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Nominal effective immunoreaction volume of magnetic beads at single bead level*#

Rui WANG[1], Yuan CHEN[1], Kai FAN[2], Feng JI[3], Jian WU[1], Yong-hua YU†‡1

([1]*College of Biosystems Engineering and Food Science, Zhejiang University, Hangzhou 310058, China*)

([2]*College of Life Information Science and Instrument Engineering, Hangzhou Dianzi University, Hangzhou 310018, China*)

([3]*The First Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou 310003, China*)

†E-mail: yhyu@zju.edu.cn

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Abstract: Immunomagnetic bead (IMB)-based enzyme-linked immunosorbent assay (ELISA) has been the tool frequently used for protein detection in research and clinical laboratories. For most ELISA reactions the recommended dosage of IMBs is usually according to their weight (mg) or mass fraction (w/v) instead of the bead number. Consequently, the processes occurring in the immediate vicinity of the IMBs have always been ignored by researchers and they cannot be revealed in detail during the ELISA reaction. In this paper, we established the relationship between number of IMBs and colorimetric results, and further proposed a new concept of "nominal effective immunoreaction volume (NEIV)" to characterize a single IMB during ELISA reaction. Results showed that the NEIV of a single IMB has a constant value, which is unrelated to the amount of beads and the concentration of antigen. Optimal results of the colorimetric ELISA are achieved when the incubation volume meets each IMB's NEIV and is no longer enhanced by increasing the incubation volume. Thus, the reliable and relatively precise number of IMBs for ELISA detection during practical application could be determined. Most importantly, a study using IMB's NEIV would lay the foundation for a kinetics analysis of IMBs and antigens for future study.

Key words: Nominal effective immunoreaction volume (NEIV); Immunomagnetic bead; Enzyme-linked immunosorbent assay (ELISA); Bead diameter; Mixing mode

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1 Introduction

Enzyme-linked immunosorbent assay (ELISA) has been the tool frequently used for protein detection in research and clinical laboratories because of its specificity, simplicity, and low cost (Monaci *et al*., 2011; Xu *et al*., 2012; Li *et al*., 2013). The standardized microplate-based ELISA suffers from many limitations, i.e. prolonged incubations, large volumes of reagents, and multiple washing steps (Wang *et al*., 2013; Kim and Choi, 2015). As an alternative method, immunomagnetic beads (IMBs)-based ELISA has been suggested to reduce the total time for analyses and improve the sensitivity of detection (Urusov *et al*., 2014; Wang *et al*., 2014; Xiong *et al*., 2014). Compared with micro-titer plate formats, IMBs have higher surface-to-volume ratio, lower mass, and faster reaction kinetics (Song *et al*., 2014; Chen *et al*., 2015). Moreover, IMBs allow for a kind of "in solution" reaction. They can be easily separated from the reaction mixtures with a magnet and redispersed immediately (Parsa *et al*., 2008; Song *et al*., 2014). Quantitative assessments of IMBs suggest that the binding

‡ Corresponding author

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ORCID: Rui WANG, http://orcid.org/0000-0002-9852-1247

property is highly important in recommending the optimal conditions for the particles' use (Kourilov and Steinitz, 2002; Makaraviciute and Ramanaviciene, 2013; Saha *et al*., 2014; Cohen *et al*., 2015). Unfortunately, current practice is solely based on the empirical comparison of experimental data from different dilutions of the IMBs' initial suspension (Tran *et al*., 2012; Shih *et al*., 2014; Yang *et al*., 2015). Final recommendations are then presented as weight (mg) or as a mass fraction (w/v) of the IMBs (Nishi *et al*., 2000; Song *et al*., 2014). The obtained parameters cannot therefore be compared with the processes occurring in the immediate vicinity of the particles (i.e. at the micro and submicron scales).

In this paper, we explored the relationship between the number of IMBs and colorimetric results in detail. We proposed a new conception of the nominal effective immunoreaction volume (NEIV) of a single IMB, and attempted to describe more clearly the immune concentrating processes during an ELISA reaction (Fig. 1). We evaluated the factors that were influenced by NEIV and optimized the reaction system of ELISA according to the NEIV parameters.

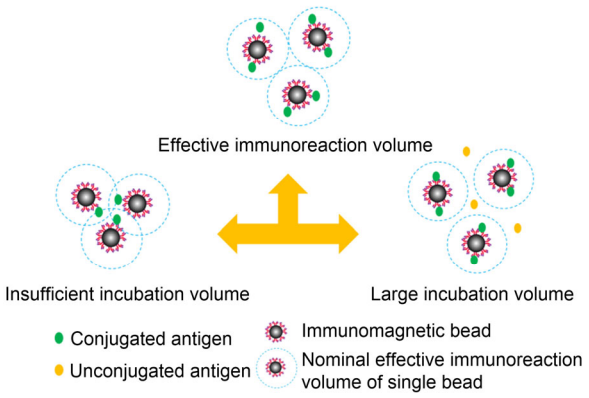

Fig. 1 Nominal effective immunoreaction volume (NEIV) of a single IMB during ELISA reactions

2 Materials and methods

2.1 Materials

Prostate-specific antigen (PSA) standard, anti-PSA primary antibody, and horseradish peroxidase (HRP)-conjugated anti-PSA secondary antibody were purchased from Longji Biotech Co., Ltd. (Hangzhou, China). Two types of Dynabeads were purchased from Thermo Fisher Scientific Inc. (MA, USA) with a diameter of 1 μm (Dynabeads® MyOne™ carboxylic acid, Type One IMBs for short) and 2.7 μm (Dynabeads® M-270 carboxylic acid, Type Two IMBs for short). *N*-Hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), and 2-morpholinoethanesulfonic acid (MES) were purchased from Sigma-Aldrich Co. LLC. (MO, USA).

2.2 Preparation of IMBs and calculation methods

Both types of Dynabeads were coated with antibody (short for "anti-PSA primary antibody") using the EDC-NHS method (Fig. S1) (Pieper *et al*., 2000; Peng *et al*., 2013). Taking Type One Dynabeads (1 μm in diameter) as an example, 50 μg of antibodies were incubated with 1 mg of Dynabeads according to the manufacturer's instructions. After incubation, the Dynabeads were washed three times to remove the uncombined antibodies. Thereafter, the non-specific adsorption sites on Dynabeads' surface were blocked with 2% (0.02 g/ml) bovine serum albumin (BSA). After blocking, the Dynabeads were washed three times and resuspended with phosphate-buffered saline (PBS) for further study. All the unconjugated antibodies were removed and all the non-specific adsorption sites on Dynabeads' surface were blocked by way of the above process, thereby avoiding any non-specific combination between IMBs and antigens. The antibody-conjugated Dynabeads are referred to as IMBs in this paper. The working concentration of Type One IMBs was 1 mg/ml, equivalent to 7.00×10^8 beads/ml. Similarly, 1 mg of Dynabeads of 2.7 μm in diameter were incubated with 20 μg of antibodies (called "Type Two IMBs"). The working concentration of Type Two IMBs was 1 mg/ml, which was nearly 6.67×10^7 beads/ml (Table 1).

Table 1 Working concentrations of two types of Dynabeads

No.	Dynabeads type	Diameter (μm)	Concentration (beads/ml)
1	MyOne™ carboxylic acid	1.0	7.00×10^8
2	M-270 carboxylic acid	2.7	6.67×10^7

Dynabeads per milligram were incubated with 50 μg antibody

2.3 Two steps of IMB-based ELISA

The first step was the combination of IMBs and antigens (Fig. S2). In preparation, IMBs in a working concentration were added to centrifuge tubes and the

supernatants were removed by magnetic separation. After PSA solutions were added, the tubes were incubated under different conditions (Sections 2.3.1–2.3.8) to form antigen-antibody conjugates (IMB-antigen). Then, the supernatants were discarded and the tubes were washed three times with 0.5% PBST (PBS with Tween 20) to remove excess antigens.

The second step involved the reaction between IMB-antigens and anti-PSA secondary antibodies (Fig. S2). Batches of 50 μl of anti-PSA secondary antibody labeled with HRP (Longji Biotech Co., Ltd., Hangzhou, China) were added into each tube and incubated at 37 °C for 10 min. Then, tubes were placed on a magnet for 3 min and the supernatants were removed. IMBs were then washed four times with 0.5% PBST. After this, 100 μl 3,3',5,5'-tetramethylbenzidine (TMB) substrates (T.J. Biotechnologies, Ltd., Tianjin, China) were added into each tube and incubated at room temperature for 5 min. Next, under magnetic separation, 50 μl solutions from each reaction tube were transferred to a 96-well plate and the chromogenic reactions were terminated by 1 mol/L HCl, followed by the measurement of absorbance at 450 nm with Spectramax M5 microplate reader (Molecular Device, CA, USA).

We focused our study largely on the first incubation step, by changing the incubation conditions in order to explore the NEIV of a single IMB during ELISA reactions, as detailed below.

2.3.1 Prerequisite establishment for NEIV study

Batches of 25 μl Type One IMBs in working concentration (7.00×10^8 beads/ml) were added into centrifuge tubes and the supernatants were removed by magnetic separation. Hence, the number of Type One IMBs in each tube was 1.75×10^7. Then, a group of 50 μl antigen solutions with the concentrations ranging from 0.0625 to 5 ng/ml was injected into each tube and tubes were incubated statically at 37 °C for 10 min.

2.3.2 NEIV exploration with different concentrations of antigen

Three different concentrations (0.1, 0.125, and 0.25 ng/ml) of antigen were detected and the concentration of 0.1 ng/ml is taken as an example. Batches of 1.75×10^7 Type One IMBs were added into twelve centrifuge tubes and the supernatants were removed by magnetic separation. Then, eleven different volumes of 0.1 ng/ml antigen (25, 50, 100, 200, 400, 600, 800, 1000, 1200, 1400, and 1600 μl) were added to each tube with one having no antigen added as a blank control. The tubes were incubated statically at 37 °C for 10 min.

2.3.3 NEIV confirmation with different amounts of IMBs

Three different amounts of Type One IMBs (3.50×10^7, 1.75×10^7, and 1.30×10^7 beads) were tested with a certain concentration of antigen under different incubation volumes. For 3.50×10^7 beads in each tube, the incubation volumes were 50, 100, 200, 400, 800, 1600, 2000, 2400, and 2800 μl. For 1.75×10^7 beads in each tube, the incubation volumes were 25, 50, 100, 200, 400, 800, 1000, 1200, and 1400 μl. For 1.30×10^7 beads in each tube, the incubation volumes were 18.75, 37.5, 75, 150, 300, 600, 750, 900, and 1050 μl. The concentration of antigen was 0.125 ng/ml for each test. Tubes with different incubation volumes were incubated statically at 37 °C for 10 min.

2.3.4 Influence of incubation duration

The incubation duration is the time for conjugation between IMBs and antigens in the first step. Different incubation durations of 3 and 10 min were tested in this assay. Here, the incubation duration of 3 min is taken as an example. In preparation, about 1.75×10^7 Type One IMBs were added to batches of centrifuge tubes and the supernatants were removed by magnetic separation. Then, different volumes of 0.125 ng/ml antigen (25, 50, 100, 200, 400, 600, 800, 1000, and 1200 μl) were added into each tube including one with no antigen added as a blank control. The tubes were incubated statically at 37 °C for 3 min.

2.3.5 Influence of incubation mode

The influence of ultrasonic vibration was studied during the first incubation step. Batches of 1.75×10^7 Type One IMBs were added into centrifuge tubes and the supernatants were removed by magnetic separation. Then, different volumes of 0.125 ng/ml antigen (25, 50, 100, 200, 400, 600, 800, 1000, 1200, and 1400 μl) were added to each tube as well as a blank control, which had no antigen added. The tubes were incubated under 40 kHz of ultrasonic vibration

(JK-2200B, Jiangxi Instrument Co., Ltd., China) at 37 °C for 10 min. Simultaneously, the water temperature was measured by thermometer and the results showed that it had changed by less than 1 °C after 10 min of ultrasonic vibration. The influence of temperature during ultrasonic vibration could therefore be ignored. In parallel, for static incubation, the tubes were placed in incubator statically at 37 °C for 10 min.

2.3.6 Influence of IMB's diameter

Batches of 25 μl Type Two IMBs in working concentration (6.67×10^7 beads/ml) were added to centrifuge tubes and the supernatants were removed by magnetic separation. The number of Type Two IMBs in each tube was therefore 1.67×10^6 beads. They were investigated to test two concentrations of antigen (0.250 and 0.125 ng/ml). Here, the detection of 0.125 ng/ml antigen is used as an example. Briefly, different volumes of 0.125 ng/ml antigen (25, 50, 100, 200, 400, 600, 800, 1000, 1200, 1400, and 1600 μl) were added into each tube with one having no antigen added as a blank control. The tubes were incubated under static condition at around 37 °C for 10 min.

2.3.7 NEIV effect on limit-of-detection

The limit-of-detection (LOD) of the IMB-based ELISA was tested with the naked eye. In each test 1.75×10^7 Type One IMBs were used and the supernatants were removed by magnetic separation after the IMBs were added. Next, three different volumes (50, 400, and 800 μl) of 0.01 ng/ml antigen were added into each tube with one having no antigen added as a blank control. The tubes were incubated statically at 37 °C for 10 min.

2.3.8 Potential application of NEIV

To test the potential application of NEIV, three incubation volumes (50, 400, and 800 μl) during the first incubation step were investigated. The incubation volume of 50 μl was taken as an example. Batches of 1.75×10^7 Type One IMBs were added into centrifuge tubes and the supernatants were removed by magnetic separation. Then, different concentrations of 50 μl antigen (ten-fold serial dilution from 1 to 10^{-5} ng/ml) were added into each tube with one having no antigen added as a blank control. The tubes were incubated statically at 37 °C for 10 min.

3 Results and discussion

First, it was investigated whether the binding sites of IMBs were sufficient as a premise for an NEIV study. Batches of 1.75×10^7 Type One IMBs were used to test 50 μl volumes of antigen, whose concentrations ranged from 0.0625 to 5 ng/ml. Results showed that the optical density (OD) values went up gradually with the increase of antigen concentrations (R_1^2=0.9844 for Type One IMBs; R_2^2=0.9812 for Type Two IMBs) and they did not become saturated when the concentrations of antigen went up to 5 ng/ml. It revealed that the binding sites of IMBs were sufficient for combination with antigen molecules. Furthermore, we tried to halve the dose of antibody binding to Type One IMBs. As shown in Fig. 2, its linear response was basically the same with that of Type One IMBs. It verified that there was an excess of binding sites in Type One IMBs. In the following assay, the concentrations of antigen (or the total amount of antigen) were all within the range (from 0.0625 to 5 ng/ml), so the binding sites of the IMBs were also sufficient in the following experiments.

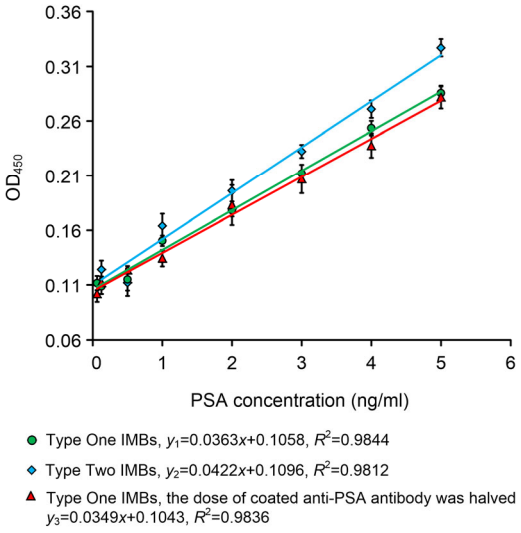

Fig. 2 Relationships between PSA concentrations and OD values

Data are expressed as mean±SD. Each sample was in triplicate

The existence of an IMB's NEIV was investigated with different incubation volumes at three different antigen concentrations (0.1, 0.125, and 0.25 ng/ml). About 1.75×10^7 Type One IMBs were used in each

test and the incubation condition was static at 37 °C for 10 min. As shown in Fig. 3, for all three curves, with the increase of incubation volumes, the responses displayed a rapid increase at the beginning and bent to be saturated afterwards. The final OD values of the three curves were different because more antigen molecules would be captured from the solutions with higher antigen concentrations. Most importantly, although the final OD values varied, the turning points of the three curves were almost the same. Since the previous experimental results had shown that there were enough binding sites on the surface of IMBs, the appearance of the plateau phase was not caused by the depletion of binding sites. Therefore, it is assumed that the existence of NEIV leads to this phenomenon. When IMBs are exposed to antigen solutions, antigen-antibody complexes will be formed due to collisions between IMBs and antigen molecules. Beads can easily collide and bind with nearby antigen molecules, but not with those in the distance. Specifically, with the increase of incubation volume, more and more antigen molecules exist beyond the IMB's maximum immunoreaction region that is NEIV, and will not be caught by IMB as predicted under the certain incubation condition; hence the plateau will emerge. The three curves reached almost the same turning point, which was 2.29×10^{-5} μl for each Type One bead. It revealed that the maximum reaction volume of each IMB was constant, which was irrelevant to the concentration of antigen under each specific incubation condition. NEIV therefore does indeed exist and has a determined value for certain magnetic beads.

To further study the NEIV, different amounts of IMBs during immunoassay for antigen detection were investigated. As shown in Fig. 4, there were 3.50×10^7 (triangle), 1.75×10^7 (circle), and 1.30×10^7 beads (square) of Type One IMBs in each test respectively. An antigen solution of 0.125 ng/ml was added in different volumes. All tubes were incubated statically at 37 °C for 10 min. Finally, the reaction volume of a single bead was obtained by dividing the total incubation volume by the number of beads to make a comparison. For the three curves, with the increase of incubation volumes, the growth trends of OD values were similar to those in Fig. 3. The maximum OD values of each curve were different. The final OD value of the triangle curve (3.50×10^7 beads) was almost twice as high as that of the circle curve (1.75×10^7 beads). It reflected that, under the same incubation conditions, IMBs in larger quantities would react with more antigen molecules. More importantly, the turning points of the three curves were all estimated to be 2.29×10^{-5} μl, which was the same value as the NEIV discovered in the aforementioned study. Therefore, as shown in Fig. 1, the IMB's NEIV has a constant value, which is unrelated to the number of beads or the concentration of antigens under the specific incubation condition. In this way, the NEIV could be used as a new parameter

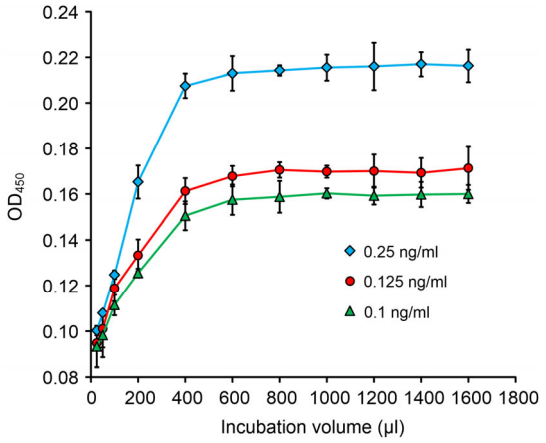

Fig. 3 Results of immunoassay with Type One IMBs for antigen detection in three concentrations

For each concentration of antigen, the incubation volumes were 25, 50, 100, 200, 400, 600, 800, 1000, 1200, 1400, and 1600 μl. Data are expressed as mean±SD. Each sample was in triplicate

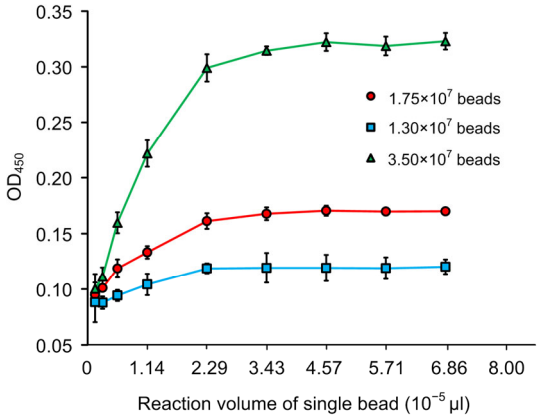

Fig. 4 Results of immunoassay with three amounts of Type One IMBs

The concentrations of antigen were all 0.125 ng/ml. The amount of beads in each test: around 3.50×10^7, 1.75×10^7 and 1.30×10^7 beads. Data are expressed as mean±SD. Each sample was in triplicate

to describe the progress of ELISA reactions and conveniently enlarge or reduce the reaction volume according to the NEIV.

To better understand the properties of the NEIV, three influencing factors were studied: incubation duration, mixing mode, and diameter of the bead. The incubation duration was investigated first. As shown in Figs. 5a and 5b, there were roughly 1.75×10^7 Type One IMBs in each sample and the concentration of antigen was 0.125 ng/ml. As shown in Fig. 5a, samples were incubated for 10 min (circle curve) and 3 min (square curve) under static incubation. Both curves displayed an ascent trend at the beginning, followed by saturation with increasing incubation volumes. The final OD values of the square curve (3 min) were smaller than those of the circle curve (10 min), which revealed that it was hard for IMBs to capture antigen molecules under short incubation duration. More importantly, the turning point of the square curve was moved left compared to that of the circle curve. It suggested that the IMB's NEIV was related to incubation duration and that longer incubation time would improve the value of the NEIV.

The second factor tested was mixing mode. As shown in Fig. 5b, tests were conducted under ultrasonic vibration incubation (square) and static incubation (circle) at 37 °C for 10 min. For the two curves, responses showed an increase at the very beginning and reached saturation afterwards. The maximum OD value of the square curve was much bigger than that of the circle curve. What mattered most was that the NEIV in the square curve was about 4.57×10^{-5} μl, which was almost twice as large as that in the circle curve. It suggested that ultrasonic vibration would produce an increase of NEIV and promote the combination of antibody-antigen effectively.

The third factor was the diameter of Dynabeads. Type Two IMBs (roughly 1.67×10^6 beads with the diameter of 2.7 μm in each test) were employed to investigate two concentrations of antigen (0.125 and 0.25 ng/ml). As shown in Fig. 5c, the responses of both curves were similar and they reached almost the same turning point at 2.4×10^{-4} μl. The NEIV of Type Two IMBs was 2.4×10^{-4} μl, which was bigger than that of Type One IMBs, which had a diameter of 1 μm. We speculate that such a discrepancy may be caused by different dynamic principles of beads with different diameters.

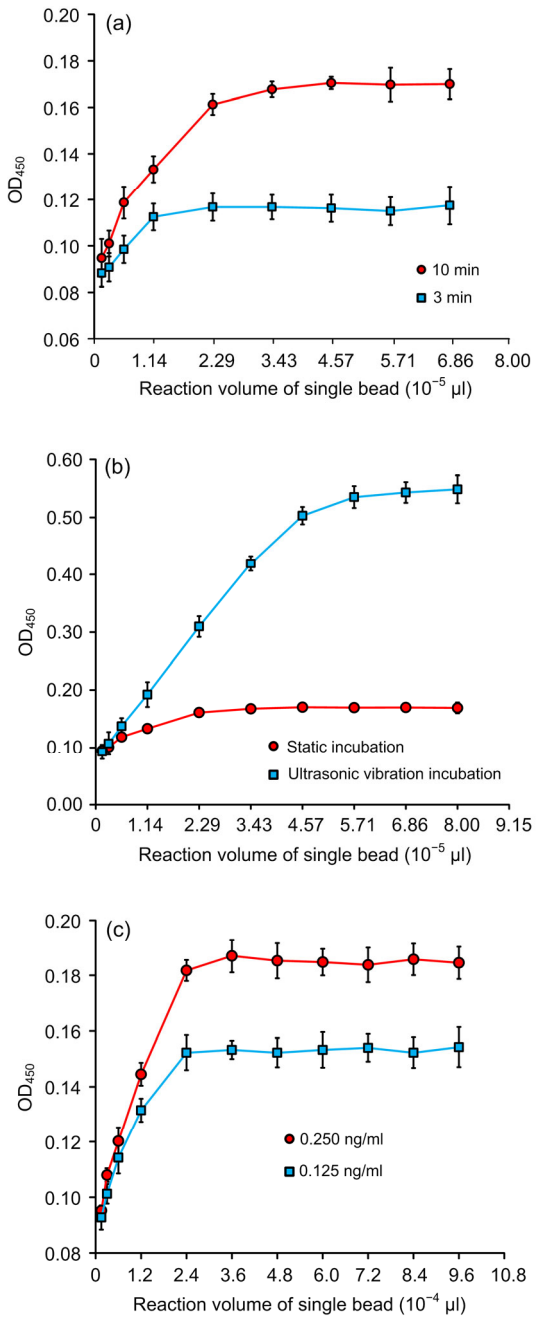

Fig. 5 Influenced factors of NEIV during immunoassay
The concentrations of antigen for each sample were all 0.125 ng/ml. (a) The influence of incubation duration. Samples were under static incubation at 37 °C for 10 and 3 min. (b) The influence of mixing mode. All samples were incubated under ultrasonic vibration incubation and static incubation at 37 °C for 10 min. (c) The influence of the diameter of IMBs. Type Two IMBs were employed to investigate two concentrations of antigen (0.250 and 0.125 ng/ml). Data are expressed as mean±SD. Each sample was in triplicate

In conclusion, under a certain incubation condition, the value of NEIV is constant and independent of the number of beads, the concentration of antigen, and the whole reaction volume. Practical applications could be better implemented with the concept of NEIV. For example, it enables the enlargement or reduction of the reaction volume according to the NEIV and the number of IMBs, which would greatly simplify experimental design during ELISA detection. Moreover, before undertaking a large sample enrichment for the trace analysis of targets (such as allergen determination), a simulation could be established according to an IMB's NEIV. This would allow the reliable and relatively precise determination of the necessary amount of IMBs needed for ELISA detection during practical applications. This essentially eliminates the need for the time-consuming and labor-intensive operation for optimizing the number of beads in an ELISA reaction. One important point to note is that the incubation duration, mixing mode and the bead's diameter are factors that could influence an IMB's NEIV. These factors therefore need to be taken into consideration before performing simulations and large sample detections using NEIV as a parameter. To the best of our knowledge, these influencing factors have never been studied at the level of a single bead during an ELISA reaction or been reported from the micro perspective.

To evaluate the potential applications of NEIV, we compared the performance of IMB-based ELISA at NEIV (400 μl for Type One IMBs) and other incubation volumes (50 and 800 μl) with a series of concentrations of PSA antigen (ten-fold serial dilution from 1 to 10^{-5} ng/ml). The colorimetric results of an immunoassay with 400 and 800 μl incubation volumes were the same for the detection of 0.01 ng/ml PSA (Fig. 6a). As shown in Fig. 6b, the limit-of-detection (LOD) of an immunoassay with 400 μl incubation volume was 0.01 ng/ml, while it was 0.1 ng/ml for that with a 50-μl incubation volume. It revealed that the colorimetric effect would not be enhanced when the incubation volume was larger than NEIV, while it would be weakened when the incubation volume was smaller than NEIV. In other words, NEIV was the optimal reaction volume for a single IMB. The colorimetric results would no longer be enhanced with an increasing amount of IMBs during an ELISA reaction. Therefore, adopting the measurement unit of NEIV as the quantity of IMBs would greatly reduce regent consumption and the cost, especially for large sample detections.

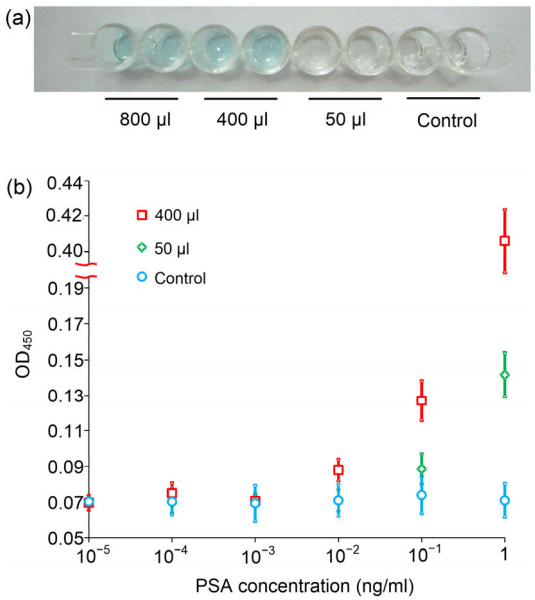

Fig. 6 Performance of IMB-based ELISA at NEIV and other incubation volumes

The amount of Type One IMBs in each sample was around 1.75×10^7 beads. (a) Photograph of colorimetric results for antigen detection in the concentration of 0.01 ng/ml. Samples were in the same colorimetric volume (50 μl) but in three different incubation volumes (800, 400, and 50 μl) with one having no antigen added as a blank control. Each incubation volume was in duplicate. (b) Results of antigen detection in the concentration of ten-fold serial dilution with different incubation volumes of 400 and 50 μl. Samples with no antigen added were as blank controls. Data are expressed as mean±SD. Each sample was in triplicate

4 Conclusions

For the first time, a specific number of IMBs were employed to describe the quantity of IMBs during an ELISA reaction instead of the frequently used weight (mg) or mass fraction (w/v). This opened up a novel microcosmic perspective for ELISA analysis and presented the possibility to track immune progress at the single bead level during ELISA reactions. This study reported the NEIV of a single IMB. This concept clarified the immune concentrating process occurring in the immediate vicinity of the particles at a micro scale. The NEIV of IMBs is at a

constant value, which is independent of the number of beads and the concentration of antigen, under the certain incubation condition. Therefore, NEIV could play an important role in IMB-based ELISA in practical applications. Enlarging or reducing the reaction volume could be conveniently done according to the NEIV and the number of IMBs. Additionally, it could effectively improve the detection efficiency and lower the cost especially for resource-limited areas. Most importantly, this study provided a new direction and reference for tracking ELISA progress at the microscopic level and demonstrated the feasibility of analyzing the immune process on the surface of a single bead. It could lay the foundations for kinetics analysis between IMBs and antigens for future research.

Compliance with ethics guidelines

Rui WANG, Yuan CHEN, Kai FAN, Feng JI, Jian WU, and Yong-hua YU declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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List of electronic supplementary materials

Fig. S1 Principle of EDC-NHS method for antibody modification of magnetic beads

Fig. S2 Principle of IMB-based ELISA assay for PSA antigen detection

中文概要

题　目：在单个磁珠的水平上探究磁珠的有效免疫反应体积

目　的：通过探究单个磁珠的有效免疫反应体积（NEIV），在微观水平上揭示磁珠的免疫反应过程，为大体积样本检测过程中磁珠的用量提供理论指导。

创新点：首次提出“单个磁珠的有效免疫反应体积”这一概念，提出在微观水平上对磁珠免疫反应进行探究，为进一步研究磁珠-抗原免疫反应的动力学过程打下基础。

方　法：首先，采用两种直径的磁珠对 50 μl 不同浓度的抗原进行比色检测，证明磁珠上抗原的结合位点过量。随后，采用定量磁珠对相同浓度不同体积的抗原进行检测，得出“单个磁珠的有效免疫反应体积”这一概念。接着，采用三种不同用量的磁珠对浓度相同、体积梯度增大的抗原进行检测，以验证所提出的 NEIV 的概念。然后，通过测定孵育时长、孵育温度和震荡与否对比色结果的影响，找到对 NEIV 影响的关键因素。最后采用定量磁珠，对梯度稀释的抗原进行检测，建立磁珠用量与比色结果的关系，对 NEIV 的实际应用提出展望。

结　论：对于同一直径的磁珠，在相同的孵育条件下，其单个磁珠的 NEIV 是恒定的，与磁珠用量和孵育体积无关。孵育温度、孵育时长和震荡与否是影响 NEIV 值的关键因素。采用 NEIV 这一参数可以在大体积样本检测之前，对磁珠的用量模拟提供有效指导。除此之外，NEIV 更从微观角度上揭示了磁珠和抗原的免疫反应过程，为进一步研究磁珠-抗原免疫反应的动力学过程奠定了基础。

关键词：单个磁珠有效免疫反应体积（NEIV）；免疫磁珠；酶联免疫吸附测定（ELISA）；磁珠直径；孵育方式