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Original Research

Optimized magnetic bead-based immunoassay for automated detection of protein toxins

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Abstract Rapid, accurate, and autonomous analysis of bioagents in the environment is critical in protecting human health from natural and intentional environmental contamination with biological toxicants. We previously developed and tested an immunoassay protocol that can be utilized for automated and simultaneous detection of selected biological agents and toxins. We adopted an antibody-based approach for the detection of pathogens and/or toxins. The fluorescent eTags™ were used as reporter molecules and the immunoassay was modified for automated field-deployed detection of pathogens and/or toxins. The present study improved the limit of detection of this system to be suitable for the detection of environmental toxins. We tested different settings to optimize the assay protocol and successfully detected 10 ng/mL (or 100 fg) of an toxin analog, ovalbumin. The developed assay represents a notable improvement from currently available assays in terms of reduced time, increased sensitivity, and automation potential. Additionally, this assay can be easily modified, with the appropriate antibodies, to detect a wide range of proteins and infectious agents.

Keywords: Immunoassay, Magnetic bead, Autonomous detection, Toxins, Fluorescent tag

Introduction

Environmental detection of biological contaminants such as protein toxins, bacterial spores, and virus particles is important for safeguarding human health and environmental health, and has various applications in the health, food, and defense industries[1,2]. The frequent recent outbreaks of viral diseases such as foot-and-mouth disease[3] and various influenzas[4] have highlighted the urgent need for the development of improved bio-detection protocols that can be used for early detection of biological contaminants. It is also desirable to develop an automated detection system that can be deployed in the field and operate without the constant need for personnel for continuous monitoring of the environment[5,6].

We previously reported the development of an automated immunoassay system that can be used for the automatic detection of pathogens[5]. The detection system comprises an aerosol collection system, three different assay trains for RNA, DNA and protein detection, and a capillary gel electrophoresis (CGE) detection system (Figure 1)[7-10]. The developed immunoassay was specifically designed for detecting protein toxins via the eTag™-based reporter system (Monogram Biosciences, CA)[11], which is a sensitive, selective, rapid, and multiplexible assay platform. In the eTag system, a fluorescence reporter consists of a fluorescent probe conjugated to a small molecule tag with a controllable migration property in CGE. The fluorescence emitting wavelength as well as the migration time in CGE can be varied in various eTags[12,13].

In performing assays, an eTag-conjugated antibody probe initially binds to the biological contaminant. Then the eTag dissociates from the antibody and is detected using CGE. A set of eTag reporters can be used to simultaneously monitor multiple pathogens and toxins. The assay can be made quantitative by comparing the peak areas of the reporter molecule with that of a known standard. Additionally, the reporter

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tags can be easily modified to be suited to a versatile detection platform, such as mass spectrometry, electrochemistry, and colorimetry. This simultaneous detection of multiple analytes using fluorescent reporter molecules offers a useful platform for multiplexed and quantitative detection of proteins[14].

In this paper, we optimized the assay performance by modifying several factor such as the ratio of capture antibody and detection antibodies, reaction time, and reaction platform[4,15]. We also tested the developed assay protocol for the adequacy for automated field-deployed detection system by measuring the robustness of the assay over time for detection of ovalbumin (Ova), a toxin analog.

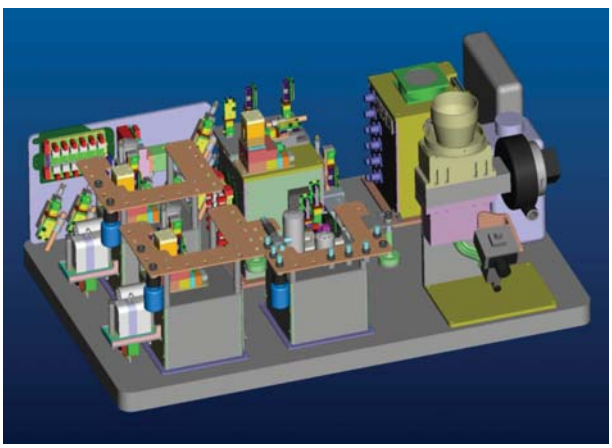


Figure 1. Shape of the automated assay platform that includes sample collector, three assay trains, and capillary electrophoresis detector.

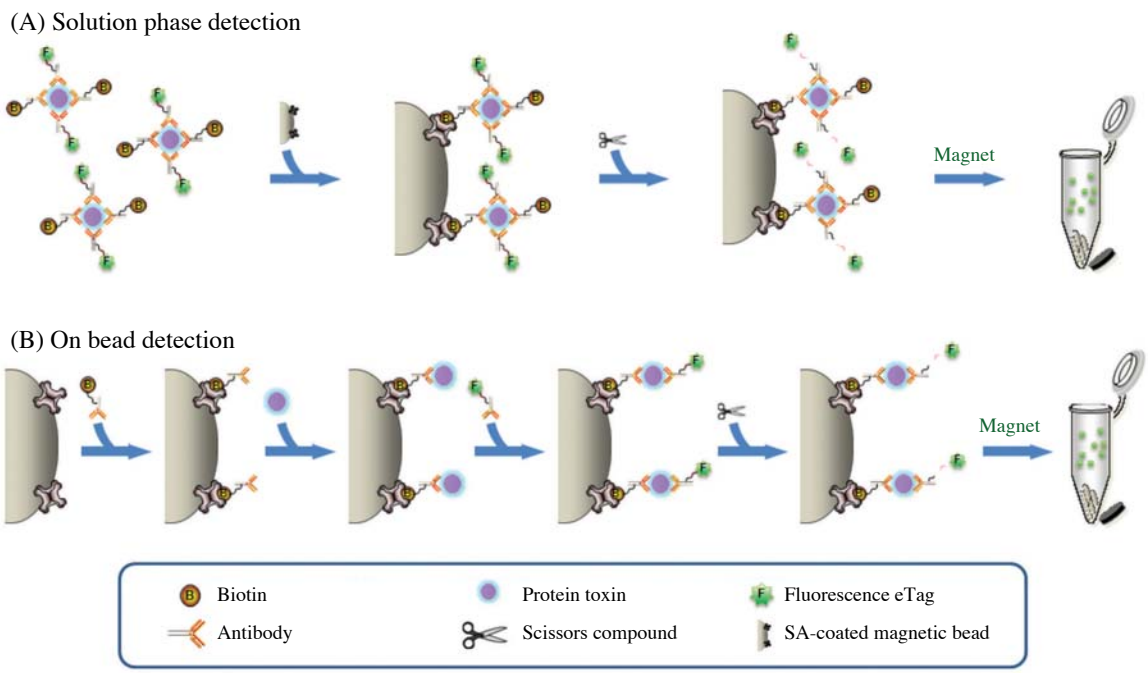
Results and Discussion

Detection of toxin analog Ova

The toxin analog Ova, a simulant of botulinum toxin, was detected using the described magnetic bead-based immunoassay. Briefly, the assay was based on antibody-antigen interaction using antibodies with two types of labels. One of the labels was a fluorescent eTag, which serves as a reporter molecule for CGE and laser-induced fluorescence (LIF) detection (Figure 1). The other label was a biotin molecule introduced to capture the toxin-antibody complex using streptavidin-coated magnetic beads. The assay was performed either in solution or on bead assay format as described in the Materials and Methods (Scheme 1).

Comparison of solution phase and bead-based assays

In order to optimize the reaction conditions to achieve a lower limit of detection, we compared the assay sensitivity in two different assay formats, in solution and on bead. In the solution assays, target was mixed with the probe antibody labeled with biotin or eTag. The mixture was incubated for 10 min for target-antibody complex formation and the streptavidin (SA)-coated



Scheme 1. Scheme of the magnetic bead based immunoassay.

magnetic beads were added to the reaction mixture to capture the target-antibody complex by biotin-SA interaction[16]. The magnetic beads were pulled-down using a particle concentrator and unreacted reagent was removed with the solution. The beads were resuspended and treated with a scissors compound to release the fluorescence tag from the antibody. Figure 2 shows representative electropherograms obtained using capillary array electrophoresis. The Pro1 peak released from the anti-Ova antibody was observed only in the presence of Ova. The limit of detection was determined for Ova (Figure 3). In the figure, the antigen concentration is shown on the X-axis and the arbitrary fluorescent intensity is plotted on the Y-axis. Each data point represents the average fluorescent intensity of three or more replicate samples. The background fluorescence was measured by performing an assay using negative control samples that do not contain the target. The limit of detection (LOD) was determined as the analyte concentration at which the average fluorescent intensity was larger than three-times that of the background. The experiment was repeated with the serial dilution of Ova solution; the LOD for Ova was 1.6 fg (20 μL of 8 ng/mL) (Figure 3). In the bead-based assay, the biotin conjugated antibody was first incubated with the SA-coated magnetic beads. Then, the target Ova and antibody-conjugated eTag were added to the bead sequentially with 10 min incubation time after each addition. The following steps were identical to the solution phase assays. The LOD was $>$125 fg and the sensitivity was decreased 10-fold as compared with the solution assay. A broad range of Ova concentrations (4 ng/mL to 500 μg/mL) was consistently detected (data not shown).

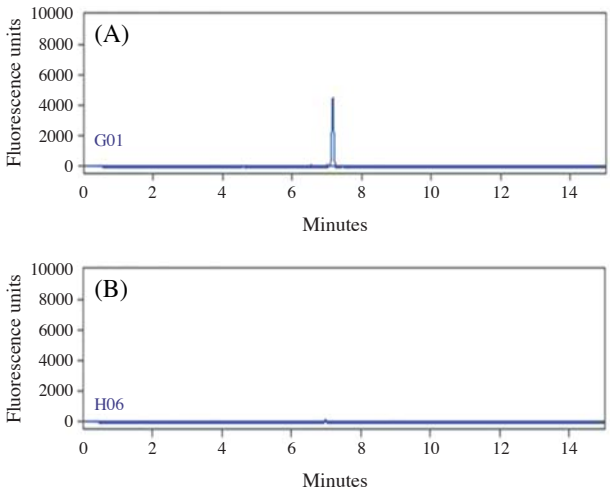


Figure 2. Electropherogram of Ova containing test solution (A) and negative control (B).

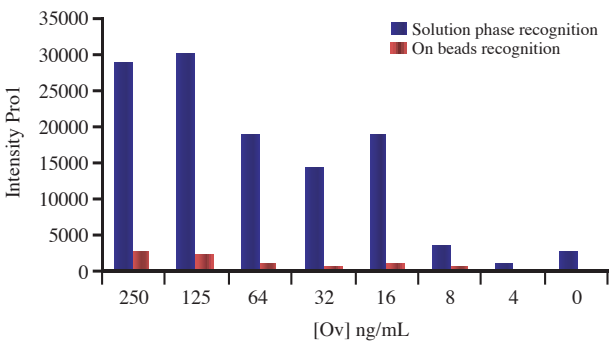


Figure 3. Efficiency comparison of solution phase protocol and on-bead protocol.

Dependence of assay efficiency on concentration of antibodies

To determine the minimum adequate amount of the reagents, the signal intensities were measured for varying concentrations of antibodies. At a high concentration (125 ng/mL), Ova samples yielded a saturated Pro1 peak when 10 μL or 20 μL antibody mixture was used for assays (Figure 4). With the target concentration lower than 64 ng/mL, the signal intensity decreased as the antibody concentration decreased, as did the background signal intensity (data not shown). The LOD was approximately 20 ng/mL when 10 μL or 20 μL of the antibody mixture was used for assays. Use of 2 μL of the antibody mixture (1/10 dilution) gave a very weak signal with all concentrations of Ova.

Dependence of assay efficiency on concentration of scissors compound

To determine the minimum adequate amount of the reagents, the signal intensity was measured for varying concentrations of the scissors compound. The signal intensity decreased as the concentration of scissors compound decreased. A high concentration of Ova (125 ng/mL) yielded a saturated Pro1 peak when 10 μL of the scissors compound solution was used for assays. The signal intensity was comparable when 20 μL of the scissors compound solution was used. When 2 μL of the scissors compound solution was used, enough signal was present for detection down to Ova concentration of 32 ng/mL, but with a weaker signal intensity. Background signal intensity decreased as the concentration of the scissors compound decreased. The LOD was approximately 20 ng/mL when 10 μL or 20 μL of the scissors compound solution was assayed.

Assay robustness

To test the suitability of this assay for field deploy-

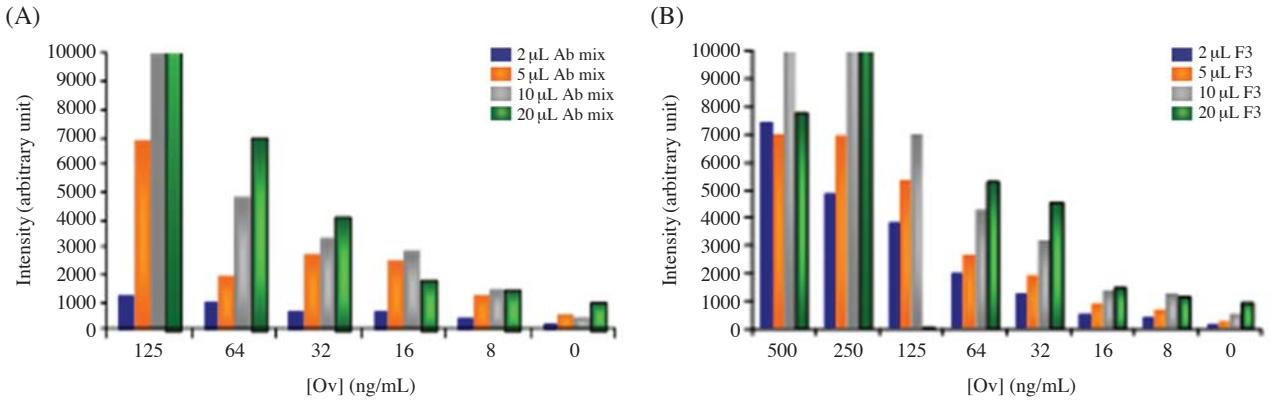


Figure 4. Dependence of assay efficiency on antibody concentration and scissors compound.

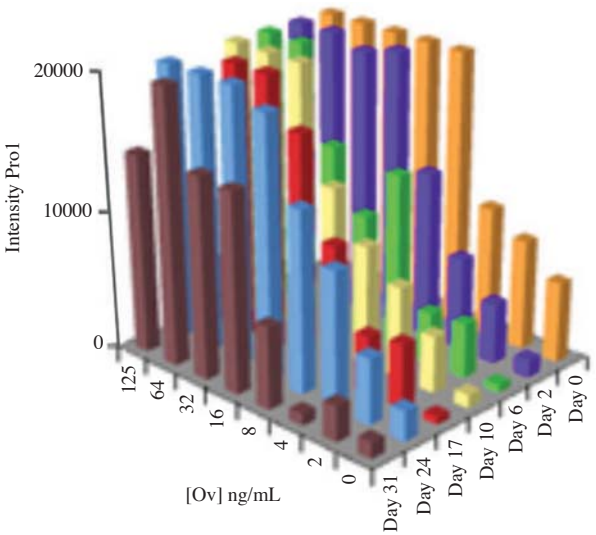


Figure 5. Assay performance monitoring over time.

ment in automated format, we tested the duration of the assay reagents maintaining the proper activity[17-19]. The reagents were stored in dark at room temperature for one month and the assays were performed with intervals. Over one month time, the assay reagents maintained fair amount of the activity but the LOD slightly increased (Figure 5). This result indicates that our assay protocol is robust and is suitable for field deployment.

Conclusions

Aiming to create a rapid and accurate analysis platform for bioagent detection in the environment, we developed an immunoassay-based protein detection system and tested its performance in various conditions. The system performed using magnetic beads and was optimized by varying the ratio of assay components. The robustness of the assay system was also tested. The platform maintained reasonable performance over one month. The developed assay is suitable as a field-deployed biosensing platform for applications including the development of a biodefense system.

Materials and Methods

Materials

The Ova toxin analog (10 g/mL) and rabbit polyclonal IgG anti-Ova antibody were purchased from the Critical Reagents Program (Frederick, MD). Sheep anti-Ova polyclonal IgG antibody was purchased from Cortex Biochem (San Leandro, CA). The antibodies were conjugated to biotin or to eTag by Monogram Biosciences (South San Francisco, CA). Wash buffer, assay buffer, Universal Standard (US1, 200 nM), Capillary Electrophoresis Standard (CES) containing two markers (M1 and M2, each 1 M) and scissors reagent (5 mg/mL) were purchased from Monogram Biosciences. Dynabeads M-280 SA (10 mg/mL) and Tris · HCl (1 M, pH 8.0) were purchased from Invitrogen (Carlsbad, CA).

Preparation of antibody solutions (S2)

Antibody solution was prepared to contain 10 nM US1, 40 nM biotin-rabbit IgG anti-Ova, and 40 nM Pro1-rabbit IgG anti-Ova.

Preparation of magnetic bead/scissors solution (S3)

Dynabeads (75 μL) were washed in a 1.5 mL microcentrifuge tube using a magnetic particle concentrator. The magnetic pellet was allowed to form by placing

each microcentrifuge tube containing magnetic beads on a magnetic particle concentrator and the soluble fraction containing unbound materials was removed with a pipette. The wash step was repeated three times using 300 μL fractions of Assay Buffer. The final bead pellet was resuspended in the particular appropriate buffer.

Detection of Ova

Solution phase detection

Assays were conducted in 96-well plates at room temperature. The 96-well plate was wrapped with aluminum foil during the assays in order to avoid unnecessary exposure to light and consequent loss of the light sensitive scissors compound. A 20 μL aliquot of sample (S1, 125 ng/mL) was mixed with 20 μL of antibody solution (S2, Ova antibody solution). The sample and antibody mixture was incubated for 10 min with shaking. After the incubation was complete 20 μL of the scissors/SA-coated magnetic bead mixture (S3) was added and incubated for an additional 30 min. The 96-well plate was then transferred to the magnetic particle concentrator, causing the magnetic particles to adhere to the sides of the individual sample wells. The soluble fraction containing unbound antibodies was removed from the wells by pipetting. The pellets were washed three times with 100 μL wash buffer, followed by resuspension in 25 μL of Tris buffer (2 mM Tris · HCl pH 8) containing 1 nM each of M1 and M2. The 96-well plate was removed from the particle concentrator and transferred to a 96-well plate illuminator that emits 680 nm light using light emitting diodes (Monogram Biosciences). The samples were illuminated for 5 min and then shaken for an additional 5 min. The soluble fraction containing freed eTag was separated from the magnetic beads using the magnetic particle concentrator and transferred to a new 96-well plate for capillary electrophoresis analysis (CGE) using an ABI3100 apparatus (Applied Biosystems, Mountain View, CA).

On bead detection

The SA-coated magnetic bead mixture (S3) was incubated with biotin conjugated anti-Ova antibody solution. S1 was added to the magnetic bead solution and incubated for 30 min. A solution of eTag-conjugated anti-Ova antibody and scissors compound was added to the mixture and incubated for an additional 30 min. Other processes were identical to the solution phase detection.

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