding might be less so. We schematically illustrated this difference in Figure 3d. It needs to be noted that this is only a cartoon representation and binding to UA would likely also include π - π stacking and other interactions,^[12a, 23] and the exact ways of binding can only be identified by structural biology methods. A single methyl group has made the RNA aptamer for theophylline 10000-fold less selective for caffeine.^[20] Here, a single oxygen can also make a difference in UA.

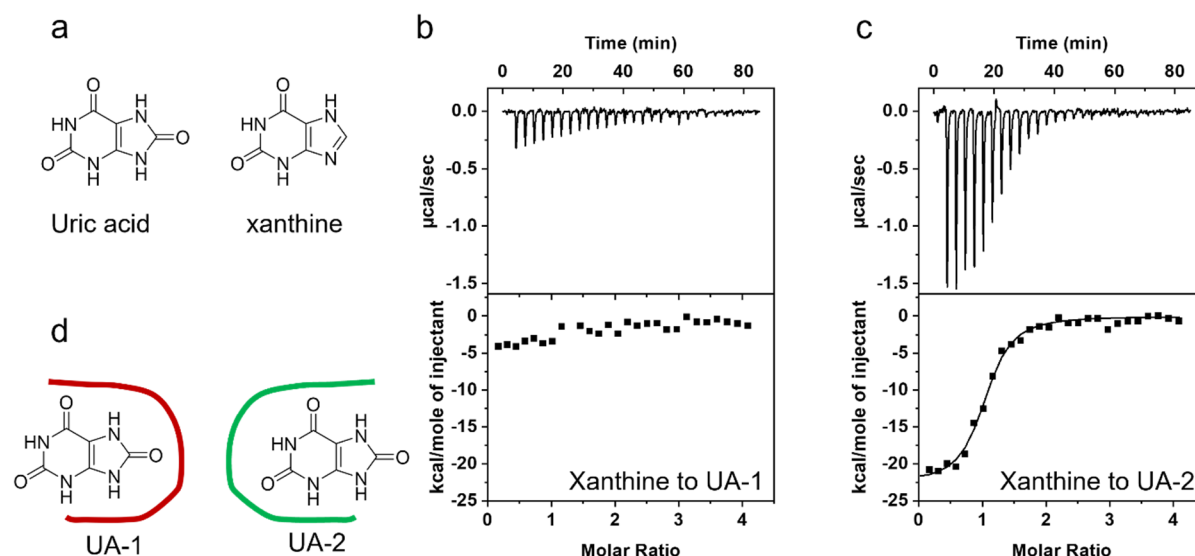


Figure 3. (a) Structures of UA and xanthine. (b) ITC traces and integrated heat of titrating xanthine (200 μ M) to (b) UA-1 and (c) UA-2 (10 μ M). (d) A schematic illustration of UA-1 and UA-2 binding to UA.

3. Truncation and mutation studies

To further understand the sequence of the aptamers, we then performed truncation and mutation studies. From the ITC studies, we know that the hairpin in UA-1 can be replaced by essentially any hairpin structure. We then tested whether the hairpin can be fully removed. Of the two truncations we made (Figure S3), interestingly, neither showed binding to UA. Therefore, a hairpin structure needs to be there, although the exact sequence is not important. We then tested a few mutants by disrupting the consensus sequences of TACGGG and GGAATT in the loop region of UA-1. As shown in Figure S4, none of these mutants showed binding to UA, indicating the importance of these consensus sequences. For UA-2, we truncated several base pair from the stem region (Figure S5a), and the binding to UA was not affected. We further mutated UA-2 for two base pairs, and no binding was observed for these mutants (Figure S5b).

4. Na⁺-dependent UA binding.

All of the ITC measurements above were performed in the buffer containing 910 mM NaCl, 10 mM MgCl₂ and 5 mM KCl. To understand the effect of salt for aptamer binding, we then decreased

the concentration of NaCl (Figure 4a). Interestingly, when the NaCl concentration was reduced, the binding was also reduced. Therefore, NaCl was required for binding. By fixing the concentration of NaCl, we then varied the concentration of Mg^{2+} , and it appeared that Mg^{2+} can be omitted as long as a high NaCl concentration was present (Figure 4b). This type of behavior is different from some other aptamers, where Mg^{2+} was more important.^[24] In addition, Na^+ cannot be replaced by Li^+ or K^+ (Figure 4c and 4d). Therefore, Na^+ participated in the binding reaction instead of simply acting as an electrolyte to screen charge repulsion. Previously, a cyanocobalamine binding RNA aptamer was shown to be dependent on a high concentration of Li^+ but independent of Mg^{2+} .^[25] Typically, G-quadruplex DNA is known for specificity for K^+ . From the selection of RNA-cleaving DNazymes, a Na^+ binding aptamer was obtained.^[26] Recently a Li^+ -specific DNzyme was reported.^[27] Nucleic acids can have good specificity for monovalent metal ions.

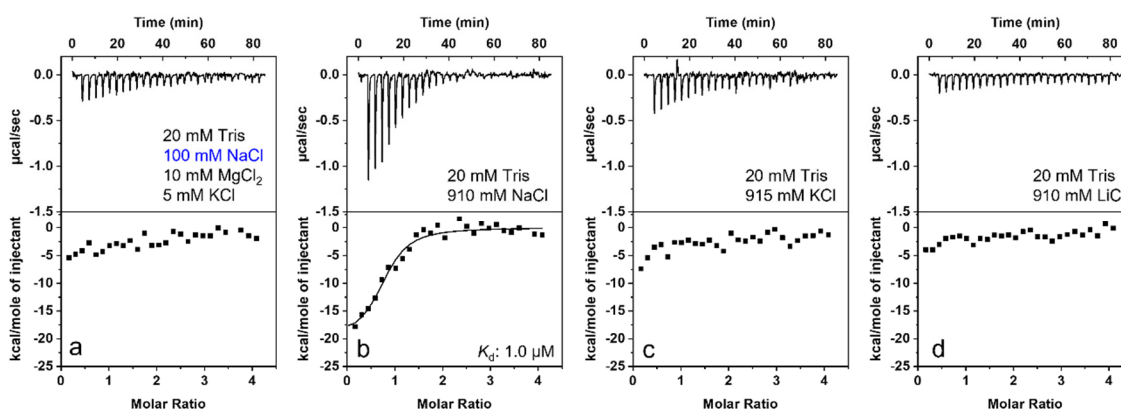


Figure 4. ITC traces and integrated heat of titrating UA (200 μM) to the UA-1 aptamer (10 μM): (a) reducing the NaCl concentration to 100 mM, (b) remove Mg^{2+} , (c) NaCl replaced by KCl and (d) NaCl replaced by LiCl.

With this interesting requirement for Na^+ , we then measured the K_d of the aptamer at different NaCl concentrations (Figure S6). The Na^+ -dependent K_d values appeared to be a sigmoidal curve, suggesting that multiple Na^+ ions were involved. A Hill coefficient of -3.9 ± 1.3 was obtained, suggesting that three to five Na^+ ions were involved in the binding of UA. The need of multiple Na^+ ions can explain the high Na^+ concentration needed.

In our UA-1 binding aptamer, the apparent K_d is higher than 300 mM Na^+ . In the Na^+ binding aptamer identified from DNAzymes, and the K_d was around 20 mM Na^+ .^[26] Thus, the role of Na^+ here is probably to facilitate binding of UA instead of directly binding to DNA, leading to a higher K_d . Since the ITC data showed that the binding stoichiometry of UA and the aptamers was 1:1, the role of Na^+ was not to recruit multiple UA molecules. Finally, the binding curve also indicated that the binding saturated beyond 730 mM NaCl (Figure S6).

5. Fluorescent biosensor for UA

After confirmed the binding of the UA aptamers, we developed an aptameric sensor using the strand-displacement fluorescence assay.^[19b, 28] We labeled a FAM fluorophore to the UA-1 aptamer (FAM-UA-1), whose fluorescence was quenched upon hybridization to a quencher labeled short DNA (Q-cDNA). When UA was added to the FAM-UA-1/Q-cDNA hybrid, target binding to the aptamer induced dissociation of Q-cDNA, resulting in the recovery of fluorescence (Figure 5a).

Due to the importance of NaCl for UA-1 binding, we optimized the buffer condition by varying the NaCl concentration from 100 mM to 1 M. The background fluorescence (from 0 to 5 min) was lower with a higher NaCl concentration due to better DNA hybridization. At 5 min, a final concentration of 100 μM of UA was added to each sample, and a higher NaCl concentration resulted in a larger increase in fluorescence (Figure 5b). We plotted the fold of fluorescence enhancement $(F-F_0)/F_0$ versus NaCl concentration (Figure 5c), and the curve was also fitted using the Hill equation, obtaining a Hill coefficient of 2.8 ± 0.2 , indicating three Na^+ ions were involved in the UA-1 binding. Both ITC and the fluorescence assays indicated that multiple Na^+ ions were involved in the binding. We believe that the fluorescence assay is more reliable due to its smaller error and reliable data in both high and low NaCl concentrations.

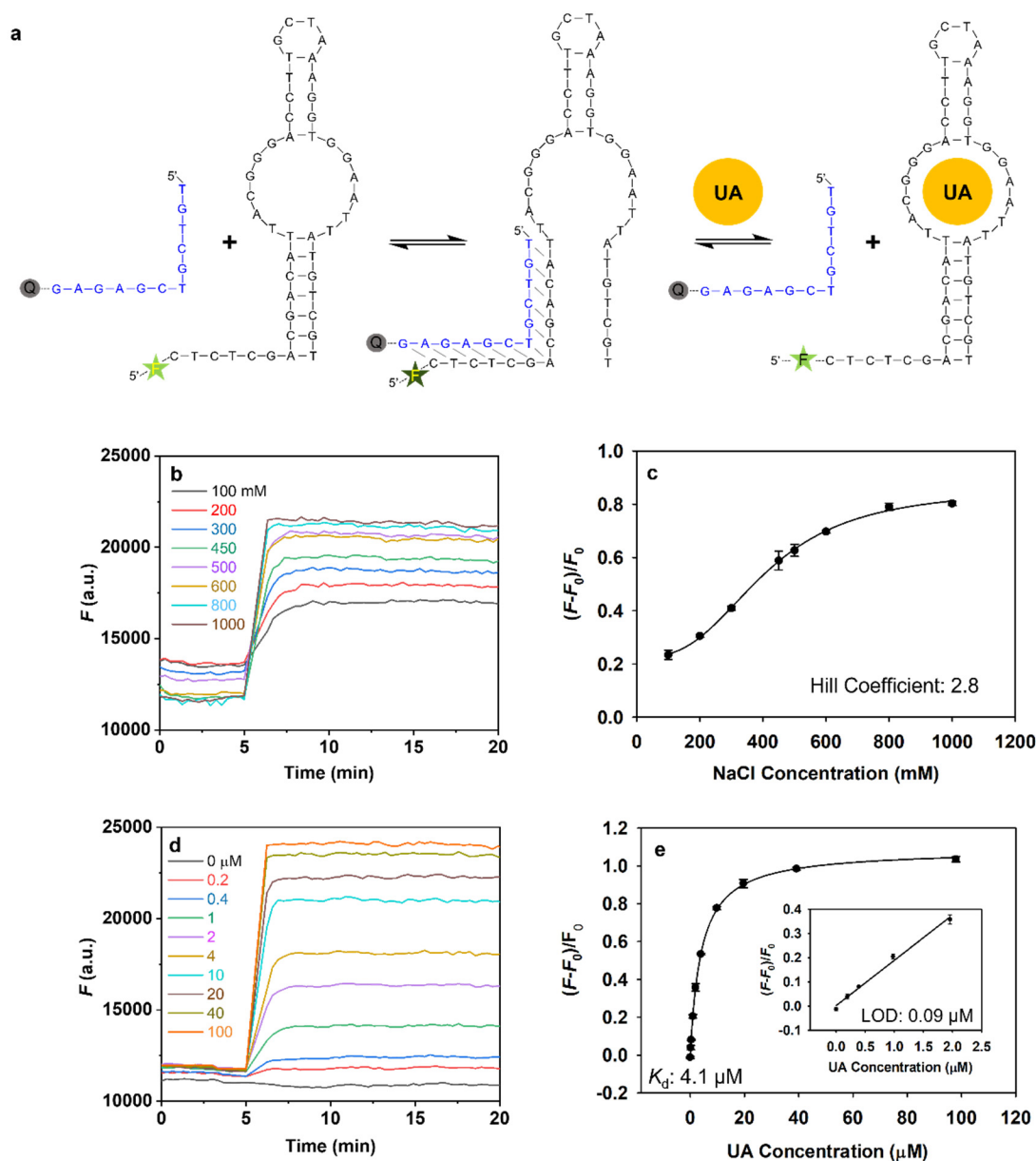


Figure 5. (a) A schematic illustration of the fluorescent sensor for the detection of UA. (b) Response of the sensor to 100 μM UA in the presence of different concentration of NaCl. (c) Fluorescence enhancement as a function of NaCl concentration. (d) Response of the sensor to various concentrations of UA in the presence of 800 mM NaCl. (e) Calibration curve of the sensor. Inset: the linear response at low UA concentrations.

Since with 800 mM NaCl, the sensor had the lowest background fluorescence and highest signal increase upon target addition, we used the buffer containing 800 mM NaCl for the detection of UA. We prepared 20 nM sensor, and then added different concentrations of UA ranging from 0.2 μ M to 100 μ M. The fluorescence change is shown in Figure 5d, and the $(F-F_0)/F_0$ was fitted to yield an apparent K_d of 4.1 μ M, which is just 3-fold higher than that obtained from ITC (1.3 μ M). The difference can be attributed to the presence of the quencher-labeled strand.^[19b, 29] The detection limit (LOD) was calculated to be 0.09 μ M based on $3\sigma/\text{slope}$ (inset of Figure 5e). This sensitivity allows the use of this sensor in biological samples.

We then tested the selectivity of the UA-1 sensor against ten small biomolecules with similar structures to UA, and each target was tested at two concentrations: 20 μ M and 200 μ M. As shown in Figure 6a, UA-1 had no response to most of the analytes except for xanthine. The apparent K_d for xanthine was 30.2 μ M from the sensor, which was 7.5-fold higher than for UA. Xanthine is not the final metabolite in human, and it can be further converted to UA by xanthine oxidase.^[30] The concentration in the human body is usually below a few micromolar.^[30-31] Since the xanthine concentration is much lower than that of UA (several hundred micromolar), xanthine is unlikely to interfere the detection of UA in human body fluids. In addition, we challenged the UA-1 sensor with glucose and urea, which also exist in high concentrations in body fluids. Neither influenced the sensor performance (Figure S7).

Detection in artificial tears.

Tears contain many biomarkers that can be detected noninvasively.^[32] Detection of UA in tears has been reported. For example, ZIF-67 Co_3O_4 gated graphene transistor was used for simultaneous detection of tear glucose and UA.^[33] Plasmonic Schirmer strips for human tear gout arthritis diagnosis was reported using surface-enhanced Raman scattering.^[4a] We prepared simulated tears containing sodium, potassium, magnesium, bicarbonate, albumin, and lysozyme at physiological levels.^[4b] After adding 20 nM UA-1 sensor in the simulated tears, we titrated UA. The fluorescence response is shown in Figure 6c, and $(F-F_0)/F_0$ is plotted in Figure 6d. By fitting the data, a K_d of 10.3 μ M was obtained. The detection limit was calculated to be 0.35 μ M UA. These figures of merit are suitable for UA detection in human tears.

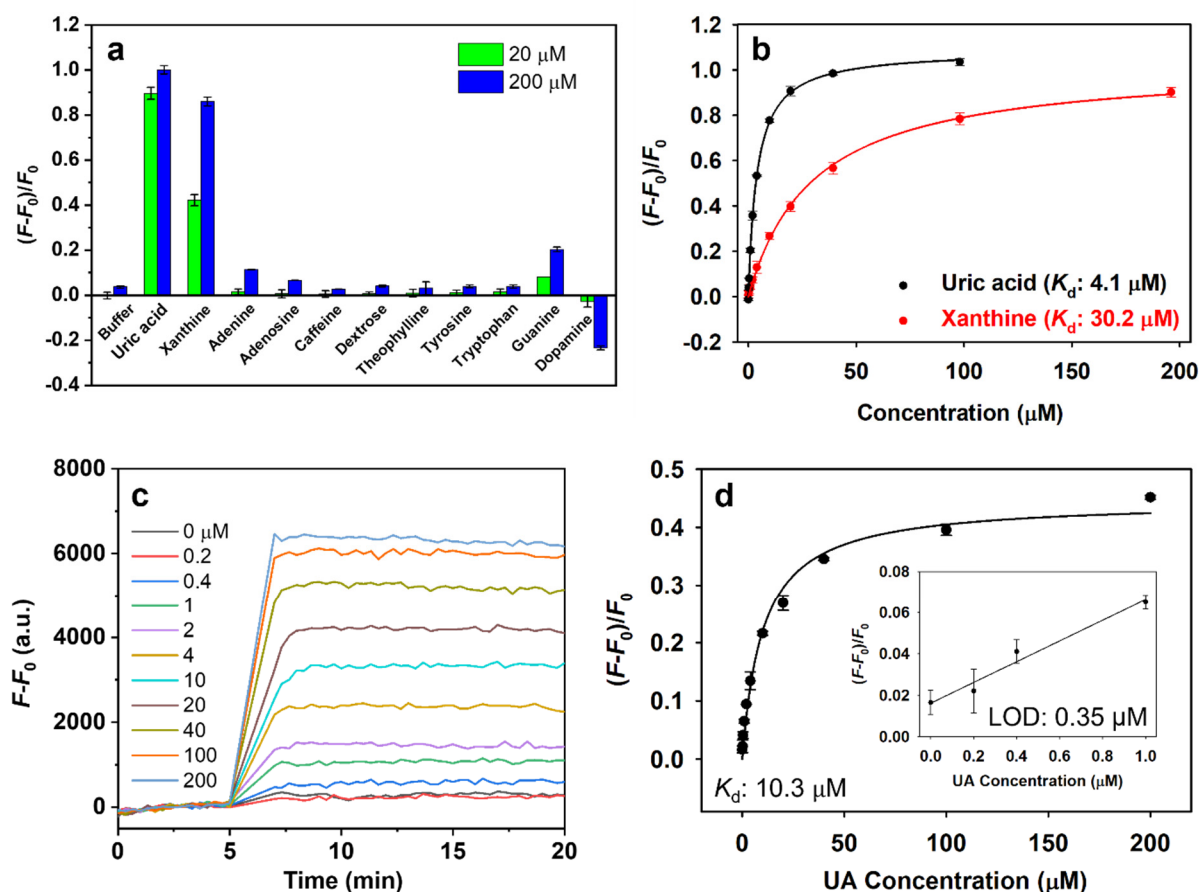


Figure 6. (a) The selectivity of the UA-1 sensor against various small molecules at two concentrations. (b) Calibration curves of the UA-1 sensor for UA and xanthine. (c) Fluorescence response of UA-1 sensor to various concentrations of UA in simulated tears. (d) Calibration curve of the sensor in simulated tears. Inset: the linear response at low UA concentrations.

Conclusions

In this work, we used the library-immobilization method to select DNA aptamers for UA, and two representative aptamers with different orientations of binding were obtained. They bind UA similarly but showed different affinities to xanthine, which differs from UA by just one oxygen atom. The binding required three Na^+ ions, which cannot be replaced by Li^+ or K^+ . In addition, the binding of UA was independent of Mg^{2+} . A fluorescent biosensor for the detection of UA was designed based on the strand-displacement strategy, where UA displaced the quencher-labeled complementary DNA. A detection limit of 90 nM UA was achieved using this biosensor. The

biosensor was also tested in simulated tears with a LOD of 0.35 μM UA. Considering the importance of UA and its role in a range of diseases, this aptamer will find important bioanalytical applications.

Materials and Methods

Chemicals

All of the DNA oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA, USA), and their sequences are listed in Table S1. UA, xanthine, adenine, adenosine, caffeine, theophylline, tryptophan, guanine, dopamine and ethidium bromide were purchased from Millipore-Sigma. Agarose and L-tyrosine were purchased from Bio Basic. Dextrose was purchased from VWR. Tri(hydroxymethyl)aminomethane (Tris), NaCl, MgCl_2 , KCl, and LiCl were from Mandel Scientific (Guelph, Canada). SsoFast EvaGreen supermixes were from Rio-Rad. Taq polymerase, dNTPs and Taq buffer were from New England Biolabs. Streptavidin-coated agarose resin (1-3 mg biotinylated BAS/mL resin) was from Thermo Scientific (IL, USA).

SELEX

The aptamer selection was carried out based on the previously described procedures with modifications.^[19a, 19b] The procedures are described in the Supporting Information.

Isothermal titration calorimetry (ITC)

ITC was performed using a VP-ITC microcalorimeter instrument (MicroCal). The cell was loaded with a 1.45 mL aptamer sample of 10 μM in buffer. UA or other molecules (280 μL of 200 μM) in the same buffer was titrated into the cell through a syringe (10 μL each injection, except for the first injection of 2 μL). The binding constant (K) was obtained through fitting the titration curves to a one-site binding model using the Origin software. The dissociation constant (K_d) values were calculated from $1/K$.

Aptamer-based biosensor assay

To form the aptamer and quencher complexation, 1 μM sensor was prepared by annealing 1 μM FAM-labeled UA-1 aptamer with 2 μM quencher-cDNA in the selection buffer. In a typical experiment, 100 μL sample with a final of 20 nM sensor was used. The background fluorescence

of the sensor was monitored for 5 min. Then 2 μ L of target solution was added. The fluorescence signal was continuously monitored for another 15 min ($E_x = 495$ nm, $E_m = 525$ nm). The experiments were run in triplicate and error bars indicate the stranding deviations. Simulated tears were prepared to contain 120 mM NaCl, 25 mM NaHCO₃, 20 mM KCl, 4 mM CaCl₂, 0.02 mg/mL albumin and 0.4 mg/mL lysozyme pH 7.4.^[4b]

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Keywords: aptamers; biosensors; SELEX; fluorescence; ITC

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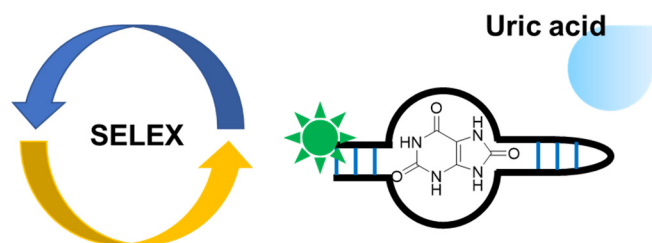


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Two types of DNA aptamers of uric acid (UA) were selected with a K_d around 1.2 μM , one of which shows better discrimination against xanthine. The binding of UA involves three Na^+ ions, which cannot be replaced by Li^+ or K^+ . A fluorescence biosensor was designed with a limit of detection of 90 nM UA in buffer and 350 nM UA in simulated tears.