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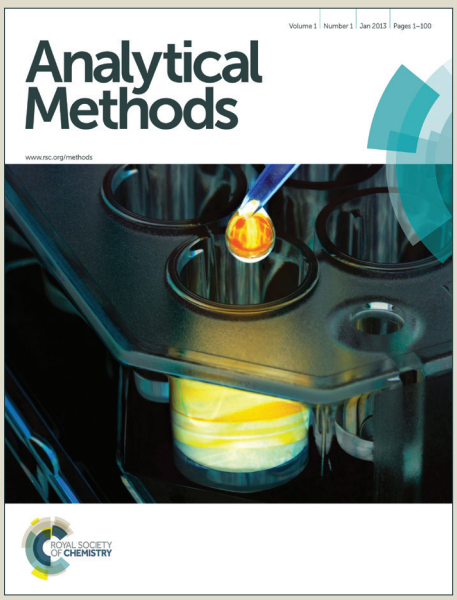

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ARTICLE

Magnetic Separation/Enrichment-mediated Signal Amplification for Simple and Sensitive Fluorometric Assay of Biotin

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Many types of separation-based sensing systems have been reported for biotin assay. Although these conventional strategies provide accurate, sensitive detection of biotin, there are still some inconveniences that exist, such as the complex sample treatment, time-consuming assay process, and technical expertise as well as the sophisticated equipment. We have addressed these limitations and report herein the proof-of-principle of a dual strategy which combines magnetic separation/enrichment with DNA/SG (SYBR Green I)-based signal amplification to develop a simple and sensitive fluorometric biotin sensing strategy. This method is based on the competition scheme where biotin and the biotinylated dsDNA compete for the binding sites of streptavidin coated on the magnetic beads surface (SA-MB). After separation and enrichment under the magnetic field, the fluorescence emission intensity or fluorescent images can be obtained by addition of SG, which is inversely related to biotin concentrations. Using the biotin solution as a model system, we demonstrated that our assay can detect biotin at a concentration as low as 1.19 ng/mL in one hour which highly excels traditional assays such as HPLC. Moreover, we also used the proposed method to measure the biotin level in actual samples for example flour and peanut, satisfactory results can be obtained.

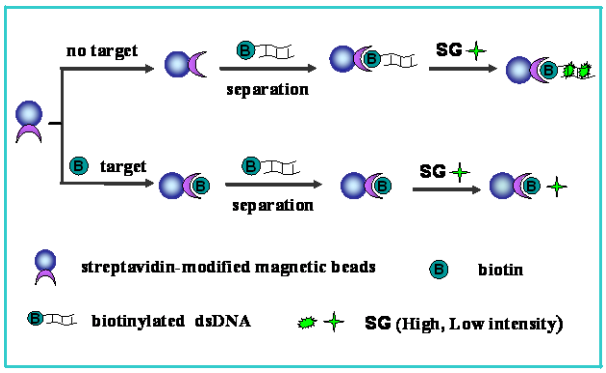
Introduction

Biotin is a water-soluble vitamin (Vitamin H), generally classified as a B-complex vitamin, playing an important biochemical role in living cells.[1-4] This vitamin is synthesized in a wide variety of bacteria and plants. However, several microorganisms as well as higher animals are notable to synthesize it and their needs in this vitamin are met by dietary intake.[2] Clinical findings of biotin deficiency may cause developmental delay in infants and children. Recent evidence suggests that biotin deficiency and the accelerated catabolism during the normal pregnancy might cause birth defects[2,4]. Furthermore, as a cofactor for necessary metabolic enzymes, the alterations in its content and the enzymes to which it is attached have been correlated with colorectal cancer and other diseases[5,6]. Consequently, it is very important to realize rapid and sensitive diagnosis of biotin deficiency and monitoring its levels in patients receiving biotin treatment, as well as the determination in foods. The detection of biotin is generally performed by use of some methods, such as polarography, thin layer chromatography, high-performance liquid chromatography and capillary zone electrophoresis[7-10]. Although these are well-established methods widely used, they are complex and time-consuming, often lack sensitivity, and sometimes rely on sophisticated and expensive instruments to process the signal. Therefore, the development of assay methods that allow rapid, simple, sensitive, selective, on-site and cost-effective detection of biotin is still highly desirable, especially in rural areas of developing countries.

To meet the challenges in biotin assay, new sensing carriers and highly sensitive signal transduction strategies should be explored and engineered together. Various functional molecules (surface anchor) can be incorporated at desirable position of a DNA sequence without compromising their functions.[11-13] While a DNA sequence can be synthesized in a large scale by a DNA synthesizer with almost no batch-to-batch variation, many organic dye molecules (signaling molecules) can be intercalated into its double-stranded structures. There signaling molecules have been widely used to develop DNA sequence-based probes for fluorescent,[14-18] quartz-crystal microbalance[19] or electrochemical[20-22] assay methods for a broad range of targets. Magnetic particles with the properties of easy separation and enrichment amplification, have also been found potential advantages in bioassay development.[20] However, to the best of our knowledge, no research efforts have yet found a way to combine these strategies into one system for the sensitive assay of biotin. So, due to the excellent properties of synthetic nucleic acid and the magnetic particles in the aspects of design, synthesis, chemical stability, separation process and enrichment, this combination might be an optimal choice for the development of satisfactory biotin assay method.

Herein we propose a simple and sensitive fluorometric assay strategy for biotin detection using magnetic separation and enrichment coupled to double-stranded DNA (dsDNA)/SG probe system (SG is DNA intercalating dyes, SYBR Green I) which activates large number of non-fluorescent SG dyes to produce

enhanced fluorescent read out signal (Scheme 1). The present strategy is based on a competition scheme where biotin and the biotinylated dsDNA compete for the binding sites of streptavidin immobilized on the magnetic beads surface (SA-MB). Specifically, the SA-MB is utilized to capture the biotinylated dsDNA and the captured dsDNA is inversely related to biotin concentrations. After separation and enrichment through using a permanent magnet, the fluorescence emission intensity or fluorescent images can be obtained by addition of SG. This assay method shows very high sensitivity and good detection limits. Moreover, we also used the proposed method to measure the biotin level in actual samples for example flour and peanut, very satisfactory results were obtained.



Scheme 1. Schematic representation of the sensing procedure for detection of biotin based on the magnetic separation/enrichment and fluorescence signal amplification strategy.

Experimental Section

Reagents and instruments

The biotinylated DNA sequence S1 (5′-CATTGAAGGATTTTCCTTGTCTCCC-biotin-3′) and its complementary sequence S2 (5′-GGGAGACAAGGAAAATCCTTCAATG-3′) were synthesi -zed by Sangon Biotechnology Co. Ltd. (Shanghai, China). Streptavidin-modified magnetic microbeads (SA-MB, 1.0 μm diameter, 4 mg/mL) were obtained from New England BioLabs (Beijing, China). SYBR Green I (SG 10,000× concentrate) and biotin were purchased from Shanghai Biological Engineering Technology & Services Co. Ltd. Glucose were purchased from Hunan Ketai Biotechnology Co. Ltd. (Changsha, China). ATP (Adenosine triphosphate) and Adenosine were purchased from Sangon Biotechnology Co. Ltd. (Shanghai, China). HAS (Human serum albumin) and IgG (human immunoglobulin G) were obtained from Dingguo Biochemical Reagents (Beijing, China). Other chemicals were of analytical grade and were used without further purification. We used an affimag 96 magnetic stands purchased from Baseline Chrom Tech Research Centre (Tianjin, China) for beads separation. Distilled water (≥18.2 M) generated by a Milli-Q water purification system (Millipore, 96 Bedford, MA, USA) was used throughout the experiments.

The fluorescence intensity assays were performed on an F-4500 FL Spectrophotometer equipped with a personal computer data processing. The fluorescence spectra were excited at 490 nm and the emission spectra from 510 to 600 nm were recorded. Both the excitation and emission slits were set for 5 nm. The fluorescence images were carried out with an Olympus IX71 Inverted Microscope (ANDOR TECHNOLOGY PLC). All measurements were carried out at room temperature unless stated otherwise.

Preparation of biotinylated dsDNA

The biotinylated dsDNA was prepared according to the following procedure: 10 μM S1 and 10 μM S2 in PBS buffer (10 mM, PH 7.3, 50 mM NaCl, 5 mM $MgCl_2$) were first heated to 95 ℃ for 5 min to ensure a single-stranded structure. Then this solution was incubated for two hours at 37 ℃ to guarantee the formation of double-stranded DNA structure. Finally, the obtained biotinylated dsDNA was cooled down to room temperature and stored at 4 ℃ for later use.

Detection of biotin

SA-MB was first rinsed twice by using PBS (10 mM, PH 7.3, 50 mM NaCl, 5 mM $MgCl_2$) and then samples with various concentrations of biotin were added into 100 μL SA-MB-contained PBS (SA-MB is 0.08 mg/mL). After incubation for 30 min at room temperature under shaking by a oscillator at a shock speed of 300 rpm, 8 μL of 1.0 μM biotinylated dsDNA was added into this mixture and another 30 min-shaking incubation process was carried out at room temperature. Finally, the reaction mixture was placed on the magnetic racks to collect the magnetic beads with the help of a permanent magnet. The obtained magnetic beads were washed three times with PBS and then resuspended in 100 μL PBS. After addition of 2 μL SG (50×) and incubated for 10 min at room temperature, the fluorescence spectra recording and fluorescence imaging were carried out by using FL Spectrophotometer or fluorescence imaging system, respectively.

Real sample assay

Two kinds of food samples, flour and peanut, purchased from a local market, were analyzed by using our proposed method. Each sample (1.0 g) was weighed and homogenized in 3 mL PBS buffer. After ultrasonic treatment for 30 min at room temperature, the homogeneous suspension of the samples was moved to 1.5 mL PE centrifuge tube to be centrifuged for 15 min at 12000 rpm. The supernatants were filtered through a 0.45 μm-filter to get rid of the large suspended materials. Taking extracting solution to detect the amount of biotin in flour and peanut respectively, according to the principle of the proposed sensing scheme. First, using extracting solution of flour and peanut 20 μL and 30 μL, respectively. Then SA-MB (0.8 mg/mL) was added 10 μL each into the two obtained sample solutions. After incubation for 30 min at room temperature, another 30 min incubation process was carried out by addition of 6 μL of 1.0 μM biotinylated dsDNA. The volume of the reaction is 100 μL by adding PBS buffer. Finally, the reaction mixture was placed on the magnetic racks to collect the magnetic beads with the help of a permanent magnet. The obtained magnetic beads were washed three times with PBS and then resuspended in 100 μL PBS. After addition of 2 μL SG (50×) and incubated for 10 min at room temperature, the fluorescence emission test or fluorescence imaging was carried out by using FL Spectrophotometer or fluorescence imaging system, respectively. In order to guarantee the reproducibility of the experiment, each sample was tested parallelly three times. The biotin concentration in food sample was ascertained from calibration curve and reported as ng biotin in 1.0 g sample.

Results and discussion

Sensing scheme

The sensing scheme of the approach is based on the competitive binding of biotinylated dsDNA to the streptavidin-modified magnetic beads and the magnetic separation/enrichment. Scheme 1 illustrates the signaling mechanism of the proposed approach for biotin assay. When biotin is not present, the biotinylated dsDNA would bind to the streptavidin-modified magnetic beads, while in the presence of biotin target the binding sites of the streptavidin-modified magnetic beads would be occupied by the target, thus the biotinylated dsDNA could not be adsorbed on the magnetic beads's surface. When magnetic separation is carried out with the help of a permanent magnet and the obtained beads is resuspended in SG-containing PBS buffer, high fluorescence signal would be obtained for the sample without biotin while the decreased fluorescence signal should be observed for the sample with biotin.

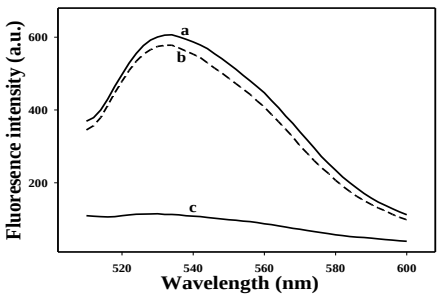


Fig. 1 Fluorescence emission spectra for the samples with or without biotin. (a) magnetic beads + PBS buffer + biotinlyted dsDNA + SG. (b) magnetic beads + no biotin + biotinlyted dsDNA + SG, and the concentration of biotin is 0 μg/mL. (c) magnetic beads + biotin + biotinlyted dsDNA + SG, and the concentration of biotin is 1 μg/mL. The detail experimental steps are described in experimental section.

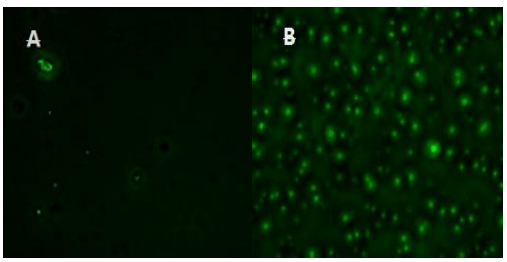


Fig. 2 The fluorescence microscopic images for the samples with or without biotin. (A) With biotin and the concentration is 10 μg/mL. (B) Without biotin. The detail experimental steps are described in experimental section.

Figure 1 shows the fluorescence emission spectra for the samples with or without biotins. One can see from the figure that no significant change in fluorescence emission intensity could be observed for the sample without addition of biotin (curve b), while a dramatically decrease in fluorescence intensity is observed when free biotin is present (curve c), indicating that the binding between biotinylated dsDNA and streptavidin-modified magnetic beads is occurred for the sample without biotin while the binding is prohibited when the free biotin is present, therefore resulting in a significant fluorescence signal response for the free biotin-containing samples. We also use fluorescence microscopic technique to further validate the principle of the proposed method. As shown in Figure 2, compared with the sample without free biotin (Figure 2B), the microscopic image for the sample with biotin is almost dark (Figure 2A). This indicates that the biotin assay can also be realized easily by using fluorescence microscope.

Optimization of the general procedures

In this study, several factors that may influence the final detection result were optimized. Since the concentration of biotinylated dsDNA is an important factor influencing the fluorescence sensing, we first explored the effect of the concentration of biotinylated dsDNA on the performance of the proposed method.

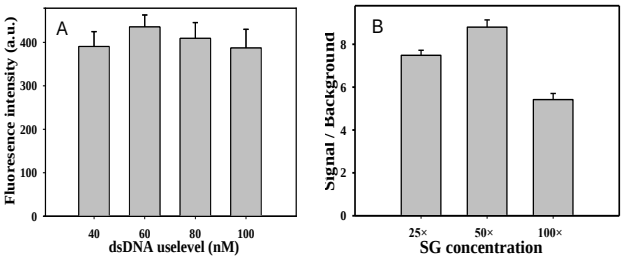


Fig. 3 (A) Fluorescence emission intensities at 532 nm of the reaction solution in the presence of biotinylated dsDNA with various concentrations. The concentration of SA-MB is 0.08 mg/mL and the used 50 × SG is 2 μL. (B) Influence of SG amount on the fluorescence response. The concentration of SA-MB is 0.08 mg/mL and the biotinylated dsDNA is 60 nM. The detail experimental steps are described in experimental section.

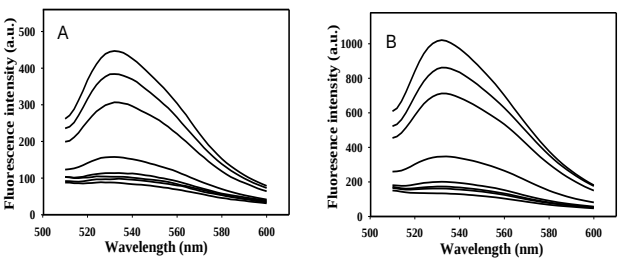


Fig. 4 Effect of SA-MB amount on the performance of the sensing detection. (A) The used SA-MB is 0.08 mg/mL and the concentrations of biotin (top to bottom: 0, 4, 10, 20, 40, 60, 80 ng/mL). (B) The used SA-MB is 0.16 mg/mL and the concentrations of biotin (top to bottom: 0, 10, 20, 40, 60, 80, 100 ng/mL). The concentration of biotinylated dsDNA is 60 nM and the amount of 50 × SG is 2 μL, which used samely for A and B. The detail experimental steps are described in experimental section.

Figure 3A shows the fluorescence emission intensities in the presence of biotinylated dsDNA with various concentrations. One can see from the histogram that the fluorescence emission intensity reached the maximum when the concentration of biotinylated dsDNA was more than 60 nM. So, 60 nM biotinylated dsDNA were used in the following experiments. In order to get a better signal/background ratio, the concentration of SG was also investigated. Figure 3B displays the influence of SG amount on the fluorescence response. The column chart revealed that 2 μL SG (50×) in 100 μL reaction solution were optimal.

Another important factor influenced the performance of the proposed method is the dosage of SA-MB. Figure 4 shows the detection range of biotin under different concentration of SA-MB. According to the linear equation calculated by the data of Figure 4 that a detection concentration range of 1.19 ng/mL to 23.90 ng/mL (F = – 16.62 C + 466.78) is obtained when 0.08mg/mL SA-MB was used, while a more wide detection range of 3.10 ng/mL to 51.66 ng/mL (F = – 18.28C + 1077.9) can be reached when 0.16mg/mL SA-MB was used, indicating that the detection range of biotin can be adjusted according to the dosage of magnetic beads.

Biotin sensing in aqueous buffer

Under optimal condition, we successively evaluated the capability of this proposed method for quantitative detection of biotin. Figure 5 shows the fluorescence emission spectra of the sensing system in PBS in the presence of different concentrations of biotin at room temperature. One can observe from Figure 5 that the fluorescence emission intensity at 532 nm is dramatically decreased upon increasing the biotin concentration, indicating that more and more binding sites of the SA-MB were occupied by the free biotin and the biotinylated dsDNA captured by the beads was decreased, thus resulting in the decrease in fluorescence emission intensity. The changes in fluorescence emission intensity at 532 nm of the sensing system were linearly related to the concentration of the biotin over the range from 0 to 24 ng/mL with a correlation coefficient (R) of 0.9971 and a linear regression equation of F= – 16.62 C + 466.78 (Inset of Figure 5), where 'F' represented the fluorescence emission intensity at 532 nm of the sensing system in the presence of different concentrations of biotin, and 'C' represented the concentration of biotin. The sensitivity and detection range of the proposed method was favorably compared to those reported in literature.[4]

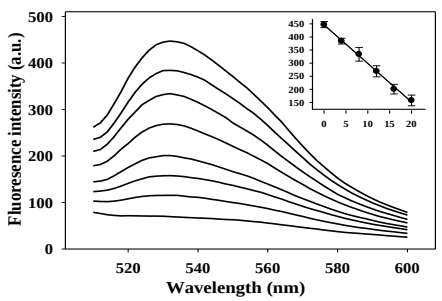


Fig. 5 The fluorescence spectra of the sensing system with different concentrations of biotin (top to bottom: 0, 4, 8, 12, 16, 20, 24 ng/mL). Inset: plot of the value of fluorescence emission intensity at 532 nm of the reaction solution as functions of the concentration of biotin. The magnitude of the error bars was calculated from the uncertainty given by three independent measurements. The used SA-MB is 0.08 mg/mL, biotinylated dsDNA is 60 nM and 50× SG is 2 μL. The detail experimental steps were described as experimental section.

Selectivity investigation

In order to confirm the change of fluorescence signal is caused by the specific binding between biotin and avidin, Glucose, ATP, Adenosine, IgG and HAS which usually exists in biological samples were chosen as chaff interferent to investigate the selectivity of the assay. Under the same experimental conditions, according to the different fluorescence signal response to the target and the interferents, the selectivity of the assay method can be examined. Fig.6 shows the selectivity histogram of the assay based on the analysis results of the samples. One can conclude from the histogram that the selectivity of this method for biotin is far greater than those interferents. It is indicated that the nonspecific binding combination of the interferent is negligible, and also confirmed the selectivity of the proposed assay is satisfactory.

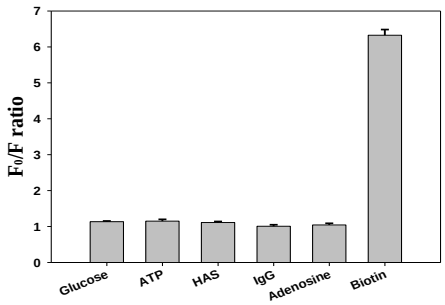


Fig. 6 Selectivity investigation of the proposed assay method. The concentration of biotin is 4 nM. The concentration of glucose, ATP and adenosine is 0.4 μM. Both HAS and IgG is 50 μg/mL. F_0/F is defined as the ratio of fluorescence emission intensity at 532 nm from the blank to that from the target or interferents.

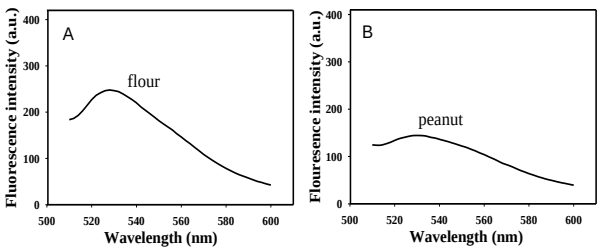


Fig. 7 The fluorescence emission spectra of the sensing system for two samples(A: flour; B: peanut). The detail experimental steps are described in experimental section.

Application of the sensing strategy in real sample

The vitamin H (biotin) is present in a wide variety of foodstuffs at low concentration levels, and majority of biotin in foods is in the free form or covalently bound to proteins of the food matrix. To demonstrate the feasibility of practical use of the proposed sensing strategy, the detection of biotin in flour and peanut was performed. The experimental process is described in experimental section and the fluorescence emission spectra of the sensing system for samples are shown in Figure 7 (A) and (B). According to Figure 7A of the obtained changes in fluorescence emission intensity at 532 nm and the above linear regression equation, the concentration of biotin is 200.77 ng/g in the flour sample. And according to Figure 7B of the obtained changes in fluorescence emission intensity at 532 nm, the concentration of biotin is 194.09 ng/g in the peanut sample. The biotin content values of the two real samples determined by the proposed methods are similar with that obtained from the foodstuffs data through the traditional detection methods.[23-25] The similar results by our proposed method and the traditional methods indicate that our proposed sensor method was feasible for biotin assay in real

samples.

Conclusion

In summary, we have developed a simple and sensitive fluorometric method for determination of biotin based on the magnetic separation/enrichment process and DNA sequence signal amplification strategy. The principle of our design is simple and can be used for actual sample assay. The determination of biotin in two kinds of food samples was successfully achieved by the proposed sensing strategy. In addition, the detection range of the sensing system can be adjusted according to the actual needs in assays. Due to the advantages of the dynamic detection range, high sensitivity and simplicity in operation, this proposed sensing strategy might find application for biotin assay in the actual samples. Moreover, the proposed sensing principle seems promising in the design of detection methods for other small molecules.

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The brief schematic diagram: