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# An RNA Aptamer-Based Electrochemical Biosensor for Detection of Theophylline in Serum

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Theophylline (1,3-dimethylxanthine) is one of the most commonly used bronchodilators and respiratory stimulators for the treatment of the symptoms of acute and chronic asthmatic conditions, and it is one of the most frequently clinically monitored drugs in the USA.[1] The plasma levels useful for effective bronchodilation action are within a narrow 20−100 $\mu$M concentration range. It is toxic at higher concentrations and can be lethal or lead to permanent neurological damage.[1] At present, measurements of serum or plasma theophylline are carried out routinely in many clinical laboratories using gas/liquid chromatography methods and commercial immunoassays.[1a,b] Both approaches suffer from interference primarily from structurally closely related caffeine and theobromine resulting in overestimated serum/plasma theophylline concentrations. Increased specificity of theophylline analysis has been achieved with theophylline oxidizing enzyme assays;[2] however, restricted availability and stability of the enzyme impedes their routine clinical application.

Here we report on the application of a readily available RNA aptamer[3] as the biorecognition unit to develop a selective and label-free electrochemical sensor for theophylline. Combination of aptamers with electrochemical detection methods allows a sensitive and versatile way of fast, simple, and cost-effective sensing of the target analyte.[4] There are several reports on successful DNA aptamer-based sensors for proteins.[4,5] In contrast, only very few electrochemical DNA aptamer-based sensors for small molecules such as cocaine,[6] adenosine,[7] and ATP[8] have been developed. In two of the approaches used, conformational changes of the aptamer upon binding of the ligand lead to a change in either the efficiency of electron transfer (eT) to a redox probe conjugated to the immobilized aptamer[6,8] or the interfacial eT resistance.[7]

This report is the first example of an RNA aptamer-based electrochemical sensor. The versatility of the RNA functionality and the ability of RNA molecules to form three-dimensional high-order architectures[9] appear to be particularly useful for the in-vitro selection of high-affinity RNA aptamers against a variety of target molecules. The vast majority of designed aptamer sequences are based on RNA,[3b,c] and they offer important applications in clinical diagnostics and treatment of viral and immune diseases.[10] However, integration of the RNA aptamers within the electrode format has not yet been probed, probably as a result of the higher susceptibility of RNA to degradation than DNA.

We have used a 33 nts RNA aptamer sequence, which has been found to recognize and selectively bind theophylline (Figure 1A).[11a] The conserved 15 nucleotide region required for high affinity theophylline binding consists of two internal loops and an internal stem. Both internal loops interact to form a well-ordered binding pocket. Theophylline binds into a triple base sandwich by intensive stacking and hydrogen bonding interactions.[11a] This RNA aptamer

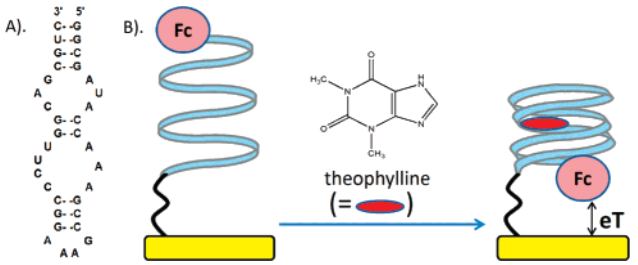


***Figure 1.*** (A) Theophylline-binding RNA aptamer sequence and (B) schematic representation of the electrochemical RNA aptamer-based sensor for theophylline (Fc = ferrocene).

displays a high affinity for theophylline with a $K_d$ of 0.3−0.4 $\mu$M, and it furthermore shows a ~10 000 times lower affinity for caffeine.[11b]

For development of the electrochemical sensor we applied an approach similar to the molecular beacon type electrochemical sensors for DNA pioneered by Heeger et al.[12] (Figure 1B). The RNA aptamer is immobilized on a gold electrode via thiol chemistry, and the other terminal position of the aptamer is labeled with a ferrocene (Fc) redox probe. In the absence of theophylline the aptamer is in an open conformation, and the Fc probe can be in various distant positions relative to the surface. In the presence of theophylline the aptamer folds into the conformationally restricted hairpin structure, and this conformational change results in the increased efficiency of eT (decrease in the average eT distance) between the Fc probe and the electrode surface. This enables calibration of the signal versus theophylline concentration. One of the major challenges in this approach compared to the DNA aptamer based sensors is the fragility of RNA toward ribonuclease digestion and chemical cleavage. The 2′-hydroxyl groups adjacent to the phosphodiester linkages in RNA can act as intramolecular nucleophiles in both base- and enzyme-catalyzed hydrolysis. Hence, the stability of RNA at electrodes under conditions of immobilization and electrochemical cell measurements is critical due to the increased risk of RNase contamination. Therefore simple and fast immobilization and on-electrode labeling methods have been used for the preparation of the sensor, and much care has been taken to avoid any contamination with RNases (Supporting Information).

The 5′-C6-disulfide- and 3′-amino-modified RNA aptamer was prepared by automated RNA synthesis. This sequence was directly immobilized onto a gold electrode. The Fc redox probe was covalently attached to the 3′-amino group of the immobilized RNA by reaction with a Fc-carboxylic acid *N*-hydroxysuccinimide ester (Supporting Information). Finally, the remaining gold surface was passivated by 6-mercaptohexanol.

This sensor was tested in a deaerated HEPES buffer, pH 7, by cyclic voltammetry and differential pulse voltammetry. It is worth mentioning that a principal similarity between the sensor response in aerated and deaerated solutions has been observed, which


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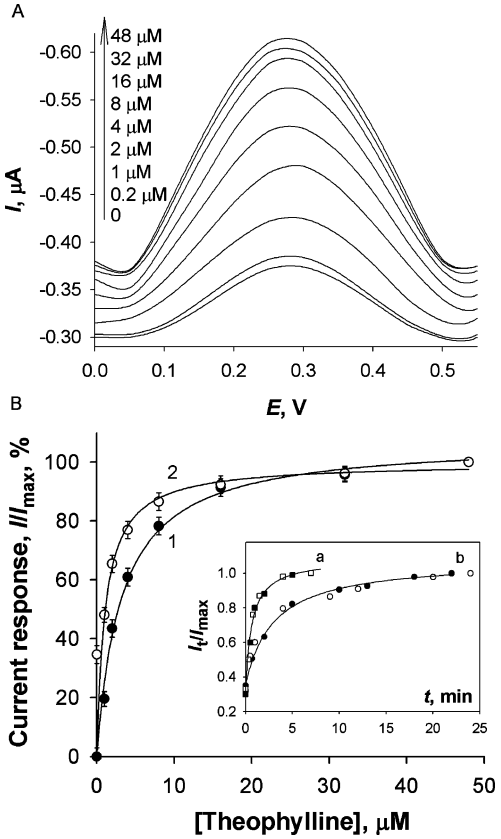


***Figure 2.*** (A) Representative differential pulse voltammograms recorded for the electrochemical RNA aptamer-based sensor after reaction with theophylline (within 0 to 48 $\mu$M concentration range) and (B) sensor response, normalized for the maximum current signal $I_{max}$ observed at saturating concentrations of theophylline, calibrated versus theophylline concentration (averaged data for 5 independent experiments), (1) corrected for 0 $\mu$M theophylline curve as a background and (2) corrected for individual backgrounds for each differential pulse voltammetry curve. Pulse amplitude 50 mV, sampling time 50 ms, effective scan rate 10 mV $s^{-1}$. Inset: Time dependence of the response of the (a) unlocked and (b) DNA-locked RNA aptamer-based sensor normalized versus maximal achieved current response $I_{max}$ during reaction with 48 $\mu$M theophylline, (■, ●) cathodic and (□, ○) anodic signals.

facilitates the routine sensor applications in aerated conditions. In the absence of theophylline a low electrochemical response at 286 mV stemming from the Fc redox couple was observed (Figure 2A, 0 $\mu$M). In analogy to the electrochemical DNA hairpin sensors[5c,8] we assume the low response is due to an unfolded conformation of the RNA sequence at the electrode surface. In another approach the open RNA conformation was locked with a complementary 10-mer DNA sequence as well, which resulted in the background signals similar to those from the RNA aptamer alone. The Fc peak currents were proportional to the potential scan rate, consistent with an immobilized redox probe, and the heterogeneous eT rate constant $k_s$[13] was found to be $0.08 \pm 0.01$ $s^{-1}$, for on average $(0.9 \pm 0.3) \times 10^{-11}$ mol $cm^{-2}$ (actual area) of Fc probes involved in this redox process.

In the presence of theophylline the aptamer adopts a locked hairpin conformation ($T_m = 72$ °C[14]), in which the Fc redox probe on average is located closer to the electrode surface, thus providing an increased electrochemical signal from the $Fc/Fc^+$ couple (Figure 2A). The voltammetric response was recorded versus concentrations of theophylline in the range 0.1 to 48 $\mu$M. The detection limit was 0.2 $\mu$M, which is consistent with previously reported sensitivity levels.[3b,11a,14] The dynamic range was within 0.2 to 10 $\mu$M (Figure 2B), which is improved compared to the 0.3−2 $\mu$M dynamic range

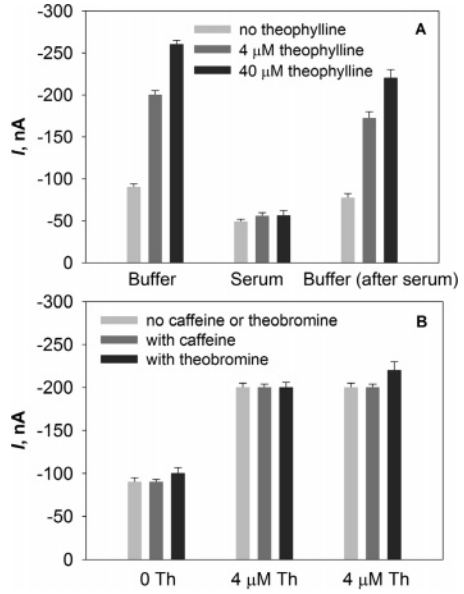


***Figure 3.*** Representative background-corrected differential pulse voltammetry responses of the RNA aptamer-based sensor to (A) 0, 4, and 40 $\mu$M theophylline in aqueous buffer, 20-times diluted serum, and blank buffer solution after exhibition to serum theophylline. In panel B sensor responses to 1 mM caffeine and 0.01 mM (1−2 bar groups) and 0.1 mM (third bar group) theobromine in the absence and in the presence of 4 $\mu$M theophylline is shown.

of fluorescent assays.[14] The RNA sensor responded quickly and specifically to theophylline additions (Figure 2B, Inset). For theophylline additions below 8 $\mu$M, 99% of the final signal was reached within 2 min and within 5 min for higher concentrations of theophylline. For the locked RNA aptamer sensor the response time at concentrations of theophylline higher than 1 $\mu$M increased from 5 up to 15 min (Figure 2B, Inset) as a result of competitive binding of theophylline to a RNA−DNA complex accompanied by the release of locking DNA, since the RNA aptamer has higher affinity for theophylline than for complementary 10 nts DNA sequence ($T_m$ of 71 °C versus 45 °C).

When the sensor is saturated with theophylline at 48 $\mu$M, we propose that all Fc probes are located close to the electrode by the ligand-induced structural rearrangement (Figures 1 and 2). The $k_s$ in this case increased to $1.6 \pm 0.3$ $s^{-1}$, reflecting a 20-fold increase in the eT efficiency characteristic for a folded beacon structure. Along with this, the number of Fc groups involved in the eT reaction increased approximately 5-fold, comprising $(4.5 \pm 0.7) \times 10^{-11}$ mol $cm^{-2}$ (actual area). This surface coverage of Fc-modified RNA sequences is consistent with previously reported densities of thiol immobilized single-stranded DNA sequences on gold.[5c,15] However, the eT rate constant is an order of magnitude lower than those reported for Fc-terminated DNA beacon systems.[5c,8,12a,15a,e] The slow eT rates can primarily be attributed to the slower kinetics of charge transfer through the passivating 6-mercaptohexanol layer at the electrode surface compared to the 2-mercaptoethanol layers (or no blocking at all) used in other works.[5c,8,12a,15a]

Next we assessed the ability of the RNA aptamer-based sensor to detect theophylline in serum, in the presence of interfering methylxanthines. The sensor responded readily to theophylline in serum, 20 times diluted by the HEPES buffer as an electrolyte and treated with a ProtectRNA RNase inhibitor. However, the signal was essentially inhibited when measurements were performed directly in diluted serum, with no signal increase at concentrations of theophylline higher than 4 $\mu$M (Figure 3A). Once transferred to the blank buffer solution, the sensor previously exposed to serum

theophylline displayed a concentration calibrated response consistent with that observed after sensor reaction with theophylline in the buffer alone. No interference from 1 mM caffeine and 0.01 mM theobromine was detected in theophylline samples (Figure 3B). The 1 mM concentration of caffeine is well above analytically important caffeine plasma levels (25 to 100 $\mu$M),[16] and thus the RNA aptamer-based sensor enabled highly selective discrimination between theophylline and caffeine. In the competitive-binding mode, no cross-interference from up to 0.01 mM theobromine was detected over the whole studied theophylline concentration range, while, in the absence of theophylline, sensor response to 4−10 $\mu$M theobromine was less than 9% from that to theophylline. That is an expected result since theobromine has higher affinity for the RNA aptamer than caffeine has (albeit 1500 times lower than theophyline[14]). It is worth stating that at a 50-fold excess of theobromine over theophylline the cross-reaction from 0.1 mM theobromine was responsible for the minor 10% increase of the sensor response (Figure 3B).

The nonligated sensor was regenerated by thorough washing and overnight storage in water or in 6 M guanidinium hydrochloride,[5e] with a 70−50% recovery of the original background signal from Fc (Figure S3, Supporting Information).

In conclusion, this label-free theophylline sensor is the first example of an electrochemical RNA aptamer-based sensor. The sensor provides sensitive and selective detection of theophylline in aqueous buffer solutions and in serum in the presence of structurally related caffeine and theobromine. The RNA aptamer-based sensor readily responded to theophylline within a few minutes. When special care is taken to provide RNase-free storage and experimental conditions, the RNA aptamer functionalized electrodes demonstrate no decrease in theophylline activity after 2 weeks of storage at 4 °C. Compatibility and stability of the RNA aptamer within the electrode format extend the existing designs of the electrochemical DNA aptamer based switches[5c,e,6,8] to the RNA aptamer electrode modifications. Electrochemical detection of theophylline by the RNA aptamer-based sensor has some advantages compared to the previously reported approaches, which do not allow direct selective detection of serum theophylline in the presence of structurally related methylxanthines.[1b] Furthermore, the electrochemical RNA aptamer-based sensor is more sensitive than fluorescence sensors, where the sensitivity is limited by significant background fluorescence in the absence of theophylline.[14] The assay time is also improved compared to an average 3 h required in chromatographic assays (including cumbersome sample pretreatment as well).[1b] The availability of the synthetic RNA aptamer for theophylline and the ease of its electrode function make the RNA aptamer-based sensor an attractive alternative to known enzyme assays, which exhibit sensitivity and selectivity levels comparable with those of the designed RNA aptamer based sensor.[2]

In perspective, RNA aptamers with high specificity and affinity can be selected for any given analyte, also for such methylxanthines as caffeine and theobromine. In this case, an RNA aptamer-based multiarray electrode format will make possible simultaneous fast and selective analysis of single clinically important methylxanthines in complex biological samples.

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**Supporting Information Available:** Materials and methods; organic synthesis details and schemes for chemical reactions involved; additional data on voltammetry and sensor regeneration. This material is available free of charge via the Internet at http://pubs.acs.org.

## References

(1) (a) Kawai, M.; Kato, M. *Methods Find. Exp. Clin. Pharmacol.* **2000**, *22*, 309−320. (b) Rowe, D. J.; Watson, I. D.; Williams, J.; Berry, D. J. *Ann. Clin. Biochem.* **1988**, *25* (Pt 1), 4−26. (c) Howard, C. E.; Capers, C. C.; Bess, D. T.; Anderson, R. J. *Am. J. Hosp. Pharm.* **1994**, *51*, 1672. (d) Herrmann, G.; Aynesworth, M. B.; Martin, J. *J. Lab. Clin. Med.* 1937, *23*, 135−148.

(2) (a) Ferapontova, E. E.; Shipovskov, S. V.; Gorton, L. *Biosens. Bioelectron.* **2007**, *22*, 2508−2515. (b) Gupta, S.; Agarwal, A.; deCastro, A. *Clinic. Chem.* **1988**, *34*, 1267. (c) McNeil, C. J.; Cooper, J. M.; Spoors, J. A. *Biosens. Bioelectron.* **1992**, *7*, 375−380. (d) Wang, J.; Dempsey, E.; Ozsoz, M.; Smyth, M. R. *Analyst* **1991**, *116*, 997−999.

(3) (a) Ellington, A. D.; Szostak, J. W. *Nat. Struct. Biol.* 1990, *346*, 818−822. (b) Hermann, T.; Patel, D. J. *Science* **2000**, *287*, 820−825. (c) Patel, D. J.; Suri, A. K. *Rev. Mol. Biotech*nol. **2000**, *74*, 39−60. (d) Tuerk, C.; Gold, L. *Science* **1990**, *249*, 505−510.

(4) Willner, I.; Zayats, M. *Angew. Chem., Int. Ed.* **2007**, *46*, 6408−6418.

(5) (a) Hansen, J. A.; Wang, J.; Kawde, A.-N.; Xiang, Y.; Gothelf, K. V.; Collins, G. *J. Am. Chem. Soc.* **2006**, *128*, 2228. (b) Polsky, R.; Gill, R.; Willner, I. *Anal. Chem.* **2006**, *78*, 2268. (c) Radi, A.-E.; Sánchez, J. L. A.; Baldrich, E.; O'Sullivan, C. K. *J. Am. Chem. Soc.* **2006**, *128*, 117−124. (d) Rodriguez, M. C.; Kawde, A.-N.; Wang, J. *Chem. Commun.* **2005**, 4267−4269. (e) Xiao, Y.; Lubin, A. A.; Heeger, A. J.; Plaxo, K. W. *Angew. Chem., Int. Ed.* **2005**, *44*, 5456−5459. (f) Xiao, Y.; Piorek, B. D.; Plaxco, K. W.; Heeger, A. J. *J. Am. Chem. Soc.* **2005**, *127*, 17990.

(6) Baker, B. R.; Lai, R. Y.; Wood, M. S.; Doctor, E. H.; Heeger, A. J.; Plaxco, K. W. *J. Am. Chem. Soc.* **2006**, *128*, 3138−3139.

(7) Zayats, M.; Huang, Y.; Gill, R.; Ma, C.; Willner, I. *J. Am. Chem. Soc.* **2006**, *128*, 13666−13667.

(8) Zuo, X.; Song, S.; Zhang, J.; Pan, D.; Wang, L.; Fan, C. *J. Am. Chem. Soc.* **2007**, *129*, 1042−1043.

(9) (a) Gilbert W. The RNA world. *Nature* **1986**, *319*, 618. Gestland, R. F.; Cech, T.; Atkins, J. F. *The RNA world*; 3rd ed.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 2005.

(10) Rimmele, M. *ChemBioChem* **2003**, *4*, 963−971.

(11) (a) Zimmermann, G. R.; Jenison, R. D.; Wick, C. L.; Simorre, J.-P.; Pardi, A. *Nat. Struct. Biol.* **1997**, *6*, 644−649. (b) Jenison, R. D.; Gill, S. C.; Pardi, A.; Polisky, B. *Science* **1994**, *263*, 1425−1429.

(12) (a) Fan, C.; Plaxco, K. W.; Heeger, A. J. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 9134−9137. (b) Lai, R. Y.; Lagally, E. T.; Lee, S.-H.; Soh, H. T.; Plaxco, K. W.; Heeger, A. J. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 4017−4021.

(13) Laviron, E. *J. Electroanal. Chem.* **1979**, *101*, 19−28.

(14) Rankin, C. J.; Fuller, E. N.; Hamor, K. H.; Gabarra, S. A.; Shields, T. P. *Nucleosides, Nucleotides Nucleic Acids* **2006**, *25*, 1407−1424.

(15) (a) Chahma, M.; Lee, J. S.; Kraatz, H. B. *J. Electroanal. Chem.* **2004**, *567*, 283−287. (b) Peterlinz, K. A.; Georgiadis, R. M. *J. Am. Chem. Soc.* **1997**, *119*, 3401−3402. (c) Petrovykh, D. Y.; Kimura-Suda, H.; Whitman, L. J.; Tarlov, M. J. *J. Am. Chem. Soc.* **2003**, *125*, 5219−5226. (d) Steel, A. B.; Levicky, R. L.; Herne, T. M.; Tarlov, M. J. *Biophys. J.* **2000**, *79*, 975−981. (e) Immoos, C. E.; Lee, S. J.; Grinstaff, M. W. *J. Am. Chem. Soc.* **2004**, *126*, 10814−10815.

(16) Turnbull, J. D.; Moshrig, R.; Gere J. A.; Kochalka, J. *Clin. Chem.* **1984**, *30* 1721.

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