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A magnetically active microfluidic device for chemiluminescence bioassays

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Abstract

Highly active horseradish peroxidase functionalized magnetic nanoparticles were prepared and packed into a microfluidic channel, producing an in-line bioreactor that enabled a sensitive chemiluminescence assay of H₂O₂. The proposed magnetically active microfluidic device proved useful for chemiluminescence assays of biomedically interesting compounds.

Micro- and nanofluidic devices are increasingly explored for chemical analysis because of the numerous advantages they promise, including the possibility to integrate sample injection, pre-concentration, digestion /cell lysing, separation, and detection onto one single chip.^{1–3} However, since the channels in microfluidic devices are very small a sensitive detection mode is required for chemical analysis performed on a microfluidic platform. Laser induced fluorescence (LIF) detection has been most often deployed to achieve the assay sensitivity desired, particularly for single cell analysis.^{4–7} Chemiluminescence (CL) detection is another sensitive detection scheme used in microscale separations such as capillary electrophoresis (CE). Compared with LIF, CL detection offers many advantages such as high detection sensitivity, a wide linear range for quantification, and no need for a light source that allows the use of a simple instrumental set-up.⁸ In addition, since a CL detector can be easily miniaturized this detection technique is unequally well suited for in-line detection in microfluidic assays. Several microchip electrophoresis (MCE) systems with CL detection were reported.^{9–12} Recently, we reported our efforts in improving MCE-CL assay sensitivity, which included intracellular CL labeling¹³ and chemiluminescence resonance energy transfer (CRET)-based CL detection.¹⁴ The sensitivity was significantly improved with limits of detection (LODs) in the range from 10^{–7} to 10^{–9} M analytes.

Luminol-H₂O₂-horseradish peroxidase (HRP) CL system is widely used in immunoassays as well as in HPLC or CE-based methods.^{15–16} In CE-CL applications, the enzyme is normally added into CE running buffer that also contains the chemiluminescence reagent. A major disadvantage of solution-phase enzymatic assays is associated with poor enzyme stability. In addition, the enzyme can hardly be re-used which increases assay cost. Immobilizing an enzyme on a solid carrier improves its stability and allows its re-use. Immobilization techniques including physical adsorption, ionic binding, covalent binding, and sol-gel entrapment were developed.¹⁷ Use of magnetically active microfluidic devices is an effective approach to achieve enzyme immobilization. In these devices, external permanent magnets are used to immobilize enzymes grafted on magnetic nanoparticles (MNPs). A

major advantage of this approach is to allow the preparation of easily replaceable enzyme microreactors in a microfluidic device.^{18–20} Herein we report the development of a magnetically active microfluidic device for CL bioassays.

In order to prepare a highly stable and active HRP-MNP conjugate, covalent bonding via a coupling agent was studied in this work. First, a conventional alcohol-based silanization protocol with 3-aminopropyl-triethoxysilane (APTES) was carried out to prepare amine-terminated MNPs. These MNPs were then activated with glutaraldehyde that served as the cross linker and spacer. An HRP solution (5 mg/mL) was added. The solution was incubated for 24 hrs. At the end of incubation, the coupling yield was found to be ~90%.

Enzymatic activity of the prepared HRP-MNP conjugate was assessed by examining its enhancing effects on luminol-H₂O₂ CL emission with a luminescence spectrometer. It was noticed that CL emission was nearly not observable from a luminol-H₂O₂ reaction solution containing 10⁻³ M luminol and 10⁻⁵ M H₂O₂ at pH 8.6. However, the CL signal became very strong from the same solution in the presence of HRP-MNPs. To investigate the reusability, the HRP-MNPs were separated from the reaction solution by using a magnet. The solution was removed with a pipette. New solutions were added to suspend the HRP-MNPs in the cell. CL emission spectrum was recorded immediately after the addition of H₂O₂. The procedure was repeated 20 times. No decrease in CL signal was observed. Reproducibility of CL intensity at 430 nm was found to be 5.2% (RSD, n= 20). HRP-MNPs were stored in a storing buffer at 4 °C. After 2 months in storage, the enzymatic activity of the HRP-MNPs remained >90% as calculated from the catalyzed luminol-H₂O₂ CL signal. Our study also found that a free HRP solution (5 mg/mL in the same storing buffer) lost about 62% of its activity after being kept at 4 °C for one week. Based on these results, HRP-conjugated MNPs exhibited high enzymatic activity on catalysis of luminol-H₂O₂ CL reaction. Moreover, immobilization of HRP onto MNPs greatly increased the stability of the enzyme and allowed its reuse in chemical operations.

Integrating an HRP-MNP bioreactor onto a microfluidic-CL platform possesses a great potential in chemical analysis. The in-channel bioreactor can be easily prepared by placing a piece of magnet underneath the microchip to retain HRP-MNPs at a specific point of a channel as shown in Fig. 1. In this work, an HRP-MNP bioreactor was prepared at the luminol-H₂O₂ mixing point on the microchip. A photomultiplier tube (PMT) was placed right above the bioreactor to collect CL signal. The HRP-MNP microfluidic CL device was found very useful for H₂O₂ determination Figure 2 shows an electropherogram obtained from consecutive injections of standard H₂O₂ solutions at concentrations ranging from 2 to 100 μM. Linear regression analysis of the results yielded the following calibration equation:

$$H=3.35 C-4.9 \quad r^2=0.996$$

where H is the peak height and C is the concentration of H₂O₂ (μM). The calibration curve showed an excellent linearity with a correlation coefficient of 0.996. Based on signal /noise ratio [S/N] = 3, the detection limit for H₂O₂ was estimated to be 0.5 μM. Assay repeatability was investigated by analyzing a standard solution of H₂O₂ at 5.0 μM 9 times. The results showed that RSD of peak heights was 3.1% (n = 9). It's worth noting that the HRP-MNP bioreactor was easily replaceable. To do this, the external magnet was removed and the channels were flushed with water by applying a vacuum at reservoir W. The magnet was then placed back to its position and reservoir R was filled with a HRP-MNP suspension. A dynamic flow from reservoir R to reservoir BW was generated and HRP-MNPs were retained at the specific point in the channel above the external magnet. This process was monitored with a microscope. When a ~1 mm long HRP-MNPs bed was obtained in the

channel, the MNP suspension in reservoir R was replaced by water, thus completing the preparation of the in-channel bioreactor.

Many enzyme-catalyzed reactions of organic compounds involve producing H_2O_2 . Therefore, the proposed H_2O_2 assay has a potential of being used for the quantification of these organic compounds. To demonstrate the applicability, glucose was selected as the proof-of-concept analyte. First, glucose oxidase (GOx) functionalized MNPs were prepared by using the same procedure used for the preparation of HRP-MNPs. GOx-MNPs were then packed into the cross intersection of the sample loading channel and the separation channel as indicated in Fig. 1. After a sample was injected, 1-min incubation was carried out before the assay was started. Figure 3 shows an electropherogram obtained from consecutive injections of glucose standard solutions and several soft drink samples. From the analysis of the standard solutions, a linear calibration curve was obtained ($H = 0.143 C + 0.43$, $r^2 > 0.98$). The assay sensitivity (LOD) was estimated to be $5.2 \mu M$ glucose. The present assay was far more sensitive than a previously reported MCE-CL assay (LOD = $0.1 mM$)²¹ and comparable with the MCE methods with electrochemical (LOD = $1.2 \mu M$)²² and confocal fluorescence detection (LOD = $0.92 \mu M$, needing pre-column tagging of glucose).²³ Wang and Kennedy et al.²⁴ recently reported a microfluidic segmented flow system to improve temporal resolution for microdialysis. With this system, high through-put assay of glucose was achieved based on a fluorescent reaction involving Amplex® red and GOx/HRP as reagents. The assay had a linear response up to $2 mM$ glucose and a detection limit of $50 \mu M$ glucose. To evaluate the repeatability of the proposed glucose assay, a standard solution of glucose ($35.0 \mu M$) was analyzed 9 times. The relative standard deviation (RSD) in peak height was found to be 2.8%.

In summary, highly active enzyme-functionalized magnetic nanoparticles (MNPs) were prepared and used to produce easily replaceable in-line bioreactors on a microfluidic chip which enabled sensitive microfluidic chemiluminescence assays. Two enzyme-MNP conjugates, i.e. HRP-MNP and GOx-MNP were studied. The immobilized enzymes on MNPs showed a good catalytic activity, durability and reusability. The proposed assays of H_2O_2 and glucose proved to be sensitive, specific, quick, and easy to carry out. To the best of our knowledge, this report is the first on a magnetically active microfluidic device developed for CL bioassays.

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Fig. 1. Schematics of the proposed magnetically active microfluidic CL assay device: a) dimensions and layout of the microchip (S: sample reservoir; B: buffer reservoir; SW: sample waste reservoir; BW: buffer waste reservoir; and R: luminol reservoir), and b) side view of the set-up. To perform a CL assay, electrical potentials were applied to the reservoirs as following: reservoir S at 700 V, reservoir B at 250 V, reservoir BW at 350 V, reservoir SW at grounded, and reservoir R floating for 20 s to load a sample, and the potentials applied were changed as following: reservoir B at 2300, S at 1400, SW at 1400, R at 350 V, and BW grounded to start the assay.

Fig. 2. Microfluidic CL responses obtained from consecutive injections of standard H_2O_2 solutions: a) 20.0 μM ; b) 10.0 μM ; c) 4.0 μM ; and d) 2.0 μM .

Fig. 3.

Microfluidic CL responses obtained from consecutive injections of standard glucose solutions and drink samples: a) 350 μM ; b) 200 μM ; c) 150 μM ; d) 40 μM ; and e) 20 μM glucose.