Antigen Detection Using Fluorophore-Modified Antibodies and Magnetic Microparticles

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Abstract

In this submission, we detail a new method for detecting mouse Immunoglobulin G (IgG) utilizing fluorophore-modified antibodies and antibody-modified magnetic microparticles. This was accomplished by adding an excess amount of fluorescein isothiocyanate (FITC)-modified goat anti-mouse IgG ($F(ab')_2$ fragment specific to mouse IgG), and allowing them to react for some time. After a given reaction time, the bound antibody could be isolated from the unbound, excess antibody via addition of goat anti-mouse IgG (Fc fragment specific)-modified magnetic microparticles. After application of a magnetic field, the free, unbound antibody could be isolated and the fluorescence intensity of the isolated solution detected. We show that the fluorescence intensity decreased linearly as antigen concentration increased, has a detection limit of 0.65 nM, and was species specific. In a subsequent experiment, we demonstrated that simple filtration could also be used to separate the bound from unbound antibodies, which enhances the simplicity and ultimate utility of the assay. We also show that the approach can also be used to detect two different analytes in a single solution, which could be easily modified to detect multiple antigens. Finally, we demonstrate that simple observation by the naked eye could be used to detect specific antigens in a sample. This sensing approach could be easily modified to detect many other antigens, even biomarkers for disease. It is this versatility, and simplicity that makes this sensing approach potentially very impactful.

Keywords: Sensors and biosensors; Magnetic microparticles; Fluorescence; Immunoglobulin G detection; Immunoassay

1. Introduction

Diagnostic tools capable of detecting increasingly lower concentrations of biomarkers for disease (e.g., antigens and antibodies) have gained in importance over the past number of decades.[1-3] This is due, in part, to the discovery of improved disease treatments, which are more efficacious when used at the early signs of disease. For example, if cancer goes untreated, it can grow, spread, and can eventually lead to death.[4] Therefore, if cancer can be detected in early stages, tumor growth can be hindered, and the risk of metastasis can be dramatically reduced.[5-7] Due to their high sensitivity and specificity, immunoassays are frequently used for quantitation of clinically relevant species like small molecules and antigenic markers.[8-10] The detection of antigens/antibodies has played a key role in early disease diagnosis and improved treatment outcomes. For example, the carbohydrate antigen 19-9 (CA 19-9), is a foreign substance released by pancreatic tumor cells, and its quantitation can be used to monitor pancreatic cancer cell growth. Therefore, one could monitor CA 19-9 levels in patients to determine the efficacy of a particular treatment.[11-12] In another example, quantitation of prostate specific antigen in bodily fluids (e.g., urine) can be used for the early detection of prostate cancer.[13] There are numerous other examples that highlight the importance of quantifying other biomarkers for disease in bodily fluids that can lead to more effective treatment, and improved quality of life.[14] Furthermore, a number of other examples exist in the literature showing sensors specific for glucose, cations, enzymes and nucleic acids.[15-18] While this is the case, there is still a need to develop novel biomolecule detection schemes that could lead to improved disease diagnostics and help improve life quality and longevity.

Antibody-based sensing schemes for the detection of specific antigens have proven to be a versatile and powerful biosensing tool. This is due to the high specificity of antibodies for the particular epitopes on corresponding antigens. Thus far, many techniques have been used for antigen/antibody detection. The most common techniques used to detect antigens/antibodies are enzyme-linked immunosorbent assay (ELISA),[19, 20] and lateral flow techniques.[21-23] These techniques are well established and are already used at hospitals, clinics, and homes. While this is

the case, ELISA protocols involve two separate binding steps followed by exposure to an organic reporting group that generates a chromophore after reaction with the enzyme-tagged secondary antibody. Since there are many steps/components to this assay, and it requires an enzymatic reaction, it is somewhat difficult to implement (especially at the point-of-care); ELISA is also requires significant time to perform. Finally, the surface chemistry needs to be carefully tuned for each analyte, which can also prohibit ELISAs utility. Compared to ELISA, lateral flow assays have many advantages, including simplicity, versatility, and low cost. Although, imprecise sample volumes are often used, which reduces the assay precision — lateral flow assays are also difficult to implement for multianalyte sensing.[24] Other techniques have been developed to detect antigens/antibodies, e.g., surface plasmon resonance spectroscopy (SPR) [25] and microfluidic-based approaches.[26] These approaches are extremely promising, however, they often require complex sample preparation procedures, bulky and expensive equipment, and professionally trained personnel to run the tests. Therefore, new sensors capable of detecting analytes in a cost effective manner that are easy to use and capable of being operated in non-laboratory settings are in demand.

In this submission, we show that Immunoglobulin Gs (IgGs) from two different species can be detected in a single solution by utilizing fluorophore-modified antibodies and magnetic microparticles. Nano/microparticles have found their way into various technologies, and have had a great impact on our everyday lives.[27, 28] One important application of inorganic nano/microparticles is their use for disease diagnosis.[29-31] The Mirkin group was among the first to demonstrate the utility of Au nanoparticles for detecting DNA/RNA in solution.[32-34] The Van Duyne group has also showed that surface bound Au nanofeatures could be used to detect biomolecules.[35-37] Magnetic microparticles have also attracted considerable interest for biosensor applications, mainly due to their ease of separation from solutions using a simple magnet.[38, 39] In one example, the Tabrizian group showed that by using an ELISA-based approach in microfluidic devices, and using magnetic field induced mixing, they decreased the volume of sample needed for analysis while maintaining high sensitivity (0.1-6.7 pM). [40] While

this approach is promising, it requires the use of microfluidic devices, which can be difficult to fabricate, especially if fabrication facilities are not readily available. In another example, Crowell and coworkers synthesized capture DNA aptamer-modified magnetic beads and reporter DNA aptamer-modified quantum dots. When specific bacteria exist in a sample, the capture DNA aptamer-modified magnetic beads, bacteria and reporter DNA aptamer-modified quantum dots form a fluorescent sandwich complex, which can be collected by an external magnet and the fluorescence detected by a fluorometer.[41] While this approach is convenient, it has been designed to detect only Campylobacter. In another example, Oplatowska and coworkers demonstrated multiplex detection of plant pathogens using microsphere immunoassay technology. In their investigation, they linked an antibody specific for each plant pathogen to separate magnetic microspheres that were modified with unique fluorophores. Individual bacteria could be detected by observing the fluorescence signature of the collected microspheres.[42] Recently, our group showed that polymer-based materials combined with the use of magnetic microparticles could be used to detect biomolecules in facile manner. [43, 44] While each of these examples has their pros and cons, there is room for more technology development in this area.

In this investigation, we developed an approach for detecting specific IgGs in solution using fluorophore-modified antibodies and antibody-modified magnetic microparticles. This sensing approach showed a limit of detection of 0.65 nM, which is comparable to the current state of the art based on carbon nanotubes (LOD of 0.1-10 nM).[45-47] The approach was also capable of detecting species specific IgGs, and was easy to implement. While the approach has been shown to be useful for detecting two IgGs in a single solution, we envision that this approach can be easily modified to detect multiple analytes in a single solution, e.g., antibodies, antigens, DNA, and other small molecules of interest.

2. Experimental Section

2.1 Materials and instruments

Mouse immunoglobulin G (IgG) (2.5 mg/mL), rabbit IgG (powder), fluorescein isothiocyanate (FITC)-modified goat anti-mouse IgG (abbreviated as FGAM, $F(ab')_2$ fragment specific to mouse IgG, emission at 520 nm, 2 mg/mL), Alexa Fluor-modified goat anti-rabbit IgG (abbreviated as AGAR, $F(ab')_2$ fragment specific to rabbit IgG, emission at 675 nm, 2 mg/mL) and phosphate-buffered saline (PBS, 1 mM KH_2PO_4, 155 mM NaCl, 3 mM Na_2HPO_4) were obtained from Thermo Fisher Scientific (Waltham, MA USA). Mouse IgG was diluted to 1 μg/μL before use. Goat anti-mouse IgG-modified magnetic beads (abbreviated as GAM@M, Fc fragment specific) and goat anti-rabbit IgG-modified magnetic beads (abbreviated as GAR@M, Fc fragment specific) were purchased from Spherotech (with an average diameter of 3.5 μm, Lake Forest, IL USA). The magnetic beads were washed with PBS buffer three times and later adjusted to the same concentration (0.5 % w/v). As characterized by the producer, 100 μL of GAM@M was capable of binding 0.64 μg of mouse IgG in maximum and 100 μL of GAR@M is capable of binding 0.56 μg of rabbit IgG in maximum. All deionized (DI) water was filtered to have a resistivity of 18.2 MΩ●cm and was obtained from a Milli-Q Plus system from Millipore (Billerica, MA).

A Photon Technology International (PTI) MP1 Fluorescence System was used to measure fluorescence spectra. An ultra high-pull Neodymium-Iron-Boron (NdFeB) magnet (5×5×1 cm) was used and purchased from McMaster-Carr Company (Elmhurst, IL). The surface magnetic strength was 1777 Gauss. An ultraviolet lamp (Mode B 100AP, BLACK-RAY CA, USA) was used to supply radiation to excite fluorescence. Photographs for each sample were obtained using a Nikon camera equipped with a 105 mm Nikon macrolens (Nikon, Ontario, Canada). The camera was placed in front of the samples.

2.2 Reaction between antigen and antibody

The approach described here can be seen schematically in Figure 1. For the mouse IgG single antigen system, 150 μL of GAM@M, 450 μL of PBS buffer solution and 1 μL (13.4 pmol) of FGAM were added in six different centrifuge tubes. Into each tube was added 0 μg (0 pmol), 0.1

μg (0.67 pmol), 0.2 μg (1.34 pmol), 0.3 μg (2.01 pmol), 0. 4 μg (2.68 pmol) and 0.5 μg (3.35 pmol) mouse IgG, respectively. The exact composition of each tube can be seen in Table S1 in Electronic Supporting Information (ESI). Each tube was wrapped in aluminum foil and gently shook for 1 h. After 1 h, the magnetic beads were separated from the solutions by exposing each tube to an external magnet, which pulled the magnetic particles to the tube's inside wall, and the supernatant solution collected. The magnetic beads were washed by adding 500 μL of PBS into the tube, mixing, magnetically separating the particles from the solution, and collecting the supernatant solution. This process was completed a total of 3 times for each tube. Finally all the aliquots from each individual washing were combined, and the fluorescence intensity measured. The excitation wavelength for FGAM was 470 nm. The emission spectra were collected from 490 nm to 650 nm, with both slit widths set at 4 nm, integration time set at 0.1 s and the step size was 1 nm.

For the mouse IgG - rabbit IgG double antigen system, 150 μL of GAM@M, 150 μL GAR@M, 300 μL PBS buffer solution, 1 μL (13.4 pmol) FGAM and 1 μL (13.4 pmol) AGAR were added to three different centrifuge tubes. Into each of the three tubes, was added 0.2 μg (1.34 pmol) mouse IgG and 0.6 μg (4.02 pmol) rabbit IgG, 0.4 μg (2.68 pmol) mouse IgG and 0.4 μg (2.68 pmol) rabbit IgG, 0.6 μg (4.02 pmol) mouse IgG and 0.2 μg (1.34 pmol) rabbit IgG, respectively. The exact composition of each tube can be seen in Table S2 in ESI. Note that GAM@M, GAR@M, FGAM and AGAR are in excess relative to mouse IgG and rabbit IgG. The tubes were wrapped in aluminum foil and gently shook for 1 h. After 1 h, the magnetic beads were separated from the solutions by exposing each tube to the external magnet, which pulled the magnetic particles to the tube's inside wall, and the supernatant solution collected. The magnetic beads were washed by adding 500 μL of PBS into the tube, mixing, magnetically separating the particles from the solution, and collecting the supernatant solution. This process was completed a total of 3 times for each tube. Finally, all the washing from each tube were combined, and the fluorescence intensity measured. The excitation wavelength for FGAM is 470 nm and AGAM 600 nm, with both slit widths set at 4 nm. The emission spectra were collected from 490 nm to

650 nm for FGAM and 620 nm to 750 nm for AGAM, with integration time set at 0.1 second and step size of 1 nm.

3. Results and Discussion

Initial experiments focused on the detection of the single analyte mouse IgG; the approach is shown schematically in Fig.1. As can be seen, fluorescein isothiocyanate-modified goat anti-mouse IgG (F(ab')2 fragment specific) (FGAM) and goat anti-mouse IgG modified magnetic microparticles (Fc fragment specific) (GAM@M) of given (and known) amounts were exposed to some amount of mouse IgG. For this assay to be successful, both the FGAM and GAM@M need to be added in known excesses relative to the amount of mouse IgG to be detected. Solutions were allowed to mix at room temperature for 2h. After 2h, the free FGAM was isolated from the rest of the components by placing the centrifuge tubes near an external magnet, which removes any free GAM@M and GAM@M bound to mouse IgG and FGAM from solution. Buffer solution was then added to wash the magnetic microparticles, followed by their isolation and rewashing. All the isolated/collected solutions were combined into one solution and the fluorescence spectrum of each combined solution acquired. Importantly, we showed that no fluorophore leached from the magnetic microparticles if they were left to incubate in buffer solution (data not shown). Additionally, no obvious morphological change of the magnetic beads was observed after their use in the assay (see scanning electron microscope (SEM) images in ESI). The fluorescence intensity depended on the amount of FGAM left in solution, which can be related to the amount of mouse IgG in the original solution — there is an inverse relationship. Specifically, higher concentration of mouse IgG in the initial solution yields lower fluorescence intensity. We note that if the fluorescence intensity from the immunoassay is not in the working range in Fig. 2, then the amount of antibody added is likely not in excess. Therefore, specific approaches can be developed to address this problem. For example, the sample to be analyzed could be split into smaller aliquots, and exposed to a range of antibody concentrations and the fluorescence intensity

from the various solutions determined. In this case, one of the solutions should have the required excess for the assay to be successful.

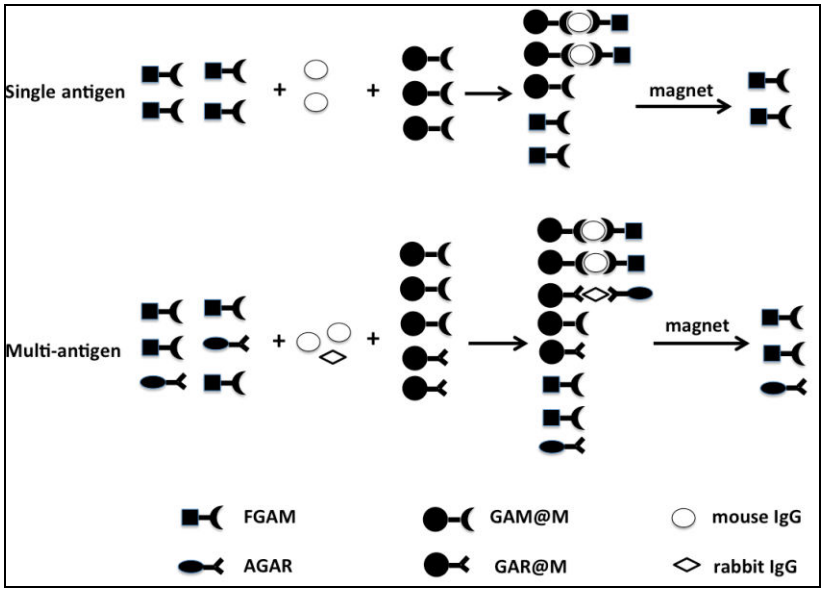

Fig. 1. Schematic representation of the approach used to detect mouse IgG (single antigen) and mouse IgG/rabbit IgG (multi-antigen). As can be seen, in each case the amount of fluorophore-modified antibody left in solution was inversely related to the concentration of the antigens in solution.

As shown in Fig. 2a, as the amount of mouse IgG in solution increases, the fluorescence intensity of FGAM in solution decreases. This is a direct result of more FGAM being removed from solution via their bond with GAM@M, which is facilitated by the mouse IgG. As can be seen in Fig. 2b, the fluorescence intensity of FGAM at 515 nm decreases in a linear fashion (with an R^2 of 0.991). Additionally, the error bars on the data points obtained from multiple experiments are extremely small, and we therefore conclude that the assay is highly reproducible and precise.

In a subsequent experiment we showed that the magnetic microparticles could be removed from solution using filtration apparatus as opposed to field. Specifically, the assay was performed using the same procedure as above, although a filter with pore size of 0.2 μm was used to remove

the particles from solution as opposed to a magnet. As can be seen in Fig. 2b, the response is also inversely related to the concentration of mouse IgG in solution, with good precision. This approach could also find utility for biosensing, and will allow other particles to be used, such as, polystyrene spheres and microgels, which will allow for easy multiplexing by using particles composed of different fluorophores.[48, 49] Finally, approaches such as centrifugation or various separations could be used to achieve similar goals.

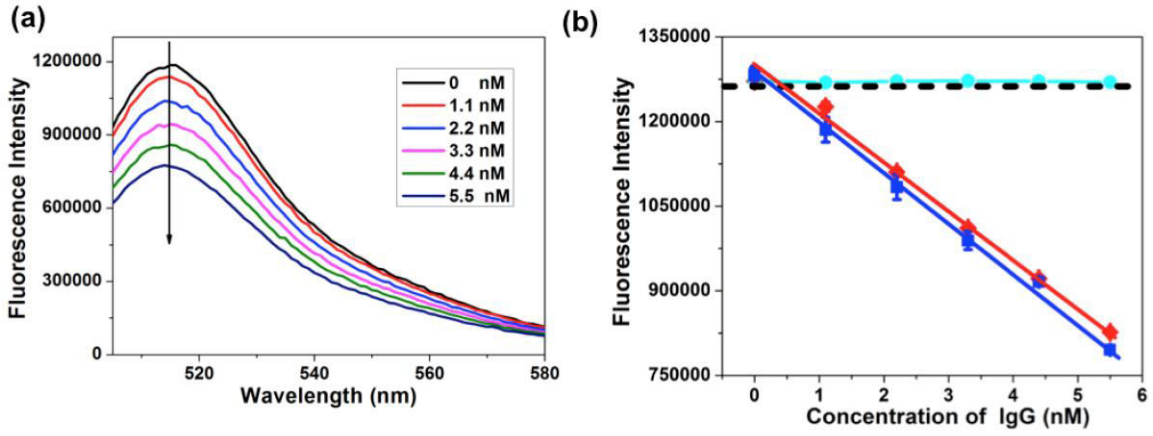

Fig. 2. (a) Fluorescence spectra of FGAM in solution after its exposure to mouse IgG followed by its magnetic field assisted isolation. The concentrations are indicated in the figure inset, and the arrow indicates increasing mouse IgG concentration. (b) Fluorescence intensity of FGAM (◆) for magnetic field-assisted separation and (■) for separation by filtration at the indicated mouse IgG concentrations. (●) Fluorescence intensity for the assay as a function of rabbit IgG concentration. The dashed horizontal line indicates the fluorescence intensity that correspnds to the limit of detection. Each data point is an average obtained from three individual experiments, and the error bars are the standard deviations of the measured values.

The selectivity of the approach was also determined by exposing FGAM and GAM@M to various amounts of rabbit IgG. Using the same approach as above for detecting mouse IgG, we isolated the remaining, unbound FGAM and measured the fluorescence intensity of the solution. As can be seen in Fig. 2b, there is almost no response of the system to rabbit IgG, proving that the assay is specific for the IgG species. Finally, the LOD for this assay was determined by averaging

the signal obtained from the control sample (fluorescence intensity obtained from sample with no IgG added, i.e., a blank) and determining the fluorescence intensity of $3\sigma_{blank}$. The LOD is indicated in Fig. 2b by the dashed horizontal line, and corresponds to a concentration of 0.65 nM (0.11 μg/mL), which is comparable (even better) that some other advanced technology based on carbon nanotubes (5 nM) [46] and ELISA (1.0 μg/mL) [50].

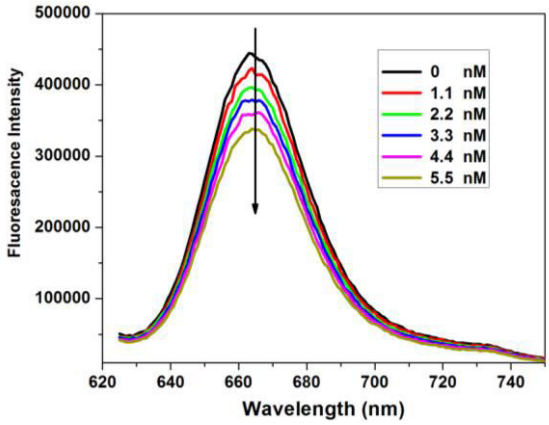

Fig. 3. Fluorescence spectra of AGAR in solution after its exposure to rabbit IgG followed by its magnetic field-assisted isolation. The concentration are indicated in the figure inset, and the arrow indicates increasing rabbit IgG concentration.

In a subsequent experiment, we showed that a similar approach could be used to quantify the amount of rabbit IgG in solution. As can be seen in Fig. 3, a trend similar to the above experiments was observed. Finally, for many practical applications, many antigens may coexist in one sample. The increase or decrease of the concentrations of some combination of antigens may be useful for identify disease states more rapidly and more accurately. To determine if multiple antigens could be detected simultaneously using our assay, we quantified both mouse IgG and rabbit IgG the same solution. As can be seen schematically in Fig. 1 (multi-antigen), FGAM, AGAR, GAM@M and GAR@M in known amounts were exposed to given amounts of mouse IgG and rabbit IgG. Again, for this assay to be successful, FGAM, AGAR, GAM@M, and GAR@M are added in known excess relative to the amount of mouse IgG and rabbit IgG to be detected. Solutions were allowed

to mix at room temperature for 2h. After 2h, the free FGAM and AGAR was isolated from the rest of the components using a magnet, which can remove any free GAM@M, GAR@M, GAM@M bound to mouse IgG and FGAM, and GAR@M bound to rabbit IgG and AGAR. After washing of the magnetic microparticles, followed by combination of the collected supernatant solutions, the fluorescence spectrum of the supernatant solution was collected. Like above, we hypothesize that the fluorescence intensity will depend on the amount of FGAM and AGAR left in solution, which can be related to the amount of mouse IgG and rabbit IgG in the original solution — again, there is an inverse relationship. Specifically, as can be seen in Fig. 4, higher concentration of mouse IgG and rabbit IgG in the initial solutions yielded lower fluorescence intensity for the FGAM and AGAR. These data suggest that the approach reported here has the potential to be used to detect multiple antigens in solution.

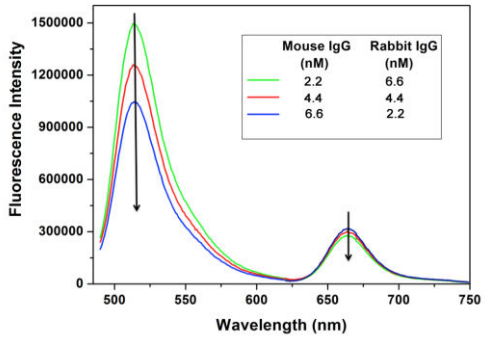

Fig. 4. Fluorescence spectra of FGAM and AGAR in solution after their exposure to a solution of mouse and rabbit IgG followed by their magnetic field assisted isolation. The concentrations are indicated in the figure inset, and the arrows indicate increasing mouse and rabbit IgG concentrations.

For many applications (e.g., point-of-care diagnostics) it is important to be able to quantify biomolecules without the use of specialized equipment. Therefore, it is important to show that this assay could work just by using the naked eye to determine fluorescence intensity. To prove that this is possible, we performed the mouse IgG assay, but instead of using a fluorometer to

determine the fluorescence intensity of the solution, we exposed the solution to UV light (365 nm), and photographed the fluorescence. The photographs can be seen in Fig. 5. As can be seen, the fluorescence from each solution can be easily seen, although the real figure of merit for this assay is how much of a change can be detected by the naked eye. While we did not investigate this in depth, one can clearly see the difference between the fluorescence of the individual solutions if the concentrations are different by an order of magnitude. While this may not be useful for quantifying sample concentrations, the visual detection could be useful as a "yes" or "no" assay.

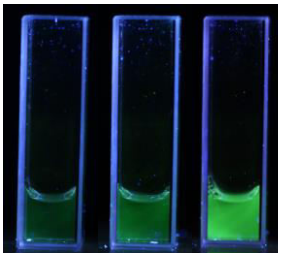

Fig. 5. Photographs of solutions containing some amount of FGAM after exposure to various concentrations of mouse IgG. The concentrations are (left) 20 μg (13.4 pmol), (middle) 5 μg (3.35 pmol) and (right) 0.1 μg (0.67 pmol).

4. Conclusions

In summary, an assay that utilizes fluorophore-modified antibodies and antibody-modified magnetic microparticles to detect mouse and rabbit IgG was developed. We showed that the assay was able to detect the respective IgGs with good LOD and selectivity. We also demonstrated that the IgGs could be detected in a single solution simultaneously, which is important for multianalyte sensing approaches. Finally, we showed that the assay could be performed without the use of a fluorometer, and by simply using the naked eye. This investigation forms the foundation for future antigen-detection approaches, and can find real-world applications for disease diagnosis.

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