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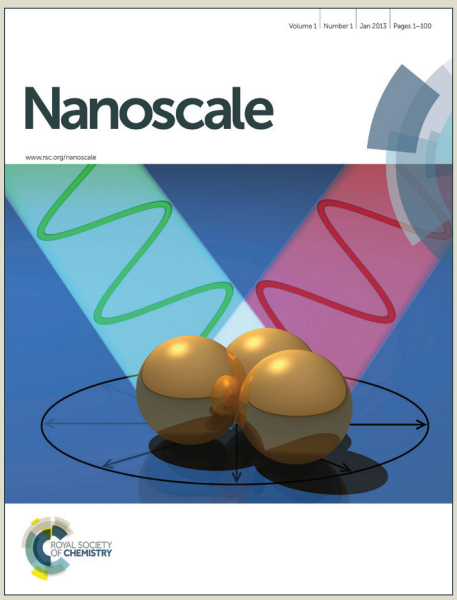

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Target-controlled formation of silver nanoclusters in abasic site-incorporated duplex DNA for label-free fluorescent detection of theophylline

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A novel, label-free, fluorescence based sensor for theophylline has been developed. In the new sensor system, an abasic site-incorporated duplex DNA probe serves as both a pocket for recognition of theophylline and a template for preparation of fluorescent silver nanoclusters. The strategy relies on theophylline-controlled formation of fluorescent silver nanoclusters from abasic site-incorporated duplex DNA. When theophylline is not present, silver ions interact with the cytosine groups opposite to the abasic site in duplex DNA. This interaction leads to efficient formation of intensely red fluorescent silver nanoclusters. In contrast, when theophylline is bound at the abasic site through pseudo base pairing with appropriately positioned cytosines, silver ion binding to cytosine nucleobase is prevented. Consequently, fluorescent silver nanoclusters are not formed causing a significant reduction of the fluorescence signal. By employing this new sensor, theophylline can be highly selectively detected at a concentration as low as 1.8 μM. Finally, the diagnostic capability and practical application of this sensor was demonstrated by its use in detecting theophylline in human blood serum.

Introduction

Theophylline is a potent bronchodilator and respiratory stimulator used for the treatment of asthma and chronic obstructive pulmonary disease.[1] However, this substance promotes permanent neurological damage at concentrations higher than the acceptable therapeutic range.[2] Therefore, the level of theophylline needs to be constantly monitored in order to prevent its toxic side effects from occurring. At present, measurements of serum theophylline levels are typically carried out in the clinical laboratory using gas and liquid chromatography, ultraviolet spectrometry, and immunoassays.[3] However, these methods possess several limitations, including the need for complex, multi-step procedures that require technical expertise to perform, as well as long analysis times. In addition, these assay procedures have a relatively low selectivity for theophylline over structurally similar substances such as caffeine and theobromine, which could lead to undesirable false positive signals.

In alternative approaches aimed at overcoming these limitations, an RNA aptamer with high binding affinity and specificity for theophylline[4] has been employed as the affinity reagent for many types of biosensor platforms with various signaling strategies including colorimetric,[5] electrochemical,[2, 6] and fluorescent read-outs.[7] However, the RNA aptamer is susceptible to both degradation by ribonucleases and chemical cleavage owing to the presence of 2'-hydroxyl groups in the ribose moiety that serve as intramolecular nucleophiles in both base and enzyme catalyzed phosphate ester cleavage reactions.[2] In addition, the difficult and expensive synthesis of RNA hinders the widespread use of the RNA aptamer approach for assaying theophylline.[8]

Recently, a new class of duplex DNA, containing an abasic (AP) site that serves as a binding cavity for target molecules, has been described.[9] This new binding motif enables strong and selective binding of target molecules and it circumvents problems associated with the instability of RNA molecules. For example, Teramae *et al.* showed that duplex DNA, containing cytosines positioned opposite the AP site, selectively binds theophylline with a dissociation constant (10 μM) that is notably lower than that for either caffeine or theobromine.[9b, 10] Based on this unique feature, they designed a novel duplex DNA probe which contains a fluorescent analog of adenine, 2-aminopurine, at a position that flanks the AP site.[10] This sensor generates a fluorescence response when the micro-environment of the 2-aminopurine fluorophore is changed by theophylline binding to the AP site.

Although interesting, this method requires modification of DNA probe in order to introduce the 2-aminopurine group, an issue that complicates the probe design and leads to higher costs associated with the analysis.[11] An alternative strategy, devised to remove this limitation, employs a ligand (riboflavin) whose fluorescence is quenched upon binding to the DNA AP site and recovered when theophylline competitively binds to this same site.[8] Although this theophylline assay procedure is both sensitive and selective, the fluorescent organic ligand used for signaling was found to have several inherent deficiencies, including most notably a lack of photostability and low emission intensity.[12] As such, there is a continuing need for measuring theophylline in a cost effective manner that relies on a highly stable and efficient signaling mechanism.

Toward this goal, we describe a new, label-free, fluorescence sensor for theophylline, which is based on target-controlled formation of fluorescent silver nanoclusters (AgNCs) from AP site-incorporated duplex DNA. The AgNCs employed in this system, comprised of a few silver atoms, offer a compelling alternative to

conventional organic fluorophores because of their outstanding spectroscopic and photophysical properties, low toxicity, and biocompatibility.[13] The general strategy relies on the formation of AgNCs taking place as a consequence of interactions between silver ions and cytosine moieties[14] that are paired with an AP site in duplex DNA and the fact that this interaction is interfered with by competitive binding of theophylline at the same site. The diagnostic capability of this strategy was successfully verified by reliably detecting theophylline present in human blood serum.

Experimental

Materials

All DNA strands used in the present study were synthesized from Integrated DNA Technologies Incorporated (Coralville, IA, USA).[15] The sequences of DNA oligonucleotides are listed in Table S1. Silver nitrate, sodium borohydride, theophylline, caffeine, theobromine, creatinine, D-glucose, human serum, sodium hydrogen phosphate, sodium dihydrogen phosphate, and sodium acetate were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of anlytical grade and used without further purification.[16] Aqueous solutions were prepared using ultrapure DNase/RNase-free distilled water purchased from Invitrogen (Carlsbad, CA, USA). The phosphate buffer (PB, 20 mM, pH 7) containing 100 mM sodium acetate was used as the binding buffer throughout this study.

Theophylline detection procedure

Solutions containing 6 μM abasic site-incorportated ssDNA and 6 μM complementary ssDNA in binding buffer were heated at 90 ℃ for 5 min, cooled slowly to 5 ℃ (0.1 ℃/s) and incubated at 5 ℃ for 20 min to form a duplex DNA. Different concentrations of theophylline and 100 μM of theobromine, caffeine, D-glucose, and creatinine were added independently to this solution, followed by incubation at 5 ℃ for 15 min. $AgNO_3$ [Ag^+ to DNA molar ratio of 6.67:1] was then added to each solution that was incubated 5 ℃ for 15 min. Finally, freshly prepared $NaBH_4$ [$NaBH_4$ to Ag^+ molar ratio of 3:1] was added to the individual solutions followed by incubation at 5 ℃ for 1 h.

Instrumentation

Fluorescence intensities were measured using a Tecan Infinite M200 pro microplate reader (Mnnedorf, Switzerland) and black, 384-well Greiner Bio-One microplates (ref: 781077, Courtaboeuf, France) at an excitation wavelength of 580 nm.[17] Field emission transmission electron microscopy (Tecnai, FEI, Netherlands), operated at an accelerated voltage of 300 kV, was employed to characterize the DNA-AgNCs. Samples were prepared by applying the synthesized DNA-AgNCs to a carbon-coated copper TEM grid followed by drying at room temperature.

Preparation and analysis of human serum samples

A solution of human serum diluted 10 times with the above mentioned binding buffer was loaded into centrifugal filter device (MWCO = 10,000 Da, Millipore). The sample was then subjected to centrifugation at 11000 g-force for 20 min.[18] Finally, theophylline was spiked into the filtered human serum at different concentrations. Each sample was analyzed by using the above-mentioned theophylline detection procedure.

Results and discussion

The key component of our theophylline sensor is an AP site paired with cytosine in the middle of the 13-mer duplex DNA (Fig. 1). Cytosine paired with the AP site in duplex DNA causes selective binding of the target theophylline through pseudo base pairing, which is stabilized by nucleobases flanking the AP site.[8,10] Importantly, the cytosine groups simultaneously serve as signaling components by inducing formation of AgNCs with intensely red fluorescence when theophylline is not present in the AP site (Fig. 1).[14f] Specific binding of theophylline, however, inhibits binding of silver ions to the cytosine nucleobases, a phenomenon that prevents formation of fluorescent AgNCs. Consequently, samples containing theophylline cause a significant reduction in the fluorescence signal arising from the AgNCs, a change that can be readily detected with the naked eye under a hand-held UV lamp.

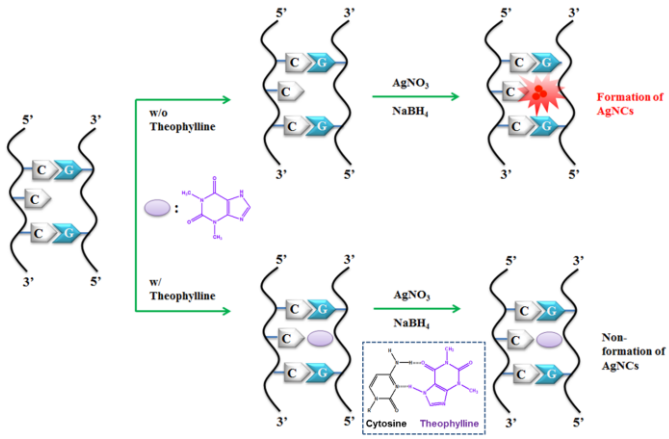

Fig. 1 Schematic illustration of theophylline detection system based on target-controlled formation of fluorescent AgNCs.

To explore the feasibility of this strategy, we first examined the effect of theophylline on the formation of fluorescent AgNCs. We found that, in the absence of theophylline, a highly intense red fluorescence signal with a maximum at 640 nm (λ_{ex} = 580 nm) is generated as a result of the formation of AgNCs by interaction of silver ions with cytosines near the AP site in duplex DNA (3, Fig. 2 (a) and (b)). The fluorescence signal of AgNCs is linearly dependent on the concentration of duplex DNA (Fig. S1, ESI†). However, the fluorescence signal is significantly reduced when theophylline is present (3, Fig. 2 (a) and (b)). Given this, we tested whether AgNCs are formed by two single stranded (ss) DNAs of duplex DNA serving as synthetic templates. The results show that the AP site-incorporated ssDNA does not promote formation of a significant amount of AgNCs (1, Fig. 2(a)). On the other hand, ssDNA containing cytosine receptor nucleobase induces production of AgNCs due to the presence of consecutively placed cytsoine within the strand as reflected in the generation of an observable fluorescence signal (2, Fig. 2 (a)).[13g,14b] However, the fluorescence signal generated from the cytosine receptor-containing ssDNA is much lower than that arising from the duplex DNA and, most importantly, no fluorescence reduction occurs in the presence of theophylline (2, Fig. 2 (a)). Overall, these observations demonstrate that AP site-incorporated duplex DNA promotes theophylline-governed formation of highly fluorescent AgNCs.

The optimal conditions required for analysis of theophylline using our method based on target-controlled formation of fluorescent AgNCs were evaluated. The results of experiments, in which the time used for silver ion reduction (Fig. S2, ESI†), the reducing agent ($NaBH_4$) to silver ion ratio (Fig. S3, ESI†), the length of the AP site-incorporated duplex DNA (Fig. S4, ESI†), and the pH value (Fig. S5, ESI†) were varied, demonstrate that a 1 h reduction time, 3:1 ratio of

$NaBH_4$ to silver ions, 13-mer duplex DNA, and pH 7 are ideal for the efficient analysis of theophylline (See ESI†). Besides, the synthesized AgNCs were characterized by transmission electron microscopy (TEM) analysis and their stability was also examined as presented in the ESI† (Fig. S6 and S7).

Observations made in further studies showed that theophylline effectively inhibits the formation of AgNCs, leading to a fluorescence intensity reduction, only when it is present before formation of AgNCs takes place (Fig. S8, ESI†). In contrast, when theophylline is applied after the formation of AgNCs, no reduction in the fluorescence signal occurs. This finding indicates that theophylline does not reduce the fluorescence intensity arising from formed AgNCs, but rather blocks the binding site of Ag^+ ions and thus prevents formation of AgNCs. The UV-vis absorption spectra in the absence and presence of theophylline were also measured, which are given in the ESI† (Fig. S9).

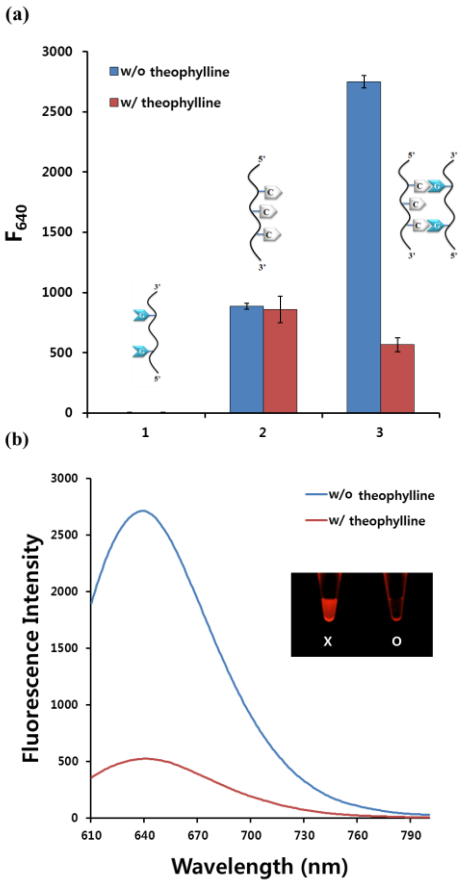

Fig. 2 Theophylline-controlled formation of fluorescent AgNCs from 13-mer duplex DNA having AP site paired with a cytosine receptor nucleobase. (a) Fluorescence intensities of AgNCs generated from 13-mer ssDNA or duplex DNA in the absence and presence of theophylline (100 μM) (1: ssDNA having AP site, 2: ssDNA having a cytosine receptor nucleobase, 3: Duplex DNA having AP site paired with a cytosine receptor nucleobase). (b) Fluorescence spectra of AgNCs generated from 13-mer duplex DNA having AP site paired with a cytosine receptor nucleobase in the absence and presence of theophylline (100 μM). Inset: Photograph images under UV light (354 nm).

An investigation was carried out to explore the effects of other nucleobases paired with the AP site in the duplex DNA on the formation of AgNCs. For this purpose, we examined fluorescence intensities resulting from AgNCs formed in four different duplexes, having identical sequences but different nucleobases, including cytosine, adenine, thymine, and guanine, paired with the AP site. The results show that only duplex DNA containing cytosine opposite to the AP site efficiently induces formation of highly fluorescent AgNCs (Fig. 3).[14f] Importantly, the other three duplex DNAs do not produce significant amounts of AgNCs. Accordingly, the theophylline-promoted reduction of the fluorescence signal is observed only in the system possessing cytosine as the receptor nucleobase (Fig. 3).

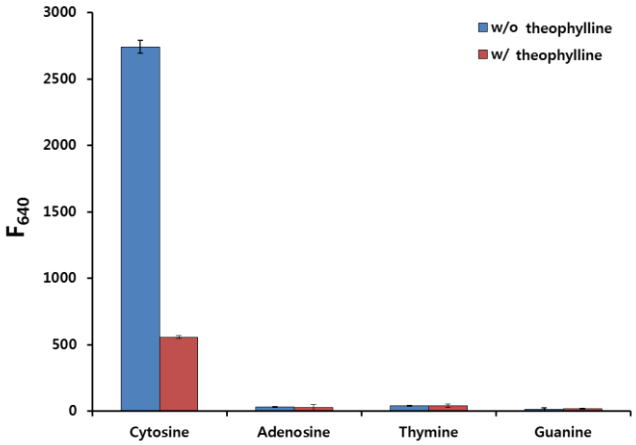

Fig. 3 Fluorescence intensities of AgNCs generated from 13-mer duplex DNA having AP site paired with four different nucleobases (cytosine, adenosine, thymine, and guanine) in the absence and presence of theophylline (100 μM).

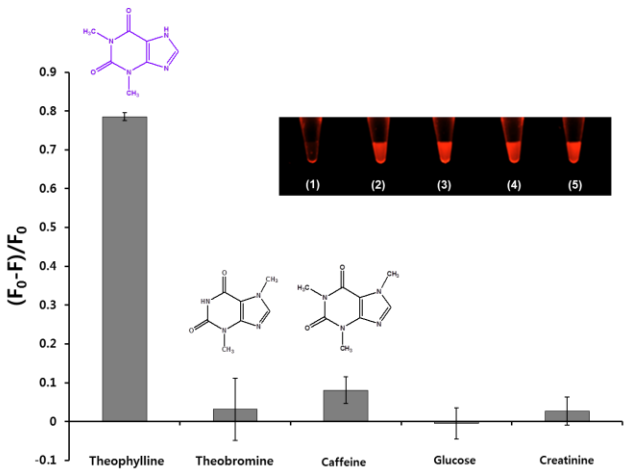

Fig. 4 Selectivity of the AgNCs based detection system. The concentrations of theophylline (1), theobromine (2), caffeine (3), D-glucose (4), and creatinine (5) are 100 μM. The degree of signal reduction was defined as $(F_0-F)/F_0$, where F_0 and F are the fluorescence intensities at 640 nm in the absence and presence of the corresponding molecule, respectively. Inset: The photograph images under UV light (354 nm)

In order to assess the specificity of our detection strategy, the abilities of structurally related methylxanthine derivatives and other molecules present in serum to inhibit the formation of fluorescent AgNCs were determined and compared with that of theophylline. As the data in the bar graphs displayed in Fig. 4 show, effective inhibition of AgNCs formation takes place only when theophylline is present in the sample. This observation is reflected in the significant reduction of the fluorescence signal to *ca.* 20 % of the value in the absence of theophylline. On the other hand, the presence of the methylxanthine derivatives, caffeine and theobromine, which

structurally differ from theophylline only by a single methyl group attached to the xanthine ring system, does not cause a significant reduction of the fluorescence signal. Also negligible reduction of the fluorescence signal is observed when creatinine and D-glucose, substances normally present in serum, are contained in the sample. These results clearly demonstrate that the new sensing method is highly selective for theophylline.

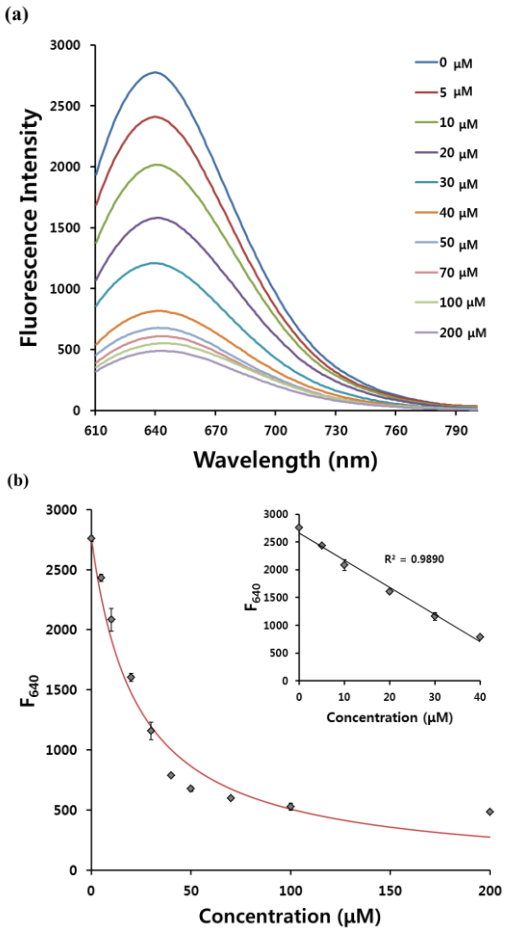

Fig. 5 (a) Fluorescence spectra of AgNCs generated from 13-mer duplex DNA having AP site paired with a cytosine receptor nucleobase in the presence of theophylline at different concentrations. (b) Theophylline concentration dependent change of fluorescence intensity (λ_{max} = 640 nm). Inset: Linear range between F_{640} and the theophylline concentration (0 - 40 μM).

The detection sensitivity of the AgNCs-based sensing system was determined by measuring fluorescence intensities at 640 nm as a function of the concentrations of theophylline in samples (Fig. 5). The results show that fluorescence intensities decrease with increasing concentrations of theophylline up to 40 μM, but plateau at concentrations greater than 40 μM. An excellent linear relationship (R^2 = 0.9890) exists in the range of 0-40 μM and the limit of detection (LOD) (3σ/slope) is *ca.* 1.8 μM, a value that is comparable to those reported for previous methods developed to detect theophylline.[2,8,19] Besides, the half maximal inhibitory concentration (IC_{50}) of theophylline for the 13-mer duplex DNA-based AgNC synthesis was 23.2 ± 4.7 μM, which was obtained by fitting a curve to the fluorescence signals using the equation $Y = B + (M - B) \times IC_{50}/(IC_{50} + X)$, where Y is the fluorescence intensity, X is the theophylline concentration, and B and M are the baseline and maximum fluorescence signals, respectively (Fig. 5 (b)).[20]

Table 1 Determination of theophylline in diluted human serum.

Added theophylline (μM)	Measured theophylline (μM)	SD[a]	CV[b] (%)	Recovery[c] (%)
5	5.11	0.28	5.50	102.2
15	15.10	0.41	2.72	100.7
25	24.55	0.57	2.32	98.2
35	33.33	1.36	4.07	95.2

[a] Standard deviation, [b] Coefficient of variation, [c] Measured value/added value.

Finally, to demonstrate its practical nature, the new sensor system was employed to detect theophylline in human serum, which contains many other biological substances.[21] As the data shown in Fig. S10 demonstrate, the pattern of fluorescence intensity reductions in response to theophylline is almost same as that seen in the analysis of artificial samples containing only theophylline. Specifically, the fluorescence intensities at 640 nm decrease with increasing spiked concentrations of theophylline in diluted human serum (1 %) and a linear correlation exists in the range from 0-40 μM (Fig. S10, ESI†). Owing to its excellent reproducibility and precision, as evidenced by a coefficient of variation (CV) less than 6 % and a recovery ratio between 95 and 103 %, the new system can be employed to determine the amounts of theophylline in serum samples in a highly accurate manner (Table 1). Consequently, the results demonstrate that the method has great potential for real clinical and diagnostic applications (see ESI†).

Conclusions

In the study described above, a novel, label-free, fluorescent sensor for the sensitive and selective detection of theophylline, was developed. Our sensor utilizes AP site-incorporated duplex DNA as a key component that serves as both a synthetic template for formation of fluorescent AgNCs and a recognition element for binding theophylline. The strategy relies on theophylline-controlled formation of fluorescent AgNCs in the AP site-incorporated duplex DNA. This new sensing method exhibits high selectivity and sensitivity, and shows promise for clinical applications, as it is capable of operating in diluted serum samples. Importantly, the assay procedure can be employed in a convenient manner because it does not require any complicated modification and post treatment procedures. Furthermore, the signals arising in the assay can be seen even with the naked eye under a hand-held UV lamp. Finally, to the best of our knowledge, this is the first example demonstrating that the formation of fluorescent AgNCs can be regulated by a theophylline drug and that this phenomenon can be used to design a new type of sensing system. As a result, the observations made in this study could serve as the basis of new strategies for the design of fluorescence based sensing methods that broaden the applications of AgNCs.

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† Electronic Supplementary Information (ESI) available. See DOI: 10.1039/b000000x/

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