A sensitive and simple enzyme-linked immunosorbent assay using polymer as carrier

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Abstract

In this study, a new and sensitive enzyme-linked immunosorbent assay (ELISA) was developed by introducing a polymer as a reaction carrier. The results suggest that the newly developed ELISA method is more convenient than the existing paper-based ELISA method and applicable to a wider range of environments. In addition, the sensitivity of the new method is much higher than that of the existing paper-based ELISA method and even higher than that of the traditional ELISA method.

Keywords

polymer, paper, enzyme-linked immunesorbent assay
1 Introduction

ELISA is one of the most effective and popular methods in qualitative and quantitative studies of trace biomarkers or other proteins. This method can amplify the detection signal through the high specific binding between antigen and antibody and the high catalytic rate of enzymes, enabling this detection method to achieve high sensitivity.1, 2) Because ELISA requires expensive equipment and a complex washing process, a new paper-based ELISA method was developed in 2010.3) Compared to the traditional ELISA method, this paper-based ELISA method is simpler to use and less expensive, but its sensitivity is not high enough.4-6) To overcome the above shortcomings, researchers have also introduced graphene oxide sheets7-9) and gold nanoparticles10,11) into the traditional ELISA method to amplify the detection signal.12-14) To some extent, these improvements have solved the problems of low detection signals, high costs and complex processes.15-18) However, these modified methods are not convenient enough, and their outdoor use is limited.19-21)

In this study, we report a new ELISA method using a polymer as a carrier (Polymer-ELISA) instead of traditional 96-microzone plates and current 96-microzone paper plates.22, 23) Polymers have many advantages over other reaction carriers, such as their abundant functional groups, numerous branched chains and large internal voids.24, 25) In addition, the polymer used in this study has no effect on the activity of biological molecules. This honeycomb-like microenvironment is the best place for antibodies to react with proteins, so it can be used as a carrier for a nanoreactor.26-28) As required, hydrophobic or hydrophilic segments can be introduced into polymer molecules to achieve specific functions. Polymer-ELISA is fast, portable, low cost and reusable. Notably, the new method is an innovation in ELISA carriers, which provides potential for its application in new environments and fields. The polymer used in this study is G3-g-PEO2900.22) The molecular weight of the dendrimer-like copolymer peripheral polyethylene oxide (PEO) is as large as 2.16×10^6, with up to 2900
outer PEO arms and an average diameter of 32.8 nm in solution. The specific preparation process of the polymers is described in the literature. As far as we know, this work is the first time that a polymer used as a carrier is introduced into ELISA to develop a new approach for wider applications.

To verify the performance of the new method, we selected GST protein and Nogo-66 as models. The polymer is a dendrimer-like structure with a uniform distribution of branches, which increases the probability of binding with antibodies. Because of the narrow molecular weight distribution and the numerous branched chains of the polymer, the formation of a compact shell can greatly reduce the loss of the binding sample and antibody. Each test zone requires only 1 μL of solution to fill, and the results can be measured using a desktop scanner, thus greatly reducing equipment costs. In addition, another advantage of using a polymer as a nanocarrier is that the sample added to the test zones is not easy to lose, so the test results are more accurate. The disadvantage of the new method is that the synthesis of polymers is slightly cumbersome. Fortunately, the synthesized polymer can be used many times.

2 Material and methods

2.1. Materials

2-(N-morpholino)ethanesulfonic acid (MES), Bovine serum albumin (BSA), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), 3,3’,5,5’-tetramethylbenzidine (TMB), N-hydroxy-succinimide (NHS), horseradish peroxidase (HRP), glutathione S-transferase (GST), GST-primary antibody (GST-Ab1) were purchased from Sigma-Aldrich Chemical (Sigma-Aldrich, USA). Second generation Hoveyda-Grubbs catalyst, alkaline phosphatise, 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium and polyethylene oxide were purchased from Beijing No.2 analytical reagent factory (China).
Deionized water (R>18 MΩ) used for all experiments was purified by a Millipore purification system (Shanghai, China). Whatman No. 1 filter paper (Whatman International, Ltd., England). Blocking buffer solution consisted of a PBS solution supplemented with 2% (w/v) BSA (pH 7.4). PBST was PBS containing 0.05% (w/v) Tween 20.

2.2 Synthesis of amphiphilic dendrimer-like copolymer (G3-g-PEO2900)

The specific synthetic route for G3-g-PEO2900 is described in detail in the literature and will not be repeated here. The general synthesis process is as follows. First, a living block copolymer (PBSt-b-PSLi) and a 3-arm star-like polymer (G1) are synthesized. Second, hydrosilylation of intermediates is carried out. Third, a third-generation dendrimer-like copolymer (G3-g-BST3060) is synthesized. Fourth, amphiphilic dendrimer-like copolymer G3-g-PEO2900 is synthesized. The G3-g-PEO2900 product was placed in a dry vacuum bottle for use. By changing the temperature, the carrier can be converted between activated and inactivated states due to the hydrolysis of the benzyl chloride in the G3-g-PEO2900 nanoreactor.

2.3 Design of polymer plate

We used a 24-microzone polymer plate with an array (3×8) of circular test zones for running parallel Polymer-ELISA (Fig. 1). The reason for this design is that it is difficult to deposit the polymer evenly on a large piece of paper. In order to ensure that the distribution of the polymer plate is consistent with the traditional ELISA method, we buckle the traditional ELISA plate on the polymer plate and press it hard to produce an obvious trace on it. The diameter of each hole was half of the microzone diameter in the traditional 96-microzone plate. Therefore, the size of the test zone of the polymer plates is a quarter of that of the traditional one, which greatly reduces the amount of sample required. In this way,
the distribution of wells in the new method is completely consistent with that in the traditional method. Because of the crosslinking of the branched chains, antibodies and proteins can be fully combined, which not only improves the utilization rate of samples but also improves the detection accuracy. Four polymer plates (8×3) can be assembled into a 96-microzone polymer plate (8×12) to confer compatibility with existing microanalytical infrastructure. Another advantage of this method is that it does not need special equipment to read the results but needs only a mobile phone to take clear pictures.

![Fig. 1. Schematic diagram of the standard Polymer-ELISA procedure.](image)

To enhance the toughness of the polymer nanocarrier, the polymer was attached to paper in this study. The advantage of this method over other methods is that it is easy to operate and that the porous and branched polymer structure provides better performance. The copolymer, paper and methanol were put into the reactor together and rotated slowly. After 30 minutes, the methanol was removed and dried, and the functionalized paper was preserved for use. Because of affinity, the polymer evenly and firmly deposits on paper.

### 3 Results and discussion

The standard procedure of Polymer-ELISA includes the following steps shown in Fig. 1: 1) preparing the copolymer carrier, 2) fabricating wells, 3) immobilizing an antigen, 4) adding the first antibody (Ab1), 5) blocking the wells to prevent nonspecific adsorption,
6) washing away the unbound antibody, 7) adding the second antibody (Ab2) modifying with horseradish peroxidase (HRP), 8) washing again, and 9) adding the enzyme substrate and measuring the optical density (OD). To speed up the test, the reacted copolymer carrier can be placed in a ventilated area. If conditions permit, a vacuum pump can be used to accelerate the volatilization of the solution. For specific testing, the process may be adjusted slightly, but the basic operation will not be greatly changed.

After the new Polymer-ELISA method was established, the following experiment, which used GST protein as a model, was conducted to examine the performance of the method. Because the gray color intensity is closely related to the quantity of detected substances, the quantity can be obtained according to the gray color intensity. First, the relationship between the gray intensity in the detection area and the sample concentration should be validated. To study the relationship between the gray intensity and the quantity to be measured, the following experiment was carried out: In detail, GST protein samples diluted 10 times in turn were diluted in 100 μL of carbonate buffer with pH 9.6 and incubated at 37 °C for 0.5 h. After 3 washes with PBST, the plates were sealed with 1% BSA and incubated at 37 °C for 1 h. After 3 washes with PBST, GST-Ab1 was added and the plates were incubated at room temperature (RT) for 0.5 h. After 3 washes with PBST, GST-Ab2 was added and the plates were incubated at 37 °C for 45 min. After three washes, color was developed using antigen/antibody recognition (ALP and BCIP/NBT).

The results showed that with increasing sample concentration, the gray color intensity gradually increased; however, there was a linear correlation only within a certain interval, not in all intervals (Fig. 2).
Fig. 2. A) Images of the Polymer-ELISA results for GST protein with different dilution multiples in each zone. B) The calibration curve between color intensity and the amount of GST protein in each microzone was determined by the Polymer-ELISA method. Each datapoint is the mean of six replicates (N=6), and the error bars represent the standard deviations of the measurements. The color intensity of each test zone was analyzed using ImageJ after scanning the whole microzone plate. In addition, a linear relationship between the concentration dilutions in the range of 1/100000-1/100 of raw liquor and the color intensity is basically established (the linear equation is y=21.316x-49.102, and the R² value is 0.9933).

The curve shows the general relationship between concentration and gray level. However, for different detectors, the relationship curve may be slightly different.

To further verify the performance of Polymer-ELISA, we used this method to detect Nogo-66 in human serum samples as well as in control samples (human serum without Nogo-66). Nogo was discovered and cloned in recent years and was found to have an inhibitory effect on the central nervous system. Nogo-66 is a 66-amino-acid domain found in the extracellular hydrophilic region of the Nogo protein and is the binding site of the Nogo receptor (NgR). Nogo-66 plays a key role in the Nogo protein, so research on Nogo-66 has attracted much attention in recent years. We examined a number of dilutions of serum
samples from HIV-1-positive patients. Specifically, the new Polymer-ELISA plates were coated with Nogo-66 diluted in 30 μL of carbonate buffer at pH 9.6 and incubated at 37°C for 0.5 h. After washes with PBST, Nogo-66-Ab1 was added and incubated at RT for 1 h. After being sealed with 1% BSA, the plates were incubated at 37°C again. After washes with PBST, Ab2-HRP was added, and the plates were incubated at 37°C. After washing, color was developed using the chromogen/substrate mixture TMB/H₂O₂.

As shown in Fig. 3, the samples in the vertical columns contained the same amount of Nogo-66, and the samples in the horizontal rows represented a 3-fold dilution series. Nogo-66 was detected in the first three columns using Polymer-ELISA (Fig. 3A), while it was detected only in the first column using the traditional ELISA method (Fig. 3C).

![Image](image1.png)

**Fig. 3.** A 3-fold dilution series of Nogo-66 was detected using Polymer-ELISA (A, B) and the traditional ELISA method (C, D). B and D are the control groups detected by the new Polymer-ELISA method and the traditional ELISA method, respectively.

The results show that the Polymer-ELISA method can be used for detection in complex samples such as human serum and that the limit of detection (LOD) of the new method is only one-ninth of that of the traditional method. In other words, the sensitivity of Polymer-ELISA is approximately 9 times higher than that obtained by the traditional ELISA method. The difference in LOD between Polymer-ELISA and traditional ELISA may be a consequence of the high reaction efficiency and low background interference caused by polymer nanocarrier.
In addition, many results show that different fold division series of Nogo-66 detected using Polymer-ELISA have better repeatability (between the sample plate and multiple plates). Therefore, the method is repeatable and can be used in practical detection. Table 1 compares the Polymer-ELISA method and the traditional method.

**Table 1.** Comparison between Polymer-ELISA and conventional ELISA.

|                        | Polymer-ELISA | conventional ELISA |
|------------------------|---------------|-------------------|
| carrier                | polymer       | plastic plate     |
| detection device       | scanner or smart phone | plate reader |
| sample volume          | 2 μL          | 100 μL            |
| LOD                    | 5.6 ng/mL     | 50 ng/mL          |
| cost                   | ~$ 0.022/sample | ~$ 1.802/sample   |
| on-site operation      | Yes           | No                |

In order to confirm the reproducibility of the fabric of the proposed polymer carrier, the following experiments are designed. To enhance the contrast, several groups of polymers were made in batches, and Nogo-66 was tested according to the same method. The same samples were tested using six separate polymers, and the results were compared. The cyan-magenta-yellow (CMY) grey value is expressed as the mean ± standard deviation (x ± SD). T-test and analysis of variance were used, and the standard calibration curve was established in Fig. 4.

![Calibration Curve](image.png)
Six parallel tests were carried out on the same Nogo-66 sample at each point, and six prepared polymers were used for each test. The samples in each point (a, b, c, d, e, f, g and h) are a series of dilutions of the original samples, and the dilution ratio of each adjacent two points is 1/10 (the concentration in point g is the highest and the concentration in point a is the lowest).

Comparative experiments show that the results obtained by different batches of polymers have good repeatability. That is to say, the new method is stable and will not lead to the instability of results due to the introduction of polymers.

The new Polymer-ELISA method has the following advantages over other detection methods. First, the amount of sample required is less, approximately one-third of that used in the traditional method. Second, the new method took less time. Complete detection took only approximately 60 minutes. Third, the cost is low. The new method does not necessitate expensive equipment, and it is suitable for outdoor use. The reasons for these advantages are as follows: As the area of the test zone is reduced to a quarter of the original area, the amount of sample required is also significantly reduced. Because polymer carriers are nanoscale materials and antibodies react rapidly with antigens, which greatly shorten the whole detection process. The new method does not require additional equipment, and the detection can be performed with only a mobile phone; thus, the cost is greatly reduced. In addition, it is worth mentioning that the nanoreactor can be used repeatedly, and the usefulness of this feature can be explored in future research.

Through the previous comparison, we know that compared with the traditional method, the new method has improved sensitivity, cost, detection time, sample quantity, applicability and so on. Because the sensitivity of paper ELISA is lower than that of the traditional ELISA method, the new Polymer-ELISA method has an obvious advantage over paper ELISA in sensitivity.
4 Conclusions

In summary, we successfully developed a new and sensitive Polymer-ELISA method based on a polymer as a carrier. Because the new method has a shorter operation process and a lower LOD than those of traditional ELISA, it might enable more extensive application, analysis and environmental monitoring. Because this method is easy to use outdoors and low in price, it can be used for outdoor detection in remote areas or in relatively poor conditions. We believe that the application of polymers in ELISA will provide a useful approach to immunoassays and that this idea will play a greater role in future immunoassays.

Acknowledgements

This work was financially supported by the Ministry of Science and Technology of the People’s Republic of China (No. 2017ZX09304026) and the Beijing Municipal Science and Technology Commission Research Platform for Development of Phase-zero Clinical Trials in Targeted New Drugs.

The fund sources did not participate in the study design, the collection, analysis and interpretation of data, etc.

Conflict of Interest

The authors declare no conflict of interest.
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