CHEMISTRY
A EUROPEAN JOURNAL

DOI: 10.1002/chem.201001856

A Sensitive, Label-Free, Aptamer-Based Biosensor Using a Gold Nanoparticle-Initiated Chemiluminescence System

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Abstract: We report a label-free, aptamer-based chemiluminescent biosensor. The biosensor relies upon the catalytic activity of unmodified gold nanoparticles (AuNPs) on the luminol–H_2O_2 chemiluminescence (CL) reaction, and the interaction of unmodified AuNPs with the aptamer. The unmodified AuNPs can effectively differentiate unstructured and folded aptamer. The binding of the aptamer with the target can induce the AuNP aggregation in the presence of 0.5 M NaCl, and after aggregation the catalytic activity of the AuNPs on the luminol–H_2O_2 CL reaction is greatly enhanced. During the assay, no covalent functionalization of the AuNPs or aptamer is required. The detection limit of thrombin was estimated to be as low as 26 fM, and the sensitivity was more than 4 orders of magnitude better than that of known AuNP-based colorimetric methods for the detection of thrombin. This aptamer-based biosensor offers the advantages of being simple, cheap, rapid, and sensitive

Keywords: aptamers · biosensors · chemiluminescence · gold · nanoparticles

Introduction

As a complement to antibody-based biosensors, molecular-probe studies based on nucleic-acid platforms are emerging.[1] One of the representative examples is the use of aptamers for protein analysis. Aptamers are nucleic acid-based reagents that bind to target molecules with high affinity and specificity.[2] The high affinity and specificity of aptamers toward targets is generally achieved by a combination of molecular shape complementarity, hydrogen-bonding interactions, and stacking interactions.[3] Target binding can induce significant conformational changes in aptamers, and, based on these conformational changes, targets can be detected. Aptamers are able to recognize a broad range of targets such as metal ions, small molecules, macromolecules (such as proteins), and even viruses and cells. Aptamers that can be elicited by the systematic evolution of ligands by the exponential enrichment (SELEX) procedure, have many advantages over antibodies, such as simpler synthesis, easier storage, better reproducibility, lower cost, and wider applicability. In the last decade, aptamers have been widely reported as highly promising recognition elements for developing optical and electrochemical biosensors.[1,4–7]

These reported aptamer-based biosensors have shown potential in many research fields and practical applications, such as in basic biological studies, high throughput drug screening, disease diagnosis, nanotechnology, and materials science. However, most of the reported electrochemical aptamer-based biosensors have employed labeled aptamers with redox-active units,[8–10] redox proteins,[11,12] or eletrocatalytic nanoparticles.[13] Most of the developed optical aptamer-based biosensors involved the use of fluorophores[14–16], quantum dots[17,18], or metallic nanoparticles[19] to tag the aptamers or capture probes. Also, catalytic nucleic acids (DNAzymes) have been used as catalytic labels to amplify aptamer-based biosensing events.[20] Such a labeling process makes the determination relatively more complex and expensive. Moreover, the target-binding sites and conformational changes of the aptamers after binding were not fully identified, and the labeled sites were difficult to observe. More importantly, labeling or modifying can weaken the binding affinity between the target and their aptamers, so as to decrease the sensitivity.[21,22] It is clear that a label-free strategy for an aptamer-based biosensor would make the above steps obsolete, making the process simple, rapid, and

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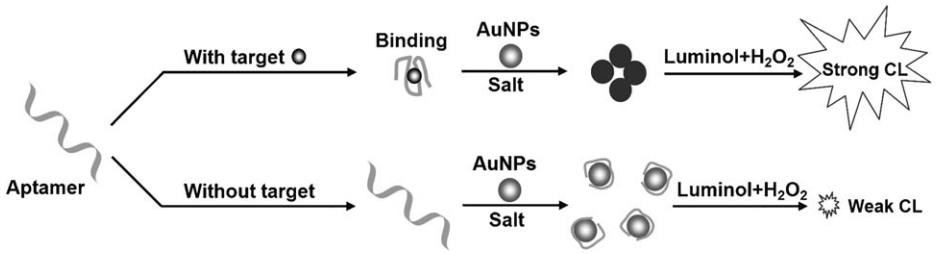
Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/chem.201001856.

low cost. Label-free, aptamer-based biosensors that use photoactive polymers, DNA-intercalating dyes, or metallic nanoparticles to report the conformational change of aptamers upon recognition of given targets have been recently fabricated.[23–26]

Chemiluminescence (CL) is known as a powerful and important analytical technique, because of its extremely high sensitivity along with its other advantages, such as simple instrumentation, wide calibration ranges, and suitability for miniaturization in analytical chemistry.[27–29] Yet, only a few aptamer-based sensing platforms have been proposed that use CL detection in recent years. Willner's group used the peroxidase-mimicking DNAzyme as an amplifying label for an aptamer and combined it with the luminol–H_2O_2 CL system to detect low-molecular-weight substrates and proteins.[30] Similarly, Dong et al. developed a CL aptamer-based sensor for thrombin by using a peroxidase-mimicking DNAzyme as the catalytic label.[31] The strategy is not a general assay and is only applicable to specific targets that have two or more sites for aptamer binding. In 2009, Lu et al. proposed an aptamer-based sensor for the CL detection of adenosine.[32] Though they did not need to label the aptamer for this strategy, the capture DNA was modified with an amino group, so as to immobilize the capture DNA on the surface of carboxyl-modified magnetic beads. In addition, 3,4,5-trimethoxyl-phenylglyoxal (TMPG) was used as the signaling molecule, a special CL regent, which reacts specifically and instantaneously with guanine (G) nucleobases to produce CL emission.[33] Therefore, considering a good response signal, the aptamer of the target is limited to a G-rich DNA sequence, and to the aptamers without G nucleobases or with few nucleobases, this assay strategy was ineffective. The assay required a magnetic separation process and, therefore, the whole assay could be completed in 4 h. Very recently, Zhang et al. designed molecular beacons as signaling probes for adenosine triphosphate (ATP) detection in cancer cells, based on CL resonance-energy transfer.[34] The presented system exhibited a good selectivity for ATP detection, but the CL aptamer-based system is rather complex. Not only was the aptamer modified with an amino group to allow the attachment of the aptamer to the surface of magnetic nanoparticles, but also HRP, LumAuNP, and fluorescein were used to label the three DNA probes.

Gold nanoparticles (AuNPs) have attracted attention in the last few years, owing to their unique structural, electronic, magnetic, optical, and catalytic properties, which have made them a very attractive material for biosensor systems and bioassays. In our recent work,[35] we found that the catalytic activity of AuNPs (≈13 nm) on the luminol–H_2O_2 CL system is greatly enhanced after they have been aggregated in the presence of 0.5 M NaCl. By taking advantage of this interesting phenomenon, and the different interactions of the single- and double-stranded oligonucleotides with AuNPs, the label-free, homogenous CL detection of DNA hybridization was proposed.[35] Inspired by the above work, we have designed a label-free, CL aptamer-based biosensor for the detection of proteins. Thrombin, an important serine protease in the blood coagulation cascade, was taken as the model target to provide the "proof-of-principle" verification of the concept. Unmodified AuNPs can effectively differentiate unstructured and folded aptamer,[36] thus unmodified AuNPs are used as signal elements in this system (Scheme 1). In the absence of the target molecule (thrombin), the unfolded aptamer (single-stranded oligonucleotides) could adsorb onto the AuNP surface against aggregation at a given high concentration of salt (0.5 M NaCl). The dispersed AuNPs induce a weak CL signal for the luminol–H_2O_2 system. In the presence of the target molecule, the aptamer folds into a specific three-dimensional conformation. The folded aptamer cannot stabilize the AuNPs at a given high concentration of salt, resulting in the aggregation of the AuNPs (at 0.5 M NaCl). The aggregated AuNPs induce a strong CL signal. In our design, the aptamer does not need any labeling or modifying, and the whole detection can be completed in less than half an hour. Furthermore, the detection limit of thrombin (3σ) was estimated to be as low as 26 fM, and the sensitivity was increased by more than 4 orders of magnitude over that of a known AuNP-based colorimetric method for the detection of thrombin.[26] Therefore, the CL method may open a new and general way to simple and highly sensitive aptamer-based biosensors for a broad range of analytes.



Scheme 1. The label-free aptamer-based CL biosensor.

Results and Discussion

Aggregation of AuNPs induced by aptamer–target binding: The prepared AuNPs (≈13 nm) in aqueous solution are stable, as a result of the electrostatic repulsion of the negative capping agent (e.g. citrate ion) against the van der Waals attraction between AuNPs.[37] The AuNPs have a surface plasma resonance absorption peak at about $\lambda = 520$ nm and appear pink.[38] The addition of enough salt (≈0.5 M NaCl) screens the repulsion between AuNPs and induces

the aggregation of AuNPs. The aggregated AuNPs have a broad surface plasma resonance absorption in the range of $\lambda = 550$–850 nm. UV/Vis spectroscopy was used to explore the change of AuNPs state induced by aptamer–target binding in this system. As shown in Figure 1, the AuNPs in the presence of aptamer and 0.5 M NaCl have a surface plasma resonance absorption peak at $\lambda = 520$ nm; after the aptamer–target binding, the absorption spectrum for the AuNPs is broad and featureless, which indicates that the aggregation of AuNPs is complete. The results were consistent with the transmission electron microscopy (TEM) images (Figure 2). The mechanism behind the phenomenon lies in the interaction between the aptamer and the AuNPs. In the absence of the target, the aptamer (single-stranded oligonucleotide) is soft and random-coil-like. As previously reported,[39,40] the coordination interaction between the nitrogen atom of the unfolded ssDNA and the bare AuNPs is stronger than the electrostatic repulsion between the negatively charged backbone and the negatively charged AuNPs. Thus, the unfolded aptamer (ssDNA) can be strongly adsorbed onto the unmodified AuNPs and help to enhance the AuNPs' stability against salt-induced aggregation. In the presence of the target, the aptamer folds into a three-dimensional conformation (e.g., G-quadruplex). As for the folded aptamer, the relatively rigid structure prevents the exposure of the DNA bases to the AuNPs and the high density of negative charges increases the repulsion between the DNA and the AuNPs.[39,41] Thus, the folded aptamer could not absorb the AuNPs and the ability to protect the AuNPs was lost. Consequently, the aptamer–target binding induced the aggregation of AuNPs.

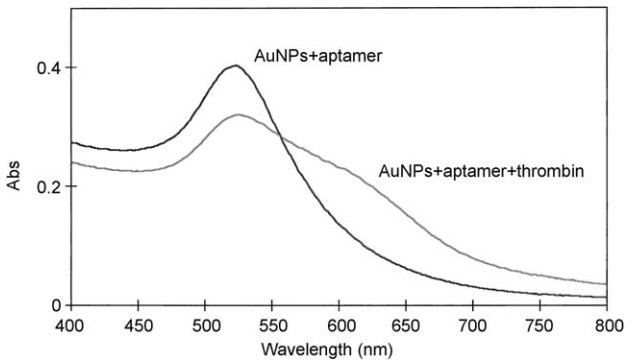


Figure 1. UV/Vis absorption spectra of AuNPs stabilized by aptamer (3.5×10^{-10} M) after the addition of salt in the absence and presence of thrombin (1.0×10^{-11} M).

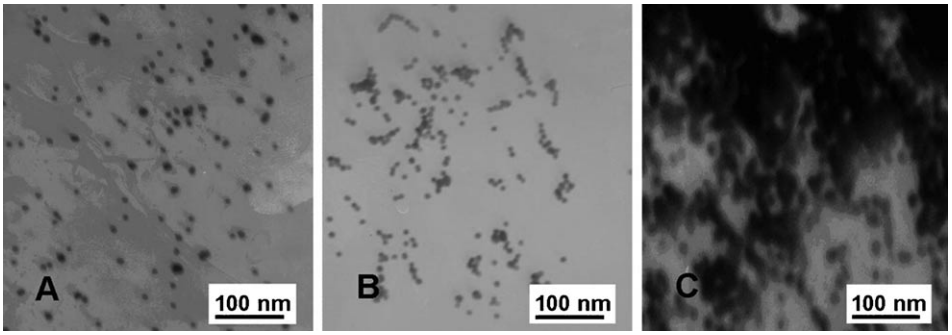


Figure 2. TEM images of A) the AuNPs, B) the AuNPs stabilized by aptamer after the addition of NaCl in the absence of thrombin, and C) the AuNPs stabilized by aptamer after the addition of NaCl in the presence of thrombin.

Enhancement of the catalytic activity of the AuNPs on the luminol–H_2O_2 CL system after aggregation: The luminol–H_2O_2 system, can be effectively catalyzed by some metal ions, metal complexes, and enzymes. Cui and co-workers first discovered the catalytic activity of AuNPs in the luminol–H_2O_2 system.[42] Some organic compounds containing OH, NH_2, or SH groups can inhibit the CL emission of the luminol–H_2O_2-AuNPs, by interacting with the AuNPs to decrease the catalytic activity.[42] We presume that the unfolded aptamer (ssDNA) absorbed onto the surface of AuNPs and influenced the catalytic activity of AuNPs in the luminol–H_2O_2 system. Therefore, we investigated the CL behavior of the luminol–H_2O_2-AuNPs system, as the surface of AuNPs (≈ 13 nm) was surrounded by ssDNA. As shown in Figure 3, the AuNPs in the presence of the aptamer show a rather weak catalytic activity, almost the same as the activity of bare AuNPs. The aggregated AuNPs after aptamer–target binding in the presence of 0.5 M NaCl, however, can enhance the luminol–H_2O_2 CL reaction more than the dispersed AuNPs alone. Based on our previous study,[35] we reasoned that the enhancement effect of the aggregated AuNPs may be attributed to the change of the AuNP surface-charge density. Analysis revealed that that the zeta potentials of the dispersed AuNPs and the aggregated AuNPs were -36.6 and -9.6 mV, respectively. It is clear that the AuNP surface negative charge density is greatly decreased after aggregation.

For the luminol–H_2O_2 CL system, the optimized pH conditions are generally about 11–12. The hydroperoxide ion (HO_2^-), easily formed in strong basic media, has an acid–base equilibrium ($pK_a = 11.7$) with H_2O_2.[43] Similarly, the luminol anion is the main molecular form of luminol in strong basic media. The anionic HO_2^- (or anionic luminol) does not easily interact with the anionic AuNPs because of electrostatic repulsion. The dispersed AuNPs, which have a high surface negative charge density, have a rather low catalytic effect on the luminol–H_2O_2 CL reaction. The aptamer–target binding induces a conformational change in the aptamer, and the folded aptamer cannot absorb the AuNPs and, therefore, loses its ability to

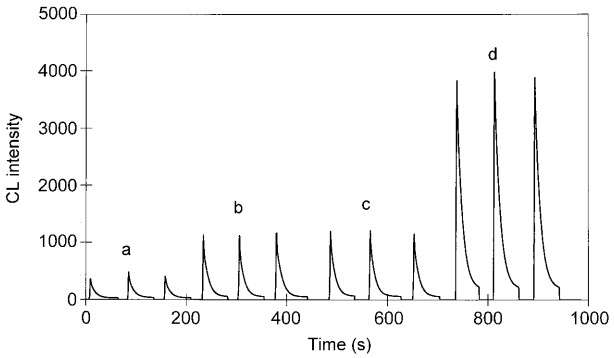


Figure 3. The effect of AuNPs on the luminol–H_2O_2 CL reaction: a) without AuNPs (aptamer 0 M; thrombin 0 M; NaCl 0 M), b) AuNPs (aptamer 0 M; thrombin 0 M; NaCl 0 M), c) AuNPs in the presence of aptamer (aptamer 3.5×10^{-10} M; thrombin 0 M; NaCl 0.5 M), d) AuNPs in the presence of aptamer and thrombin (aptamer 3.5×10^{-10} M; thrombin 3.5×10^{-10} M; NaCl 0.5 M). The voltage in the photomultiplier tube was 780 V.

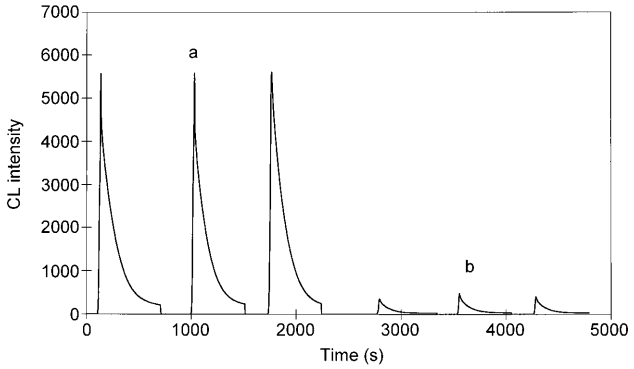


Figure 4. The effect of cationic AuNPs and anionic AuNPs on the luminol–H_2O_2 CL reaction: a) cationic AuNPs, b) anionic AuNPs. Conditions: luminol, 5×10^{-4} mol L^{-1}; H_2O_2, 1.0×10^{-3} mol L^{-1}. The voltage in the photomultiplier tube was 630 V.

protect the AuNPs. Salt in high concentration can screen the repulsion between the negatively charged AuNPs and can lead to the aggregation of the AuNPs. The aggregated AuNPs with low negative charge density can more easily interact with the anionic HO_2^- (or anionic luminol), resulting in a higher catalytic effect on the luminol–H_2O_2 reaction (Figure 3). It is clear that the conversion of aggregation state before and after aptamer–target recognition would be easily distinguishable by a CL analysis of the luminol–H_2O_2 CL system. Hence, we reasoned that the decrease of the surface negative charge density of AuNPs resulted in the enhanced catalysis of the aggregated AuNPs.

According to the above proposed mechanism for AuNP catalytic activity enhancement, we speculated that the cationic AuNPs would show a higher catalytic activity than the anionic AuNPs, because the anionic HO_2^- (or anionic luminol) can easily interact with cationic AuNPs. To validate the scenario, we prepared cationic AuNPs by reducing $HAuCl_4$ with $NaBH_4$ in the presence of 2-aminoethanethiol.[44] The zeta potential of the cationic AuNPs was +19.6 mV. When the luminol–H_2O_2 solution was injected into the cationic AuNP solution, the stronger CL emission was observed (Figure 4). Therefore, we reasoned that the decrease of the surface negative charge density of AuNPs would result in an increase of the catalytic activity of the AuNPs on the luminol–H_2O_2 CL system.

Optimization of assay conditions: Based on the principle of the aptamer-based sensing for thrombin, there are two main interactions: aptamer–target binding and aptamer–AuNPs binding. Two strategies were developed for the analysis: Strategy 1: The aptamer and the thrombin solution were first mixed and allowed to incubate for a set period, and then the AuNPs solution was added into the mixed solution. Strategy 2: The aptamer and the AuNP solution were first mixed and allowed to interact for set period, then the thrombin solution was added into the mixed solution. Analysis of the experimental results revealed that the sensitivity of the CL aptamer sensor (aptasensor) in Strategy 1 was higher than that of Strategy 2. In this CL aptasensor for thrombin, the aptamer–thrombin binding is the molecular recognition process and is the key factor for the detection of thrombin. In Strategy 1, the aptamer interacts with thrombin under the optimum conditions. In Strategy 2, the aptamer and AuNPs are mixed, and the aptamer is absorbed onto the surface of the AuNPs; after the addition of thrombin, the aptamer needs to de-absorb from the AuNPs, which might then influence the molecular recognition process. This may be the reason why the sensitivity in Strategy 2 is lower. Thus, we chose Strategy 1 (shown in Scheme 1) as the analytical strategy for the assay.

With a fixed strategy, the performance of the developed CL aptasensor is still strongly influenced by the assay conditions, such as aptamer concentration, AuNP concentration, binding time, binding temperature, and the CL reaction conditions. Different assay conditions were investigated in our studies.

The amount of aptamer, a vital parameter for thrombin detection, was explored. Notably, the high concentration of thrombin itself could effectively stabilize the AuNPs under the conditions we used, maybe because, as a protein, thrombin can be absorbed onto the surface of the AuNPs. Therefore, to guarantee that the addition of thrombin leads to AuNP aggregation, the aptamer concentration should be enough high to decrease the free thrombin in solution. However, if the aptamer concentration is too high, it is unsuitable for the detection of small amounts of thrombin. The experimental results showed that the optimum aptamer concentration was 3.5×10^{-10} M.

A long binding time for the aptamer and thrombin is expected to yield a high CL signal. The response of the sensor was recorded for increasing reaction times. The results showed that the CL signal increased with increasing binding time, reached a maximum at 20 min, then continued at an almost constant value. Thus, 20 min was selected as the optimum time for aptamer–thrombin binding. Naturally, the binding temperature also affects the aptamer–thrombin

binding event. We compared two different temperatures, 37 °C and room temperature (≈20 °C), and the results showed that the higher binding temperature could shorten the binding time. Taking into account operational convenience, room temperature (20 °C) was chosen as the operational temperature for all experiments. In this system, the media pH for the aptamer–thrombin binding reaction was 7.4. The media pH also influenced the interaction between the AuNPs and the aptamer (ssDNA). The results showed that acidic media is not favorable for the adsorption of the aptamer on 13 nm AuNPs, and under the neutral and basic media the aptamer could be strongly absorbed on the surface of 13 nm AuNPs. The results agreed with the previous studies on ssDNA/AuNPs.[35,45]

In this system, AuNPs are the signal element and the size of the AuNPs influence the aptamer–AuNP binding. Simultaneously, the size of the AuNPs also affects the catalytic activity of the AuNPs in the luminol–H_2O_2 CL reaction. The study by Cui and co-workers showed that in the range 9–38 nm, the increasing size of the AuNPs resulted in an increase in catalytic activity.[42] For this CL aptasensor, the CL signal from the dispersed AuNPs is taken as the background. Therefore, the small-sized AuNPs should be used to achieve high sensitivity. Moreover, 13 nm AuNPs can sensitively differentiate the folded aptamer and the unfolded aptamer.[36,41] Therefore, we used the 13 nm AuNPs in this CL aptasensor. The sensitivity of the CL aptasensor is also influenced by the AuNPs concentration. If the AuNP concentration is too high, the system is not sensitive enough to detect small amounts of thrombin and does not differentiate them from the background. In this system, we used 17 nM of AuNPs (≈13 nm) for all experiments. Furthermore, we investigated the effect of the age of the AuNPs. For the same concentration of thrombin, the signal did not change for the fresh AuNPs over one week, but the sensitivity decreased markedly if the AuNPs were stored for a month or more. The reason is that the AuNPs themselves aggregate during the stored time. For this reason we prepared very dilute AuNP solutions on a weekly basis for our experiments.

The important experimental parameters influencing the CL reaction of luminol–H_2O_2 and 13 nm AuNPs, including the concentrations of luminol and H_2O_2, and media pH, were optimized. It was found that both signal and background increase as the luminol concentration is raised, and the signal/noise ratio was the highest at 0.5 mM luminol. The effect of the H_2O_2 concentration on the CL was studied in the range 0.01–20 mM, and the maximum CL emission was observed at 1.0×10^{-3} M H_2O_2 solution. The experimental results showed that the optimal pH for this CL system was 12.0, which is in agreement with the results of previous studies.[35,42]

Analytical performance of the CL aptasensor for thrombin: Under the optimized conditions, experiments were carried out by adding increasing amounts of thrombin to the CL aptasensor to examine whether the CL change could be used for thrombin quantification. As shown in Figure 5, the CL intensity increases with the increasing thrombin concentration, to reveal a linear relationship in the thrombin concentration range 6.3×10^{-14}–6.3×10^{-11} M. The relative standard deviation (RSD) was 4% for 4.3×10^{-12} M thrombin, calculated after studying six replicate injections of luminol–H_2O_2 CL reagents. The detection limit is an estimate of the concentration at which we can be fairly certain that the compound is present, concentrations below which may not be detected; for this experiment it was found to be 2.6×10^{-14} M. The increase in sensitivity of this system is more than 4 orders of magnitude more than that of the known AuNP-based colorimetric method for the detection of thrombin.[26] Conversely, the detection limit is 1000-fold lower than that reported for an aptamer-based CL sensor for thrombin.[31]

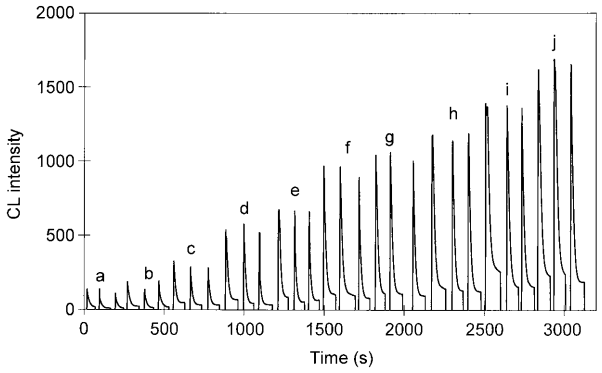


Figure 5. Representative recorder outputs of the CL system in the presence of different concentrations of thrombin. The concentrations of thrombin: a) 0, b) 6.3×10^{-14} M, c) 3.6×10^{-13} M, d) 6.3×10^{-13} M, e) 8.2×10^{-13} M, f) 1.2×10^{-12} M, g) 6.3×10^{-12} M, h) 1.2×10^{-11} M, i) 3.6×10^{-11} M, j) 6.3×10^{-11} M. The voltage in the photomultiplier tube was 520 V.

As to the determination of thrombin, its selectivity was evaluated by detecting the CL signals arising from aptamer/thrombin, aptamer (without thrombin), aptamer/BSA, and control random sequence/thrombin. The concentrations of the aptamer and control random sequence were kept at 3.49×10^{-10} M, thrombin and BSA were kept at 1.6×10^{-11} M, and the other experimental conditions were also kept the same. If thrombin was absent, or was displaced by BSA, or the aptamer was displaced by a control random sequence, the CL intensity was not greater than 5% of that with the aptamer/thrombin. The results showed that the determination of thrombin has a high selectivity (see the Supporting Information Figure S1).

Detection of K^+ by a CL aptasensor: As a further step, we attempted to prove the general applicability of our strategy by using a K^+ aptamer. By using the K^+ aptamer at a concentration of 5.0×10^{-5} M, the CL intensity increased with increasing K^+ concentration (see the Supporting Information, Figure S2). The CL aptamer-based strategy can detect K^+ with a detection limit of 1.6×10^{-8} M (3σ). The CL signal exhibited a linear correlation to the K^+ concentration through a range of 7.4×10^{-8}–3.2×10^{-5} M. The RSD was 3.6% at a K^+ concentration of 5.6×10^{-7} M with six replicate injections of the luminol–H_2O_2 CL reagents. The selectivity of the K^+

determination was investigated by using Li^+, Na^+, Rb^+, NH_4^+, and two physiologically relevant metal ions (Mg^{2+}, Ca^{2+}) to displace K^+, and one experiment used a control random sequence instead of the aptamer. The concentration of K^+ and other ions were kept the same (2.6×10^{-6} M) and, additionally, the concentrations of both the control random sequence and the aptamer were kept the same (1×10^{-5} M). The experimental results showed that the well-defined CL response was observed only for K^+ (see the Supporting Information, Figure S3). The CL response arising from the other ions was significantly smaller and the control random sequence yielded an unmistakably small CL response. The results indicate that the determination of K^+ has a high specificity.

Conclusion

In summary, a novel and sensitive strategy to convert the aptamer–target recognition event into chemiluminescence (CL) signals by employing unmodified AuNPs as signaling probes is proposed in this study. This strategy has several significant advantages: 1) The aptamer does not need labeling or modifying, so that the affinity and specificity of the original aptamer are not changed by a label, and the method is simple, cheap, and easy to operate. 2) The assay is homogenous and occurs in the liquid phase, which makes it easy to automate by standard robotic manipulation of microwell plates. 3) The assay avoids the separation and washing steps, and the whole detection can be completed in 30 min. In addition, given the simplicity and sensitivity of CL detection, this study may pave a general way to the development of aptamer-based CL sensors for a broad range of analytes.

Experimental Section

Chemicals and apparatus: All oligonucletides used in the present study were synthesized and purified by Beijing Dingguo Biotechnology Co. Ltd. (Beijing, China). Their base sequences are as follows: 15-mer thrombin-binding aptamer, 5′-GGT TGG TGT GGT TGG-3′; K^+-binding aptamer, 5′-GGG TTA GGG TTA GGG TTA GGG-3′; control random sequence, 5′-CTC ACG TAA ACT CAC GTA AA-3′. Thrombin and bovine serum albumin (BSA) were also purchased from Beijing Dingguo Biotechnology Co. Ltd. The chemical $HAuCl_4{\cdot}4H_2O$ was purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Sodium citrate and sodium chloride were purchased from Tianjing Chemical Reagent Company (Tianjing, China). Unless otherwise indicated, all reagents and solvents were purchased in their highest available purity and used without further purification. The luminol stock solution (2.5×10^{-2} M) was prepared by dissolving luminol (4.43 g) in 0.10 M NaOH (20 mL) and then diluting to 1 L with water. The luminol solution was stored in the dark for one week prior to use to ensure that the reagent had stabilized. Working solutions of luminol were prepared by diluting the stock solution. Working solutions of H_2O_2 were prepared fresh daily from 30% (w/w) H_2O_2. The oligonucleotide stock solutions (25 μM) were prepared with a TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.5) and kept frozen. In the study, a solution of the aptamer sequence was heated to 95 °C for 10 min, and then immersed in water (4 °C) immediately to unwind the single-strand oligonucleotide before use. Doubly distilled and deionized water was used throughout.

The CL intensity was measured and recorded with a model IFFL-D Chemiluminescence Analyzer (Xi′an Ruimai Electronic Sci. Tech. Co. Ltd., Xi′an, China). UV/Vis absorption spectra were recorded by using a Hitachi U-3900H UV/Vis Spectrophotometer (Tokyo, Japan). The transmission electron microscopy (TEM) images of AuNPs were taken by using a Hitachi H-600 TEM (Tokyo, Japan). An HH-1 thermostatic water bath (Beijing Kewen Instrumental Factory, Beijing, China) was used to control the temperature at 0.1 °C intervals. The X-ray photoelectron spectra (XPS) analysis of AuNPs before and after the CL reaction was performed by using an ESCALab 220I-XL spectrometer. The determination of zeta potential was carried out by using a Malvern Zetasizer 2000.

Synthesis and characterization of AuNPs: All glassware and magnetic stirrer bars used for the synthesis were thoroughly cleaned in aqua regia ($HCl/HNO_3=3{:}1$, v/v), rinsed in distilled water, and then oven-dried prior to use, to avoid unwanted nucleation during the synthesis, as well as aggregation of gold colloid solutions. The AuNPs (≈13 nm) were synthesized by reducing $HAuCl_4$ with trisodium citrate.[37] A $HAuCl_4$ solution (100 mL, 1.0 mM) was boiled and stirred vigorously to which a trisodium citrate solution (10 mL of 38.8 nM) was added quickly with a concomitant color change from light yellow to wine red, indicative of the formation of AuNPs. The solution was maintained for 10 min at boiling and then removed from the heating mantle. Stirring was continued for another 15 min, and after cooling to temperature, the prepared AuNP solution was stored in the refrigerator (4 °C). The synthesized AuNPs were characterized by using UV/Vis spectroscopy and TEM in order to measure the NP size. The concentration of the AuNPs was calculated by using UV/Vis spectroscopy, based on an extinction coefficient of $2.7\times10^8\,M^{-1}cm^{-1}$ at $\lambda=520$ nm for 13 nm particles.[38]

The cationic AuNPs were prepared according to the procedures given in references [46,47]. In brief, a 2-aminoethanethiol solution (0.2 mol L^{-1}, 0.4 mL) was added to a $HAuCl_4$ solution (0.014 mol L^{-1}, 40 mL). After stirring for 20 min, a $NaBH_4$ solution (0.001 mol L^{-1}, 1 mL) was added dropwise for 2 min, and the mixture was stirred for 12 h. The resulting solution was stored in brown glass bottles at 4 °C and used within 1 month.

Procedure for the CL detection of thrombin: A typical CL analysis was realized by following the procedure given in Scheme 1. First, 15-mer thrombin-binding aptamer solution (60 μL, 3.5×10^{-10} M) was mixed with thrombin (60 μL) of the appropriate concentration, and the mixed solution was incubated for 20 min at room temperature, leading to the formation of a thrombin–aptamer complex. Second, a solution of AuNPs (100 μL) was added to the prepared solution, and the solution was allowed to react for 5 min at room temperature, then a 0.5 M NaCl solution (30 μL) was added to induce the aggregation of the AuNPs. Finally, an aliquot of the above aptamer/thrombin/AuNPs solution (50 μL) was pipetted into a 40×14 mm quartz tube (used as the CL reactor), then luminol–H_2O_2 CL solution (200 μL, the volume ratio of 5×10^{-4} M luminol and 1.0×10^{-3} M H_2O_2 was 2:1) was injected, and the CL signal was measured and recorded by using the IFFL-D Chemiluminescence Analyzer. The concentration of thrombin was quantified by the CL intensity. The CL experiments in this work were not consecutive measurements.

Acknowledgements

This work was supported financially by the National Natural Science Foundation of China (No. 21075080), the Key Project of Chinese Ministry of Education (No. 109150), and the Fundamental Research Funds for the Central Universities (Program No. GK20091004).

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Received: July 1, 2010
Published online: December 7, 2010

