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Chemiluminescence flow biosensor for hydrogen peroxide using DNAzyme immobilized on eggshell membrane as a thermally stable biocatalyst

Weiwei Chen, Baoxin Li*, Chunli Xu, Lei Wang

Key Laboratory of Analytical Chemistry for Life Science of Shaanxi Province, School of Chemistry and Materials Science, Shaanxi Normal University, Xi'an 710062, PR China

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ABSTRACT

A DNAzyme-based chemiluminescence (CL) biosensor for sensitive detection of hydrogen peroxide (H_2O_2) has been developed. The complexation of hemin with a guanine-rich single-stranded nucleic acid yields the DNAzyme with peroxidase catalytic activity. The DNAzyme catalyzes the oxidation of luminol by H_2O_2 and the generation of CL. The DNAzyme is immobilized on eggshell membrane, and then packed into mini-column as CL flow cell. In the presence of luminol, H_2O_2 passed through the CL flow cell to produce CL emission, and then was sensed. The response to H_2O_2 concentration was linear in the range of 1×10^{-5}–1×10^{-7} M with a detection limit of 5×10^{-8} M (3σ). Compared to the HRP-based biosensor for H_2O_2, the DNAzyme possessed high catalytic activity in strong alkaline medium, which was well compatible with the luminol CL system. The immobilized DNAzyme could be stored at room temperature (~20 °C) and exhibited good stability with a shelf life of at least 3 months. A complete analysis, including sample and washing, could be performed in 1 min with a relative standard deviation of less than 2%. This biosensor for H_2O_2 has the potential to serve as a general platform for design sensors for other molecules (such as glucose and uric acid).

1. Introduction

Natural enzymes could accelerate the multitude of reactions upon which life depends, and they play central roles in biochemistry (Zou and Zhu, 1997). Natural enzymes are biological catalysts bearing some excellent properties such as high substrate specificities and high efficiency under mild conditions. So, natural enzymes have significant practical applications in medicine, chemical industry, food processing and agriculture. Almost all natural enzymes are proteins. Thus, like all protein, natural enzymes can be easily denatured by environmental changes because the minor alteration of their native protein conformation could result in loss of their catalytic activity. The preparation, purification and storage of natural enzymes are usually time-consuming and expensive. Furthermore, natural enzymes can be digested by proteases. Therefore, the serious disadvantages of natural enzymes limit their practical uses.

For years, the construction of efficient artificial mimics of natural enzymes has been a challenging topic for scientists from a variety of disciplines (Kirby, 1996; Zen and Kumar, 2001; Wulff, 2002; Groves, 1997). Mimetics of many natural enzymes (such as cytochrome P450 (Aissaoui et al., 1998), serine proteases (Ghosh et al., 1999), dioxygenase (Chen and Que, 1999), phosphodiesterase (Molenveld et al., 1999), ligase (Han et al., 2000) and methanogenesis (Signor et al., 2000)) have been developed. Among these, a lot of research has been focused on peroxidase mimetics including hemin (Wang et al., 2007a; Fruk and Niemeyer, 2005), hemoglobin (Li et al., 2001b), and porphyrin (Sono et al., 1996). Unexpectedly, Yan and his coworkers have found that Fe_3O_4 magnetic nanoparticles exhibit an enzyme mimetic activity similar to peroxidase (Gao et al., 2007), and Wang et al. recently used the Fe_3O_4 magnetic nanoparticles as peroxidase mimetics to detect H_2O_2 and glucose (Wei and Wang, 2008). These artificial mimics of peroxidase have been widely employed in analytical chemistry, but their catalytic activity is all much lower than that of horseradish peroxidase (HRP). A lot of effort has been made to improve the activity of mimetic peroxidase (Chen et al., 2009; Castriciano et al., 2008; Yang et al., 2004; Huang et al., 2003).

The discovery of catalytic RNAs (ribozymes) in cells has sparked scientific activities directed to the preparation of new non-protein biocatalysts (Cach et al., 1981; Guerrier-Takada et al., 1983; Nissen et al., 2000). Analogously, deoxyribozymes (catalytic DNAzymes) are not found in nature, but extensive research efforts have demonstrated the successful synthesis of catalytic deoxyribozymes for many chemical transformations (Breaker, 1997, 2000). DNAzyme is one kind of functional nucleic acids, and is found with a promising methodology termed in vitro selection or SELEX (systemic evolution of ligands by exponential enrichment). One interesting example of DNAzymes that reveals peroxidase-like activity includes a supramolecular complex between hemin and a single-stranded guanine-rich nucleic acid (aptamer) (Travascio et al., 2001). It was

* Corresponding author. Tel.: +86 29 85308184; fax: +86 29 85307774.
E-mail address: libx29@hotmail.com (B. Li).

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suggested that the intercalation of the hemin into the guanine quadruplex docked layers of the nucleic acid leads to the biocatalytic structure (Travascio et al., 1998). Willner and his coworkers used the DNAzyme as a label for the amplified detection of Pb^{2+} ions or L-histidine (Elbaz et al., 2008), protein (Li et al., 2007a) and DNA, or for the analysis of telomerase activity in cancer cells (Pavlov et al., 2004; Xiao et al., 2004; Niazov et al., 2004; Gill et al., 2006). Compare with protein enzymes (such as HRP), the DNAzyme is more stable and relatively less expensive to produce by the polymerase chain reaction. It is considered that, unlike proteins, the DNAzyme can be denatured and renatured many times without losing activity. Therefore, the DNAzyme has shown great promise for constructing biosensor.

Research on enzyme-based biosensor is an active, exciting and innovative area in analytical chemistry with more than 1000 papers being published annually (Wang, 2008; Borisov and Wolfbeis, 2008). For a reusable enzyme-based biosensor, most commonly, an enzyme is required to be immobilized on a suitable insoluble solid support. The DNAzyme with peroxidase-like activity has been used in analytical chemistry (Elbaz et al., 2008; Li et al., 2007a; Pavlov et al., 2004; Xiao et al., 2004; Niazov et al., 2004; Gill et al., 2006; Willner et al., 2008). However, to the best of our knowledge, its application as an immobilized DNAzyme in biosensor has not been investigated in detail. In this work, a G-rich nucleic acid sequence bound hemin to form a biocatalytic complex (DNAzyme) with peroxidase activity, and the DNAzyme was immobilized on eggshell membrane, and the immobilized DNAzyme could be reused for more than 200 times. Eggshell membrane (approximately 65–96 μm thickness (Liong et al., 1997)) is a light pink double-layered membrane inside the eggshell, and it is mainly composed of biological molecules and highly cross-linked protein fibers, and it possesses excellent gas- and water-permeability (Takiguchi et al., 2006). Choi and his coworkers immobilized α-chymotrypsin and alcohol oxidase onto an eggshell membrane to fabricate an aspartame optical biosensor (Xiao and Choi, 2002). In our recent study (Li et al., 2008), eggshell membrane was used to immobilize glucose oxidase and HRP, and one stable glucose biosensor was constructed. The use of eggshell membrane as enzyme immobilization platform offers several advantages: (i) eggshell membrane as byproduct is very cheap and can be easily made up into various forms; (ii) eggshell membrane is non-toxic and environment-friendly; (iii) the preparation procedure or process using eggshell membrane as a carrier for the immobilization of enzyme is simple and easy; (iv) eggshell membrane is one biomaterial and can keep the activity of enzyme.

Chemiluminescence (CL) has been known as a powerful and important analytical technique because the analytical performance of CL detection is better than that of other common spectroscopic detection methods (such as spectrophotometry and fluorescence), higher sensitivity, lower detection limits and wider linear ranges can be achieved with simpler and cheaper instrument (no excitation light source and no spectral resolving system) (Li et al., 2008; Wang et al., 2007b; Zhang et al., 2008; Miao et al., 2008). Nowadays, many CL enzyme-based biosensors have reported (Li et al., 2001a; Zhang et al., 2005). Luminol system is the most popular CL system in these reported CL biosensors, and its optimum pH is 10–11, while the optimum pH for natural enzyme reaction is about 7. The incompatibility of the CL reaction condition and the enzyme reaction condition would decrease the sensitivity of the CL enzyme-based biosensors. In this study, the experiments showed that at strong basic media (pH 11) the DNAzyme carrying peroxidase activity could catalyze the CL reaction of H_2O_2 and luminol. A new DNAzyme-based CL flow biosensor for H_2O_2 is proposed. The principle is schematically shown in Fig. 1. It was based on that the DNAzyme carrying peroxidase activity. The DNAzyme immobilized on eggshell membrane was packed into a mini-column as the CL flow cell. In the presence of luminol, H_2O_2 passed through the CL flow cell to produce CL emission, and then was sensed. Detection of H_2O_2 is, in practice, important because it is the product of reactions catalyzed by a large number of oxidase enzymes, and it is essential in food, pharmaceutical and environmental analysis. On the other hand, the combination of biosensor with flow injection analysis makes it possible to control all of the stages of the reagent addition, improves the sample throughput, and achieves completely automated determination along with sensitive detection, quick response, and repeated use of the immobilized DNAzyme.

2. Experimental

2.1. Chemicals and materials

Luminol stock solution (2.5×10^{-2} M) was prepared by dissolving 4.43 g luminol in 20 mL of 0.10 M NaOH and then diluting to 1 L with water. Hemin was purchased from Beijing Chemical Reagent Company (Beijing, China) and used without further purification. Hemin stock solution (5 mM) was prepared in DMSO and stored

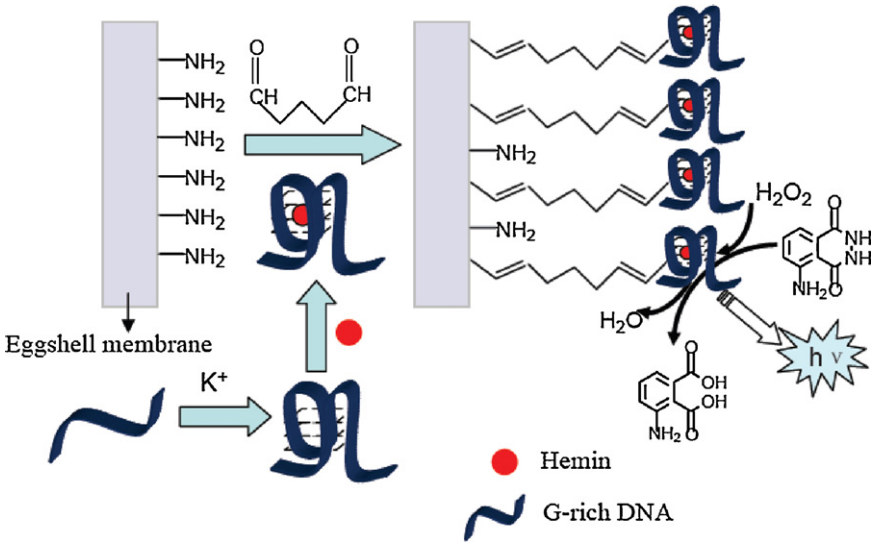

Fig. 1. Principle of the DNAzyme-based biosensor for H_2O_2.

in the dark at −20 °C. HRP (type VI, 330 U mg^{-1}) were obtained from Sigma (St. Louis, MO, USA). 30% H_2O_2 was purchased from Xi'an Reagent Plant (Xi'an, China), and 25% glutaraldehyde was purchased from Beijing Chemical Reagent Company (Beijing, China). DNA oligonucleotide (5′-GTG GGT AGG GCG GGT TGG-3′) was synthesized by Beijing Dinguo Biotechnology Co. Ltd. (Beijing, China). The purchased DNA oligomer was dissolved in Tris–HCl buffer solution (20 Mm, pH 7.4) and stored at 4 °C.

All other chemicals were of analytical reagents grade. The deionized and distilled water was used for the preparation of solution. The eggs were obtained from a local supermarket and stored at 4 °C until use.

2.2. Apparatus

The CL measurements were conducted on the IFFL-E Chemiluminescence Analyzer (Xi'an Ruike Electronic Science Tech. Co. Ltd., Xi'an, China). The CL signal was recorded and processed by a personal computer equipped with IFFL-D software running under Windows 2000. The pH measurements were carried out on model PHS-3E digital ion analyzer (Jiangsu Instruments, Jiangsu, China). UV–visible spectra were recorded on a TU-1901 UV-Vis Spectrophotometer (Beijing Purkinje General Instrument Co., Ltd., Beijing, China) at room temperature. SEM images were taken using a Quanta 200 Scanning Electron Microscopy (FEI Company, The Netherlands).

2.3. Preparation of the DNAzyme

A solution of 200 μM G-rich DNA oligomers was heated at 90 °C for 10 min to dissociate any intermolecular quadruplex. The solution was allowed to cool back to ambient temperature, and then an equal volume of the buffer solution (20 mM Tris–HCl, 40 mM KCl, 0.06% (v/v) Triton X-100, pH 7.4) was added. Then, the DNA solution was sit for 40 min at room temperature to allow appropriate folding. Hemin solution (40 μM) was added to an equal volume of the prepared DNA solution (40 μM), and the mixture was allowed to sit for 2 h at room temperature. Under these conditions, the G-rich DNA oligomer assembled hemin to form the DNAzyme with G-quardruplex structure (Pavlov et al., 2004).

2.4. Immobilization of the DNAzyme on eggshell membrane

An eggshell membrane was carefully peeled off from a broken fresh eggshell after the albumen and yolk had been removed. It was then cleaned with a copious amount of deionized water to completely remove the albumen from the eggshell membrane. The cleaned eggshell membrane was finally stored in a phosphate buffer (pH 7.4, 0.025 M) until further use.

The eggshell membrane was removed from the phosphate buffer. The membrane was placed in a clean glass slide and cut into a small piece (15 mm × 20 mm, ca. 0.02 g). The 100 μL DNAzyme (20 μM) was dropped onto the surface of the eggshell membrane. After approximately 20 min, 10 μL of 1% glutaraldehyde solution as a cross-linking agent added and spread evenly on the membrane surface with a small glass rod. The membrane was retained for 10 h at 4 °C. Finally, the DNAzyme-immobilized membrane was rinsed with and immersed in a phosphate buffer (pH 7.4) three times alternately to remove the un-cross-linked and unabsorbed DNAzyme.

The DNAzyme-immobilized membrane was packed into a colorless glass column (55 mm length, 3 mm i.d.) furnished with some sponge at both ends to retain the membrane. The DNAzyme reactor (see Supporting Information Figure S1) was used as the CL flow cell. The DNAzyme reactor was stored at room temperature. Both the ends of the reactor were sealed to avoid the membrane losing most of the water and causing cracking during storage period.

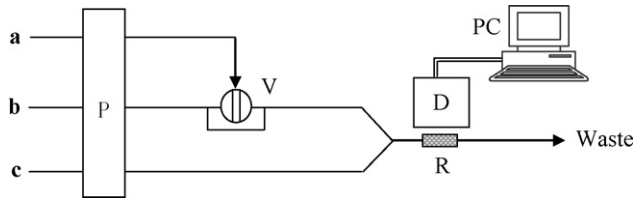

Fig. 2. Schematic of the DNAzyme-based CL flow biosensor for the determination of H_2O_2. (a) Sample; (b) buffer solution; (c) luminol; (P) peristaltic pump; (V) injection valve; (R) DNAzyme reactor; (D) detector; (PC) personal computer.

2.5. Procedure

A diagram shown in Fig. 2 illustrates the DNAzyme-based CL flow biosensor for H_2O_2. Flow lines were inserted into the luminol solution, buffer solution, and sample solution, respectively. The pump was started to wash the whole system until a stable baseline. The 120 μL of sample solution was injected by a six-way injection valve into carrier stream (0.025 M phosphate buffer solution). This stream was merged with luminol and then reached the DNAzyme reactor, producing CL emission. The concentration of sample was quantified by the CL intensity.

3. Results and discussion

3.1. DNAzyme formation from porphyrin-binding DNA aptamer

A guanine-rich nucleic acid (as porphyrin-binding DNA aptamer) can fold into a G-quadruplex structure, and bind hemin to form a new kind of synthetic DNAzyme (Travascio et al., 2001, 1998; Elbaz et al., 2008), possessing high peroxidase activity as well (Li et al., 2007a; Pavlov et al., 2004; Xiao et al., 2004; Niazov et al., 2004; Gill et al., 2006). As a prophyrin anion, hemin is subject to aggregation in aqueous buffer to form the corresponding dimers, which would influence the formation of the DNAzyme. The aggregation of hemin could be effectively avoided by preparing samples in the presence of 0.03% Triton X-100. So, we added 0.03% Triton X-100 into the buffer solution. We examined the interaction of hemin with an 18-mer DNA aptamer (Travascio et al., 2001) using absorption spectroscopy. The experimental results (Fig. 3) showed that with increasing DNA concentration, the absorbance of hemin at about 400 nm increased and the absorption peak showed a light red shift from 392 to 404 nm. We also investigated the

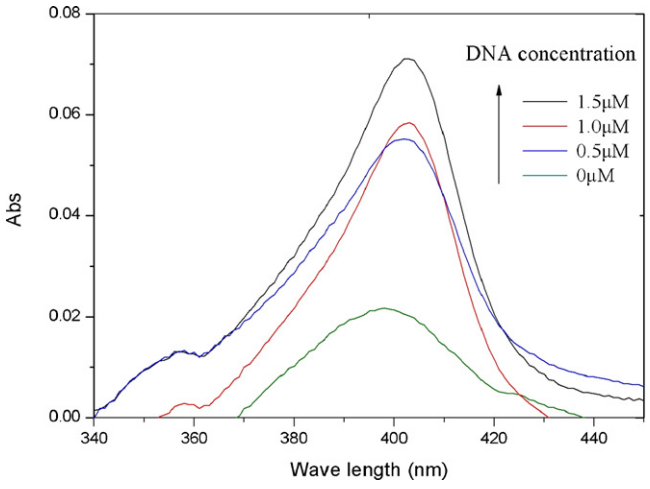

Fig. 3. UV–vis absorption spectra of hemin in the presence of different concentration 18-mer DNA aptamer. Hemin, 1 μM; Buffer solution (20 mM Tris–HCl, 20 mM KCl, 0.03% (v/v) Triton X-100, pH 7.4).

effect of interaction time (see Supporting Information Figure S2 and Figure S3). It was found that the absorbance at about 400 nm increased with increasing time in the range 0–20 min, and for more than 20 min reaction time, the absorbance remained almost constant. The increasing absorbance of hemin has been considered an indicator of the hydrophobic nature of the hemin-binding site (Ito and Hasuda, 2004). Presumably, this is also the case in the present case with regard to the hemin-binding site with the G-rich DNA. On the other hand, the interaction was monitored by absorption change of the G-rich DNA aptamer. When hemin was added into the DNA solution, the absorption peak of the DNA at 257 nm increased significantly (Supplementary Information), which indicated the formation of G-quadruplex structure (Ito and Hasuda, 2004). Therefore, these experimental results showed the DNAzyme formation from porphyrin-binding DNA aptamer in the presence of hemin. Hemin is present as an anion porphyrin with two carboxyl groups, and it is easy to penetrate into the G-quartet fold, where the intermolecular hydrogen bonds are formed and hold the DNAzyme stable. According to the previous report (Li et al., 1996), the DNA aptamer could be combined with other anion metalloporphyrine as well, but the neutral or positive species were not bond. This indicated that the negatively charged carboxyl groups hemin were beneficial to the stability of the DNAzyme.

3.2. Immobilization of DNAzyme on eggshell membrane

Covalent bonding is usually used to achieve the immobilization of DNA on a suitable solid matrix. It is often necessary to modify 5′-end or 3′-end of DNA with thiol or amino group, and then a cross-linking agent is used to bind DNA on solid supports. In this biosensor, we found that the DNAzyme was not modified and could be directly immobilized on the eggshell membrane in the presence of glutaraldehyde. In order to know the microstructure of the eggshell membrane without and with the immobilized DNAzyme, the eggshell membranes (3 mm × 1 mm) with and without immobilized DNAzyme were prepared for scanning electron microscopy (SEM). The membranes were coated with a thin layer of gold using a spray gun, and were then observed under the scanning electron microscope. The surface morphologies of eggshell membrane are shown in Fig. 4A and B. A network-like structure without any aggregation is observed on the cleaned eggshell membrane surface (Fig. 4A), which indicates that the eggshell membrane consists of highly cross-linked protein fibers (ca. diameter 1–3 μm) and cavities. From Fig. 4B, some clusters of DNAzyme can be clearly seen on the protein fibers. Since the SEM images cannot visualize the individual size of DNAzyme, the immobilized DNAzyme can only be served as some clusters on the fibers. It is clearly shown that the DNAzyme was successfully immobilized on the eggshell membrane. In this DNAzyme, the 18-mer DNA aptamer (5′-GTG GGT AGG GCG GGT TGG-3′) could fold into the three-layer G-quartets, and hemin could penetrate into the G-quartet fold. The intermolecular hydrogen bonds are formed between 12 guanine bases, and the amino groups of adenine bases and cytosine base do not participate in formation of intermolecular hydrogen bonds of G-quartets. So, we thought that the free amino group of adenine bases or cytosine base in this DNAzyme could react with glutaraldehyde. There are many amino groups on eggshell membrane, and the amino groups on eggshell membrane also could react with glutaraldehyde. It can be imagined that three types of immobilized DNAzymes may occur within the eggshell membrane: The first type is the chemical cross-linking between a DNAzyme molecule with another DNAzyme molecule or a DNAzyme molecule with a protein fiber of eggshell membrane. The second type is the electrostatic interaction between a DNAzyme and the macromolecular net-vein of the eggshell membrane. And the third type is DNAzymes that are not cross-linked to the other DNAzymes or protein fibers. Thus the DNAzyme could be directly immobilized on the eggshell membrane with no requirement for modification of DNAzyme, making this biosensor simple, easy and convenient.

3.3. Optimization of the immobilization condition of DNAzyme

The DNAzyme concentrations in feed solution for immobilization experiment affected the response of the biosensor. The experimental results (see Supporting Information Figure S4) showed that the response of the biosensor increased with increasing the DNAzyme concentrations in feed solution. This was attributed to increasing the amount of immobilized DNAzyme on the eggshell membrane. When the DNAzyme concentration was 20 μM, the biosensor response was high enough. Taking cost of the biosensor, 20 μM DNAzyme solution was chosen as the feed solution for all subsequent experiments. The effect of DNAzyme concentrations in feed solution on the biosensor (see Supporting Information Figure S4) showed that the response at 30 μM DNAzyme is about 150% as that at 20 μM DNAzyme. Therefore, when 30 μM DNAzyme would be chosen to used in feed solution for immobilization experiment, the sensitivity of the biosensor would be greatly improved.

Without glutaraldehyde, the response of the biosensor was very weak, and declined rapidly. The reason is that the amount of the DNAzymes physically adsorbed on the eggshell membrane is rather small, and the absorbed DNAzymes easily dropt from the eggshell membrane. So glutaraldehyde was used as the cross-linking agent for DNAzyme immobilization. The response of the biosensor was studied when the DNAzyme-immobilized membrane was cross-linked by various concentrations of glutaraldehyde in feed solution. The results showed that the CL intensity increased with increasing glutaraldehyde concentration in the range of 0–1.0% (w/w). But

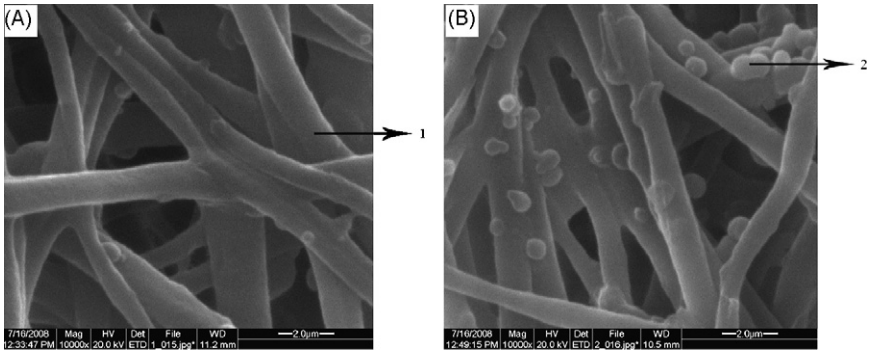

Fig. 4. Scanning electronic micrographs of the eggshell membrane. (A) Cleaned eggshell membrane and (B) eggshell membrane with immobilized DNAzyme; (1) protein fiber and (2) immobilized DNAzyme.

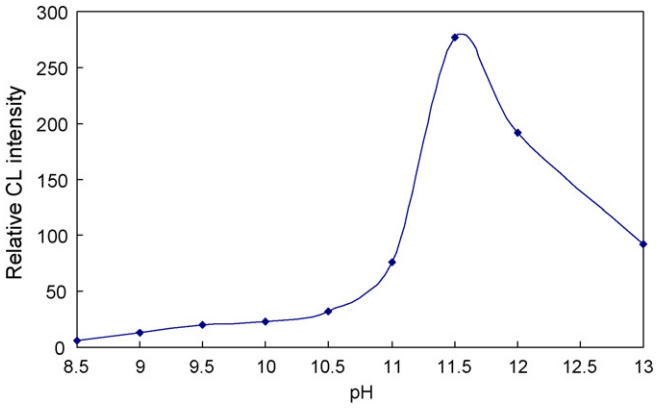

Fig. 5. Effect of pH of buffer solution on the response of the biosensor. Luminol, 5×10^{-4} M; H_2O_2, 1×10^{-5} M.

higher concentration glutaraldehyde (>1.0%) resulted in lower CL intensity because higher glutaraldehyde concentration may change the conformation of DNAzyme and lead to the decrease in sensitivity of the biosensor. As a result, 1.0% (w/w) glutaraldehyde solution was chosen as the optimum cross-linking agent for DNAzyme immobilization of this biosensor.

The duration of DNAzyme immobilization on eggshell membrane would affect the amount of immobilized DNAzyme on the eggshell membrane, which is related to the sensitivity of the biosensor. So, the immobilization time from 2 to 24 h was also investigated in our studies. The result was shown that the CL intensity increases with increasing the immobilization time from 2 to 10 h because the amount of immobilized enzymes on the eggshell membrane increases. But above immobilization time of 10 h, the CL intensity declined. The possible reason is that the long-term contact of the DNAzyme with glutaraldehyde in feed solution would destroy the intermolecular hydrogen bonds of the DNAzyme, decreasing the activity of the DNAzyme. Thus, 10 h of enzyme immobilization time was used throughout our studies. Of course, the temperature of immobilization influenced the effect of immobilization time, and for experimental convenience, adding reagent was at room temperature (about 20 °C).

3.4. Optimization of CL detection condition

As the luminescence reagent in this system, luminol concentration affects the biosensor response. The effect of luminol concentration in the range of 5×10^{-6}–2×10^{-3} M was investigated. The result showed that the optimal concentration of luminol was 5×10^{-4} M.

In this CL flow system, the carrier solution (0.025 M phosphate buffer solution) is the medium of enzyme reaction and the CL reaction. The experimental results (Fig. 5) showed that the maximal response of the biosensor was obtained at pH 11.5. It is obvious that the DNAzyme possesses high peroxidase-like activity in strong alkaline medium. Travascio et al. (1998) investigated in detail the pH dependences of the DNAzyme with 2,2′-azinobis(3-ethylbenzothiozoline)-6-sulfonic acid (ABTS) as the substrate, and they found the activity of the DNAzyme at pH 9 was higher than that of it at pH 6–7, which agreed with the result in our experiment. In view of the nature of luminol CL reaction, which is more favored under basic conditions, an alkaline medium would improve the sensitivity of the system. HRP is often used in CL biosensor (Li et al., 2008), and the optimal pH value for HRP is 6–7, which would limit the sensitivity of CL biosensor. Thus the DNAzyme could substitute HRP to be used in CL biosensor.

Flow rate is a key factor for this flow sensor, which decides the analytical speed efficiency and sensitivity of the biosensor. The influence of flow rate on the sensor response was investigated. The experimental results showed that the CL response increased with increasing flow rate in 0.5–3.0 mL/min range, probably because the luminol CL reaction was a fast process. However, above the flow rate of 3.0 mL/min, the CL response declined. This was attributed to the fact that the higher flow rate with shorter contact time of H_2O_2 and the immobilized DNAzyme could result in insufficient reaction of DNAzyme immobilized in the CL flow cell. Therefore, a flow rate of 3.0 mL/min was selected as optimum.

3.5. Lifetime of the biosensor for H_2O_2

The lifetime of this biosensor mainly depended on the enzymatic stability and activity. The operational stability of the biosensor was studied by recording over 200 successive assays of 1×10^{-5} M H_2O_2, and the relative standard deviation (RSD) was less than 2%. The storage stability of the immobilized DNAzyme reactor at 4 °C and room temperature (20 °C) was examined by checking periodically its relative activity. We found that after 100 days the activity of immobilized enzymes dropped to approximately 87% of its initial value, and the response of this biosensor for same H_2O_2 solution almost kept constant over 3-month storage period. Moreover, the storage stability of the DNAzyme reactor at room temperature (20 °C) was almost same as it at 4 °C. Therefore, room temperature (20 °C) was chosen as the storage temperature for this DNAzyme reactor, making this biosensor simple and convenient. Of course, it is necessary to bear in mind that both the ends of the immobilized DNAzyme reactor were sealed to avoid the membrane losing most of the water and causing cracking during storage period at room temperature (20 °C). Obviously, the high stability and high activity of DNAzyme immobilized on eggshell membrane were obtained. The remarkable stability of the DNAzyme immobilized on eggshell membrane is possibly related to the biological compatibility of the eggshell membrane. The eggshell membrane with net-veined structure and the gas-permeable hydrophilic property is mainly composed of biological molecules and protein fibers, and eggshell membrane can provide a biocompatible micro-environment. On the other hand, the G-quartet-based DNAzyme formed by hemin and 18-mer DNA aptamer is rather stable at room temperature (Li et al., 2007b), which is other reason for good stability of this biosensor.

3.6. Performance of the flow biosensor for H_2O_2 measurements

Under the selected conditions, response to H_2O_2 concentration was linear in the range of 1×10^{-5}–1×10^{-7} M with a detection limit of 5×10^{-8} M (3σ). The regression equation was $I = 6.44 + 2.99 \times 10^{7}$ [H_2O_2] (M) with a correlation coefficient of 0.9979 ($n = 7$). A complete analysis for the determination of H_2O_2 could be performed in 1 min, including sampling and washing, giving a throughput of ca. 60 h^{-1}. The relative standard deviation was less than 2% for 1×10^{-6} M H_2O_2 ($n = 11$). Furthermore, we prepared the three DNAzyme reactors with the same process, and the relative standard deviation of the three DNAzyme reactors was 9% for 1×10^{-6} M H_2O_2.

The apparent Michaelis–Menten constant ($K_{M,app}$), which gives an indication of the enzyme-substrate, can be obtained from the Michaelis–Menten equation: $v = v_{max}[S]/(K_{M,app} + [S])$, where v is rate of reaction, v_{max} is the maximum rate of reaction measured under saturated substrate condition, and [S] is the bulk concentration of the substrate. The $K_{M,app}$ was determined by analysis of the slop and intercept for the plot of the reciprocals of the rate of reaction versus substrate concentration. In this system, the $K_{M,app}$ of the DNAzyme immobilized on eggshell membrane was 1.03×10^{-4} M. For the control experiment, the $K_{M,app}$ of the HRP

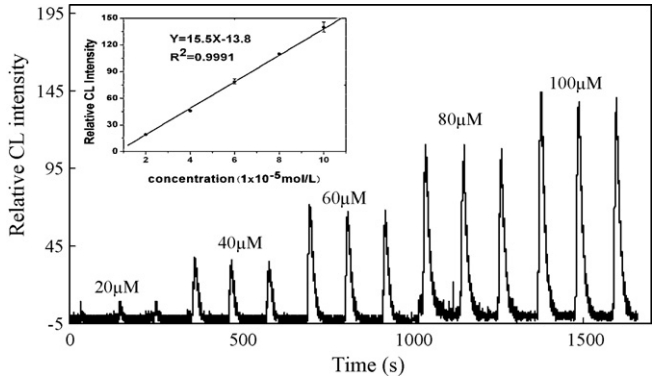

Fig. 6. Measurement results of a series of glucose standard solutions with the biosensor.

immobilized on eggshell membrane was also measured, and it was 5.12×10^{-4} M. The $K_{M,app}$ of the DNAzyme was smaller than it of HRP in this condition, indicating better affinity to H_2O_2, and high enzymatic activity for H_2O_2 reduction. Because hemin could not be immobilized stably on eggshell membrane, we compared the peroxidase activity of hemin with that of the DNAzyme in solution. In the presence of the G-rich DNA oligomers, the CL intensity of luminol–H_2O_2–hemin was enhanced more than 25-fold (see Supporting Information Figure S5). This suggested that the DNAzyme (complexes of hemin and 18-mer DNA aptamer) had significantly higher peroxidase activity than hemin alone.

In this study, influences of foreign species on the determination of 1×10^{-6} M H_2O_2 to which increasing amount of interfering species were added. The tolerable amounts of interfering species were added. The tolerable limit of a foreign species was taken as a relative error not >5%. The tolerable ratio of foreign ions to 1×10^{-6} M H_2O_2 was over 1000 for Na^+, K^+, Al^{3+}, Cl^-, F^-, Br^-, NO_3^-, HCO_3^-, Ac^- and SO_4^{2-}, 100 for Ba^{2+}, Ca^{2+}, Zn^{2+}, Mg^{2+}, HPO_4^{2-}, $C_2O_2^{2-}$ and NH_4^+, 50 for I^- and NO_2^-, 20 for Fe^{3+} and SO_3^{2-}, 10 for Fe^{2+} and S^{2-}. Equal amounts of Mn^{2+}, Co^{2+}, Cu^{2+} and Cr^{3+} positively interfered with the determination of hydrogen peroxide because these metal ions could catalyze the CL reaction of luminol–H_2O_2. The interference of these metal ions could be eliminated by adding EDTA into sample if necessary. Organic molecules, such as ethanol, methanol, formaldehyde, benzene, toluene, petroleum ether, acetone, phenol, dichloromethane and chloroform, did not affect the CL intensity.

3.7. Glucose detection with this DNAzyme-based biosensor and glucose oxidase

It is obvious that the determination of H_2O_2 is of great importance in enzyme reaction. The biosensor for H_2O_2 coupled with enzymatic molecular recognition could be applied to many important substrates. In order to demonstrate the present biosensor available for the determination of H_2O_2 production during the process of the enzymatic catalytic reaction, the enzymatic reaction of glucose oxidase (GOD) catalyzing glucose oxidation and H_2O_2 production was applied. GOD was also immobilized on eggshell membrane, and the enzyme column was prepared with our reported method (Li et al., 2008). The immobilized GOD column was placed in the sample stream for the detection of glucose. In Fig. 6, we can see that the CL intensity increases gradually with increasing glucose concentration. The emission intensity (I) versus glucose concentration (C) was linear in the range of 1×10^{-5}–1×10^{-3} M. The calibration equation in 1×10^{-5}–1×10^{-4} M range was $I = 15.5C - 13.8$ (glucose concentration, $\times 10^{-5}$ M) with a correlation coefficient of 0.9995 ($n = 5$); the calibration equation in 1×10^{-4}–1×10^{-3} M range was $I = 409.8C - 29.4$ (glucose concentration, $\times 10^{-4}$ M) with a correlation coefficient of 0.9987 ($n = 5$). The RSD was 3.4% for 1×10^{-4} M glucose ($n = 11$). The immobilized GOD mini-column could be reused for more than 100 times. Interference studies showed that foreign species present in serum such as uric acid, urea, ascorbic acid, cholesterol and lactic acid would not interfere with the determination of glucose at their normal concentration levels. It indicates that the biosensor can also be used to monitor the enzymatic catalytic reaction.

The biosensor was used to detect glucose in serum samples. The serum samples from some volunteers were collected from Shaanxi Normal University Hospital and used as testing samples. The fresh serum samples were first analyzed with a BT3000 Biochemical Analyzer (a commercial colorimetric assay kit using spectrophotometric method) in Shaanxi Normal University Hospital, and the samples were then re-assayed with the proposed biosensor. The sample was diluted 100 times by water to yield a testing sample solution. The response of the sample solution was measured and compared with that a set of glucose standard solutions. The results with the present biosensor agreed with those obtained by the hospital method (see Supporting Information Table S1).

4. Conclusions

In this paper, a DNAzyme-based CL flow biosensor for H_2O_2 was proposed. The peroxidase-like DNAzyme formed by hemin and G-rich DNA aptamer was successfully immobilized on eggshell membrane, and one immobilized DNAzyme reactor could be reused for more than 200 times. Compared to the HRP-based biosensor for H_2O_2, the DNAzyme possessed high catalytic activity in strong alkaline medium, which was well compatible with the luminol CL system. Moreover, the DNAzyme reactor could stored at room temperature (20 °C) with good stability over 3-month storage period. We anticipate that the lifetime should be much longer as we are still in the progress of monitoring its lifetime monthly. The biosensor exhibited high reproducibility, fast response, slight lower detection, and simple sensor assembly. Coupled with enzymatic molecular recognition, the biosensor could be act as stable recognition and catalytic units for the detection of various analytes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2009.01.010.

References

Aissaoui, H., Bachmann, R., Schweiger, A., Woggon, W.D., 1998. Angew. Chem., Int. Ed. 37, 2998–3002.
Borisov, S.M., Wolfbeis, O.S., 2008. Chem. Rev. 108, 423–461.
Breaker, R.R., 1997. Chem. Rev. 97, 371–390.
Breaker, R.R., 2000. Science 290, 2095–2096.
Cach, T.R., Zhang, A.J., Grabowski, P.J., 1981. Cell 27, 487–496.
Castriciano, M.A., Romeo, A., Baratto, M.C., Pogni, R., Scolaro, L.M., 2008. Chem. Commun., 688–690.
Chen, K., Que, L., 1999. Angew. Chem. Int. Ed. 38, 2227–2229.
Chen, X., Zhao, T., Zou, J., 2009. Microchim. Acta 164, 93–99.
Elbaz, J., Shlyahovsky, B., Willner, I., 2008. Chem. Commun., 1569–1571.
Fruk, L., Niemeyer, C.M., 2005. Angew. Chem. Int. Ed. 44, 2603–2606.
Gao, L., Zhuang, J., Nie, L., Zhang, J., Zhang, Y., Gu, N., Wang, T., Feng, J., Yang, D., Perrett, S., Yan, X., 2007. Nat. Nanotechnol. 2, 577–583.
Ghosh, M., Conroy, J.L., Seto, C.T., 1999. Angew. Chem. Int. Ed. 38, 514–516.

Gill, R., Polsky, R., Willner, I., 2006. Small 2, 1037–1041.
Groves, J.T., 1997. Nature 389, 329–330.
Guerrier-Takada, C., Gardiner, K., Marsh, T., Pace, N., Altman, S., 1983. Cell 35, 849–857.
Han, M.J., Yoo, K.S., Chang, J.Y., Ha, T.K., 2000. Angew. Chem. Int. Ed. 39, 347–349.
Huang, Y.P., Ma, W.H., Li, J., Cheng, M.M., Zhao, J.C., Wang, L.J., Yu, J.C., 2003. J. Phys. Chem. B 107, 9409–9414.
Ito, Y., Hasuda, H., 2004. Biotechnol. Bioeng. 86, 72–77.
Kirby, A.J., 1996. Angew. Chem. Int. Ed. 35, 707–724.
Li, B., Lan, D., Zhang, Z., 2008. Anal. Biochem. 374, 64–70.
Li, B., Zhang, Z., Jin, Y., 2001a. Anal. Chem. 73, 1203–1206.
Li, B., Zhang, Z., Zhao, L., 2001b. Anal. Chim. Acta 445, 161–167.
Li, D., Shlyahovsky, B., Willner, I., 2007a. J. Am. Chem. Soc. 129, 5804–5805.
Liong, J.W.W., Frank, J.F., Bailey, S., 1997. J. Food Prot. 60, 1022–1028.
Li, T., Dong, S., Wang, E., 2007b. Chem. Commun., 4209–4211.
Li, Y., Geyer, C.R., Sen, D., 1996. Biochemistry 35, 6911–6922.
Miao, J., Cao, Z., Zhou, Y., Lau, C., Lu, J., 2008. Anal. Chem. 80, 1606–1613.
Molenveld, P., Engbersen, J.F.J., Reinhoudt, D.N., 1999. Angew. Chem. Int. Ed. 38, 3189–3192.
Niazov, T., Pavlov, V., Xiao, Y., Gill, R., Willner, I., 2004. Nano Lett. 4, 1683–1687.
Nissen, P., Hansen, J., Ban, N., Moore, P.B., Steitz, T.A., 2000. Science 289, 920–930.
Pavlov, V., Xiao, Y., Gill, R., Dishon, A., Kotler, M., Willner, I., 2004. Anal. Chem. 76, 2152–2156.
Signor, L., Knuppe, C., Hug, R., Schweiger, B., Pfaltz, A., Jaun, B., 2000. Chem. -Eur. J. 6, 3508–3516.
Sono, M., Roach, M.P., Coulter, E.D., Dawson, J.H., 1996. Chem. Rev. 96, 2841–2888.
Takiguchi, M., Igarashi, K., Azuma, M., Ooshima, H., 2006. Cryst. Growth Des. 6, 2754–2757.
Travascio, P., Li, Y., Sen, D., 1998. Chem. Biol. 5, 505–517.
Travascio, P., Witting, P.K., Mauk, A.G., Sen, D., 2001. J. Am. Chem. Soc. 123, 1337–1348.
Wang, J., 2008. Chem. Rev. 108, 814–825.
Wang, Q., Yang, Z., Zhang, X., Xiao, X., Chang, C.K., Xu, B., 2007a. Angew. Chem. Int. Ed. 46, 4285–4289.
Wang, X., Na, N., Zhang, S., Wu, Y., Zhang, X., 2007b. J. Am. Chem. Soc. 129, 6062–6063.
Wei, H., Wang, E., 2008. Anal. Chem. 80, 2250–2254.
Willner, I., Shlyahovsky, B., Zayats, M., Willner, B., 2008. Chem. Soc. Rev. 37, 1153–1165.
Wulff, G., 2002. Chem. Rev. 102, 1–27.
Xiao, D., Choi, M.M.F., 2002. Anal. Chem. 74, 863–870.
Xiao, Y., Pavlov, V., Gill, R., Bourenko, T., Willner, I., 2004. ChemBioChem 5, 374–379.
Yang, W., Zheng, H., Yuan, W.T., Xu, J.G., 2004. Anal. Sci. 20, 1265–1270.
Zen, J.-M., Kumar, A.S., 2001. Acc. Chem. Res. 34, 772–780.
Zhang, Y., Phillips, G.J., Yeung, E.S., 2008. Anal. Chem. 80, 597–605.
Zhang, Z., Zhang, S., Zhang, X., 2005. Anal. Chim. Acta 541, 37–46.
Zou, G.L., Zhu, R.F. (Eds.), 1997. Enzymology. Wuhan University Press, Wuhan, pp. 1–29.