Naked-Eye Detection as a Universal Approach to Lower the Limit of Detection of Enzyme-Linked Immunoassays

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Abstract:

Colorimetric biosensors for the detection of analytes with the naked eye are required in environmental monitoring, point-of-care diagnostics, and analyses in resources constrained settings, where detection instruments may not be available. However instrument-based detection methods are usually more adequate for detecting small variations in the signal compared to naked-eye detection schemes, and consequently the limit of detection of the latter is usually higher than the former. Here we demonstrate that the limit of detection of colorimetric enzyme-linked immunoassays can be decreased several orders of magnitude when using naked-eye detection instead of a spectrophotometer for detecting the signal. The key step to lower the limit of detection is adding a small volume of chromogenic substrate during the signal generation step. This generates highly colored solutions that can be easily visualized with the naked eye and recorded with the camera of a mobile phone. The proposed method does not require expensive equipment or complex protocols to enhance the signal, and therefore it is a universal approach to lower the limit of detection of colorimetric enzyme-linked immunoassays.

Keywords: ELISA, antibody, biosensor, immunosensor, smartphone, naked-eye detection.

Signal generation mechanisms for the detection of analytes with the naked eye are becoming increasingly popular in biosensing [1]. These approaches are particularly advantageous in those situations in which instrumentation is not available, for example in-field studies [2, 3], point-of-care diagnostics [4, 5], and analyses in resource-constrained settings [6]. Among the different approaches for naked-eye detection, colorimetric transduction mechanisms are often preferred due to the facile detection that only requires comparing the color of control and test samples. Furthermore colorimetric signals can be easily recorded by taking a picture with a mobile phone, which is a widespread and portable technology that can be found anywhere [7, 8]. Enzyme-linked immunosorbent assay (ELISA) is one of the most popular approaches for colorimetric detection. This approach combines the outstanding selectivity and specificity of antibody biorecognition elements with the biocatalytic signal amplification mechanism of enzyme labels, which enables the detection of a target molecule with high sensitivity and specificity [9]. Although the signal in colorimetric ELISA is usually quantified by measuring the absorbance with a plate reader or spectrophotometer, the presence of the analyte in the sample can also be detected with the naked eye when changes in the color intensity of the test and control samples are clearly distinguishable. Other colorimetric signal generation mechanisms rely in changing the tonality of the solution in order to enable the detection of analytes with the naked eye [1]. This often requires controlling the state of aggregation of plasmonic nanoparticles, which induces a shift in their surface plasmon resonance (LSPR) that may become visible with the naked eye [1, 10]. This approach can be used in conjunction with enzyme-linked immunoassays when the enzyme controls the state of aggregation of the nanoparticles via kinetically controlled

nanoparticle growth pathways [11, 12] or by means of generating nanoparticle cross-linkers [13].

Regardless of the method chosen for the detection (change in color intensity or change in tonality), naked-eye detection schemes usually show higher limits of detection compared to traditional instrument-based detection methods. This is because small changes in color intensity or tonality are difficult to appreciate with the naked eye, and the interpretation of the test results may be different depending on the observer. As such only samples that yield clearly distinct color changes compared to control samples can be considered as above the limit of detection. However, naked-eye detection schemes have an advantage that is often overlooked. While conventional plate readers and spectrophotometers require a relatively large volume of colored sample to quantify the signal, changes in the color of a solution can be easily detected in a tiny volume of sample with the naked eye. Here we propose that this inherent advantage of naked-eye detection can be used to lower the limit of detection of enzyme-linked immunoassays. The key step of the proposed methodology is adding a small volume of enzyme substrate during the signal amplification step. This concentrates the enzyme and the product of the enzymatic reaction, therefore enhancing the colorimetric signal (Figure 1). Instrument-based detection methods would require diluting the sample to detect color changes in such small volume of sample. However differences in the color of these solutions can be easily detected with the naked eye and recorded with the camera of a mobile phone. Following this simple procedure we were able to lower the limit of detection of detection of an enzyme-linked immunoassay 2 orders of magnitude. The proposed method does not require expensive equipment or complex protocols to enhance the

signal, which makes it a universal approach to decrease the limit of detection of colorimetric enzyme-linked immunoassays.

Experimental Section:

The enzyme-linked immunoassays were performed on magnetic beads using a 96-well plate as a container. Two samples of 300 µL of streptavidin-coated magnetic beads (10 µm, Sigma) were washed twice with phosphate buffered saline pH 7.4 (PBS, tablets, Sigma) containing bovine serum albumin (BSA) with a concentration of 1 $g \cdot mL^{-1}$ (PBS-BSA). 10 µL of biotinylated monoclonal anti-human IgG (Fc specific) produced in mouse (Sigma) was added to one of the samples for 1 h. 10 µL of PBS-BSA was added to the other sample (control samples). After 1 h both samples were washed with PBS-BSA 3 times. In the mean time, a 96-well plate was blocked with 300 µL of PBS-BSA for 1 h followed by washing 5 times with PBST (PBS containing 0.1% Tween-20) in order to avoid non-specific interactions with the analyte in posterior steps. IgG from human serum (> 95%, Sigma) was serially diluted in PBS-BSA to yield solutions with different final concentrations. 100 µL of each human IgG solution and 2 µL of antibody-beads or control beads were added to each well as a triplicate for 2 h. After the antibodies on the beads captured human IgG, the beads were gathered with a magnet and washed 5 times with PBST. HRP-labeled anti-human IgG (γ-chain specific), $F(ab')_2$ fragment was diluted $1:10^4$ in PBS-BSA and 100 µL added to each well for at least 2 h. The beads were then gathered with the magnets and washed 5 times with PBST. After concentrating the beads with a magnet, 100 µL the enzyme substrate (TMB, Pierce™ TMB Substrate Kit (Thermo)) was added and the colorimetric signal was recorded by measuring the absorbance with a Jasco V-660 spectrophotometer. Naked-eye detection experiments in Figure 3 were

performed with the exact same method but adding 2 μL of TMB instead of 100 μL to the magnetic beads. After 15 minutes the beads were concentrated with a magnet and the colored solutions were pipetted into a new tube. The colorimetric signal was recorded by taking a picture with a mobile phone (HTC One).

Results and Discussion

Human immunoglobulin G (human IgG) was chosen as the model analyte to test the hypothesis proposed in Fig. 1. Determining levels of IgG is required for the diagnosis of infectious diseases [14], autoimmunity [15], and blood cancers [16], which makes this molecule a relevant clinical target to test the proposed methodology. The immunoassay was performed on streptavidin-coated magnetic beads as substrates, which is already routine practice in many ELISA laboratories [17–19]. The capture antibody was a biotinylated anti-human IgG (Fc specific), and the detection antibody was a peroxidase-labeled anti-human IgG recognizing the γ chain of the $F(ab')_2$ fragment. The capture antibody was immobilized on the streptavidin-coated magnetic beads. Control experiments were performed with magnetic beads that were not modified with this capture antibody. These control experiments enabled estimating the contribution of non-specific binding to the colorimetric signal. Samples at different concentrations were obtained by serially diluting a commercial standard solution. Each sample was assayed in triplicate. Figure 2a shows a picture of the colored solutions generated by different concentrations of human IgG. In this Figure the sample that yields a colorimetric signal that can be easily differentiated from the blank signal is 10^{-8} g·mL^{-1} (see also Fig. S1 in the Supporting Information). In Figure 2b the absorbance was plotted against the concentration of human IgG in semi-logarithmic scale. The resulting calibration curve shows the sigmoidal curve typically found in immunoassays. Control experiments in the absence of the capture antibody

yielded no signal, which confirmed that the observed variations in absorbance in the presence of human IgG were not originated by non-specific interactions. The limit of detection, defined as the sample that yields a signal higher than three times the standard deviation of the blank (3σ, 99% confidence) is 10^{-9} g·mL^{-1}.

To study the possibility of decreasing the limit of detection by means of using a lower volume of enzyme substrate and naked-eye detection, the immunoassay was performed in exactly the same conditions but adding 2 μL rather than 100 μL of enzyme substrate during the signal generation step. Adding such a small volume of TMB is uncomplicated when the beads are gathered at a specific point of the plate or tube with a magnet. The same magnetic beads, antibodies, dilution factors, incubation times and washing procedures were used. The experiments were performed three times on different days and under different light conditions (although always with natural illumination). Figure 3 shows photographs of the colored solutions generated by different concentrations of human IgG. The sandwich immunoassay yields solutions whose color intensity increases as the concentration of human IgG increases. Control experiments performed in the absence of the capture antibody always yield transparent solutions with no visible green-blue hue, which demonstrates that the colorimetric signal is specific. The limit of detection for naked-eye detection, defined here as the sample that generates a solution clearly different from the blank and control samples in three independent experiments, is 10^{-11} g·mL-1, which is 2 orders of magnitude lower than the limit of detection obtained with instrument-based approaches in Fig. 2b, and 3 times lower than the limit of detection for naked eye detection in Fig. 2a. The only difference between the immunoassays in Fig. 3 and the ones in Fig. 2 is that a lower volume of chromogenic substrate was added to the magnetic beads during the signal generation step, which validates our hypothesis in

Fig. 1 that combining a reduced volume of enzyme substrate with naked-eye detection can lower the limit of detection of enzyme-linked immunoassays.

Conclusion

In conclusion we have demonstrated that the limit of detection of colorimetric enzyme-linked immunoassays can be decreased several orders of magnitude by simply adding a small volume of chromogenic substrate during the signal generation step and detecting the resulting color changes with the naked eye. The obtained limit of detection for naked eye detection is comparable to the limit of detection of recently reported ultrasensitive detection systems that require complex instrumentation to read the signal [20]. The colorimetric signals can be recorded by taking a picture with a mobile phone. The uncomplicated methodology proposed here can be easily implemented in any laboratory at no cost, which makes it a universal approach to decrease the limit of detection of enzyme-linked immunoassays. Since the proposed signal transduction mechanism only requires a mobile phone or the observation of samples with the naked eye it is also inexpensive and portable, and therefore it is suitable for the detection of analytes at the point of need or in resource-constrained settings, where specialized equipment may not be available [1, 8, 21–24]. Quantification of the color in the samples could be performed with image processing software, for example with a mobile app that quantifies color levels [8, 25, 26].

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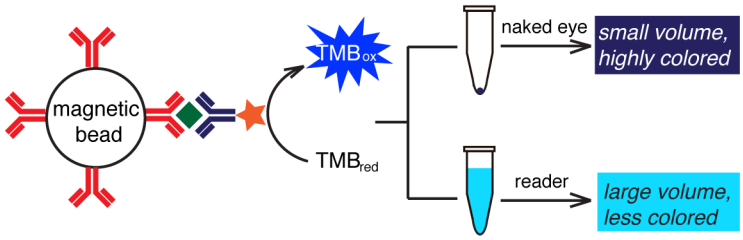

Figure 1. Schematic representation of the proposed method for decreasing the limit of detection of enzyme-linked immunoassays; after the immunoassay (sandwich format) the enzyme generates blue-colored TMB molecules; when the reaction takes place in a small volume the blue-colored molecules are highly concentrated and changes in the color of the solution can be easily detected with the naked eye; instrument-based detection requires a larger volume of enzyme substrate, and consequently the colored molecules generated by the enzyme are more diluted.

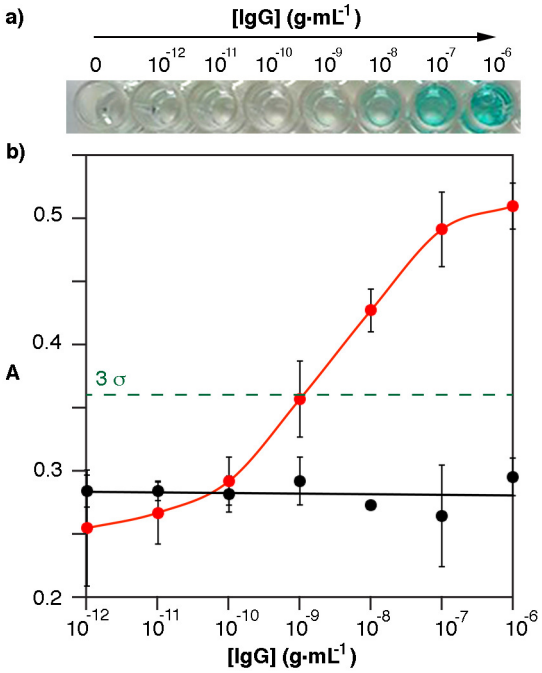

Figure 2. Traditional ELISA for the detection of human IgG; a) photograph showing colored solutions originated by different concentrations of analyte; b) calibration curve showing the variation of the absorbance (A) with respect to the concentration of human IgG (semi logarithmic scale); red dots: sandwich immunoassay; black dots: control experiments performed in the absence of capture antibody; the limit of detection (3σ, 99% confidence) is shown as a discontinuous green line. Error bars are the standard deviation (n = 3).

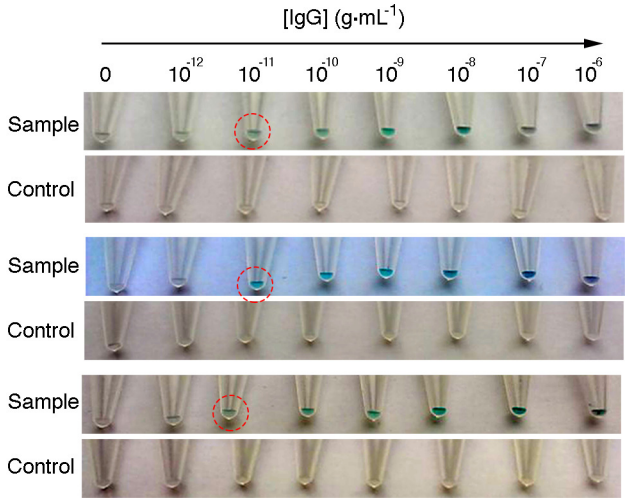

Figure 3. Naked-eye detection of human IgG with via enzyme-linked immunosorbent assay; control experiments were performed with magnetic beads that were not modified with capture antibodies.