J Fluoresc (2011) 21:339–346
DOI 10.1007/s10895-010-0721-2

ORIGINAL PAPER

Studies of Fluorescence Immunosensor Using Eggshell Membrane as Immobilization Matrix

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Received: 28 May 2010 / Accepted: 8 September 2010 / Published online: 2 October 2010

Abstract A novel immunosensor using eggshell membrane for determining the human immunoglobulin M (HIgM) in serum was developed. The immunosensor was fabricated by immobilizing goat anti-human IgM antibody on the eggshell membrane with glutaraldehyde. Based on the immunoreactions of goat anti-human IgM (primary antibody), HIgM (target antigen) and the goat anti-human IgM (secondary antibody), the sandwich complex were formed on the eggshell membrane and fluorescein isothiocyanate (FITC) labeling secondary antibody could be employed to detect the target antigen. Under the optimized conditions, the linear range for determining HIgM is 5–60 ng mL^{-1} and the detection limit is 4.3 ng mL^{-1}, which are comparable with the results obtained by general immunonephelometric method. Meanwhile, this proposed sensor also exhibited remarkable storage stability, permeability and highly biocompatibility. The effects of temperature and pH value on eggshell membrane were investigated. Therefore, the proposed immunosensor, by using eggshell membrane as immobilization platform of antibody, offers an excellent fluorescence response to HIgM. The immunosesor provided a new alternative to determine antigens and other bioactive molecules.

Keywords Eggshell membrane · Immunosensor · Human immunoglobulin M · Fluorescence

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Introduction

IgM is the heaviest immunoglobulin (Ig) with molecular weight of approximately 900,000 Da. IgM contributes about 6% of the total content of Ig and exists in the normal human serum at an average concentration of 0.6 to 1.8 g L^{-1}. IgM is very efficient agglutinates of bacterial cells and cytolytic agents. So IgM forms the most immediate and effective first line defence against bacteraemia. Since IgM appears in response to infection it is mostly confined in the blood stream. Therefore, the IgM is an important marker for many diseases and the level of the IgM in the human blood is related to the diseases such as malaria, cirrhosis, chronic hepatitis, Waldeström macroglobulinemia, intrauterine infections and HIV infections [1, 2].

Over the years, researches in the development of chemosensors and biosensors have been very fast growing. The performance of the immunoaffinity-based biosensors is dependent on the specificity and sensitivity of antibodies, and the shelf-life of immunoaffinity-based biosensors is dependent on how long the biological activity of antibodies can be retained [3]. For an immunosensor, the antibody is required to be immobilized on, or in close proximity to, the surface of a matrix. As a consequence, immobilization matrices for antibodies are of paramount importance in order to preserve their biological activity. As is known, the most commonly immobilization matrix is polystyrene such as ELISA plate, which is a very useful and powerful matrix for estimating antigen-antibody reactions [4–6]. Despite its usefulness, the plate used in ELISA has several disadvantages. On one hand, the polystyrene matrix has poor biocompatibility and easy to inactivate the reaction of antigen-antibody. On the other hand, the interaction between antibody and matrix is mainly non-covalent adsorption, which is not very stable and accordingly

impacts the measurement results. The novel solid phase supports such as a magnetic particle [7–10], nanometer particles [11–14] and self-assembly films [15–18] were developed in order to overcome these drawbacks. Recently, some biomaterials, for example, silk [19–21], collagen [22–24], bamboo inner shell membrane [25] and eggshell membrane [26–30], have attracted more interest. It has been reported that these biomaterials were employed as platforms for the immobilization of proteins and the life-times of the immobilized proteins could be prolonged. Among the materials, eggshell membrane is a common biomaterial, easily obtainable and nontoxic. Eggshell membrane is a light pink double-layered membrane that is discernible as shifts in fiber position and changes in fiber size [31]. Eggshell membrane as a good biocompatible material is mainly composed of biological molecules and highly cross-linked protein fibers [32]. The special performance of eggshell membrane is due to its special composition and structure. It is reported that eggshell membrane is semipermeable, through which the small molecules can be selectively permeated, and the large ones cannot, such as glucose [33]. The eggshell membrane possesses excellent gas and water permeability, and most importantly, due to the surface amino-groups of eggshell membrane, it is easy to combine with many biomolecules, such as enzymes, proteins and antibodies. Therefore, the eggshell membrane may be used as an ideal bio-platform for antibody immobilization. Recently, the application of eggshell membrane has attracted considerable interest as its unique virtue. Choi and his coworkers used eggshell membrane as an enzyme immobilization platform to fabricate some electrochemical biosensors, wherein enzymes catalase, glucose oxidase, myrosinase, uricase, amino acid oxidase were immobilized using glutaraldehyde [34–36]. Li et al. immobilized glucose oxidase and horseradish peroxidase on the eggshell membrane to develop a new chemiluminescence flow-through biosensor for glucose [37].

Previously, our lab has preliminarily studied the sandwich immunoassay using eggshell membrane as the stable and long-lived supporting matrix [38]. In this work, the effect of some experimental condition such as temperature and pH value on properties of eggshell membrane, storage stability of the proposed HIgM sensor and analytical performance for determining HIgM were more investigated in detail. The experimental results further suggested that it is possible to achieve the simultaneously multi-immunoglobulin determination with different fluorescent markers. The detection scheme was based on a sandwich immunoreaction of primary antibody on the eggshell membrane, HIgM (or sample serum), and the secondary antibody labeled with FITC. The effects of cross linker concentration, primary and secondary antibodies concentration on the responses of the immunosensor were also investigated. The proposed method was successfully applied to the determination of HIgM in serum sample, and the result was satisfactory compared with that obtained by immunonephelometric method [39, 40], which was a routine method in clinical diagnosis.

Materials and Methods

Reagents and Materials

Human immunoglobulin M (HIgM), goat anti-human IgM (primary antibody) and goat anti-human IgM/FITC (secondary antibody) were purchased from Beijing Biosynthesis Biotechnology Co., Ltd. (Beijing, China). Quality control serum was purchased from National Center for Clinical Laboratory (Beijing, China). All the antibody and antigen solutions were prepared by the phosphate buffer solution (PBS, 10 mmol L^{-1}, pH 7.4). Ethanolamine was obtained from Tianjin Guangfu Fine Chemical Industry Institution (Tianjin, China). Glutaraldehyde (25%, *w*/*w*) was from Beijing Chemical Reagent Corporation (Beijing, China). Chicken eggs were purchased by local markets. All other chemicals were of analytical grade and used without further purification, and all the aqueous solutions were prepared with distilled water.

Apparatus

Fluorescence measurements were performed on RF-5301PC fluorescence spectrophotometer (Shimadzu, Japan) equipped with a xenon lamp using right-angle geometry. The eggshell membrane with sandwich complex was placed on the solid sample holder and the excitation and emission wavelengths are 495 and 520 nm, respectively. Morphological characterization of eggshell membrane was recorded with JEOL JSM-6700F scanning electron microscope. The pH value was measured by a Delta 320 pH meter. Unless otherwise stated, all the experiments were carried out at room temperature and atmospheric pressure.

Preparation of Eggshell Membrane

Whole eggshell membrane was carefully peeled from fresh eggshell which incubated in acetic acid at 4°C [41]. The obtained eggshell membrane was cut into four equal parts and then cleaned with a large amount of distilled water. Further cleaning steps were necessary according to the reference [42] in order to completely remove the albumen from the eggshell membrane. Finally the eggshell membrane was stored in PBS at 4°C.

Immobilization of the Goat Anti-Human IgM Antibody on the Eggshell Membrane

The eggshell membrane was taken out from the PBS, placed on a clean small watch glass and acted cut into a small piece (10×20 mm). First, 40 μL of the goat anti-human IgM solution was dropped onto the surface of the eggshell membrane and kept for 20 min at 4°C for adsorption. Next, 10 μL of 2.5% (*w*/*w*) glutaraldehyde solution was added and spread evenly on the membrane surface with a small glass rod. After incubation at 4°C for 2 h, the immobilized membrane was rinsed with distilled water and immersed in PBS three times alternately to remove the excessive antibody and cross-linking reagent. And then 40 μL of the ethanolamine solution was added and incubated at 37°C for 20 min. Finally, the resulting in eggshell membrane was rinsed with distilled water and immersed in PBS for 5 min three times. It should be note that the position of the membrane is on the central of the spot.

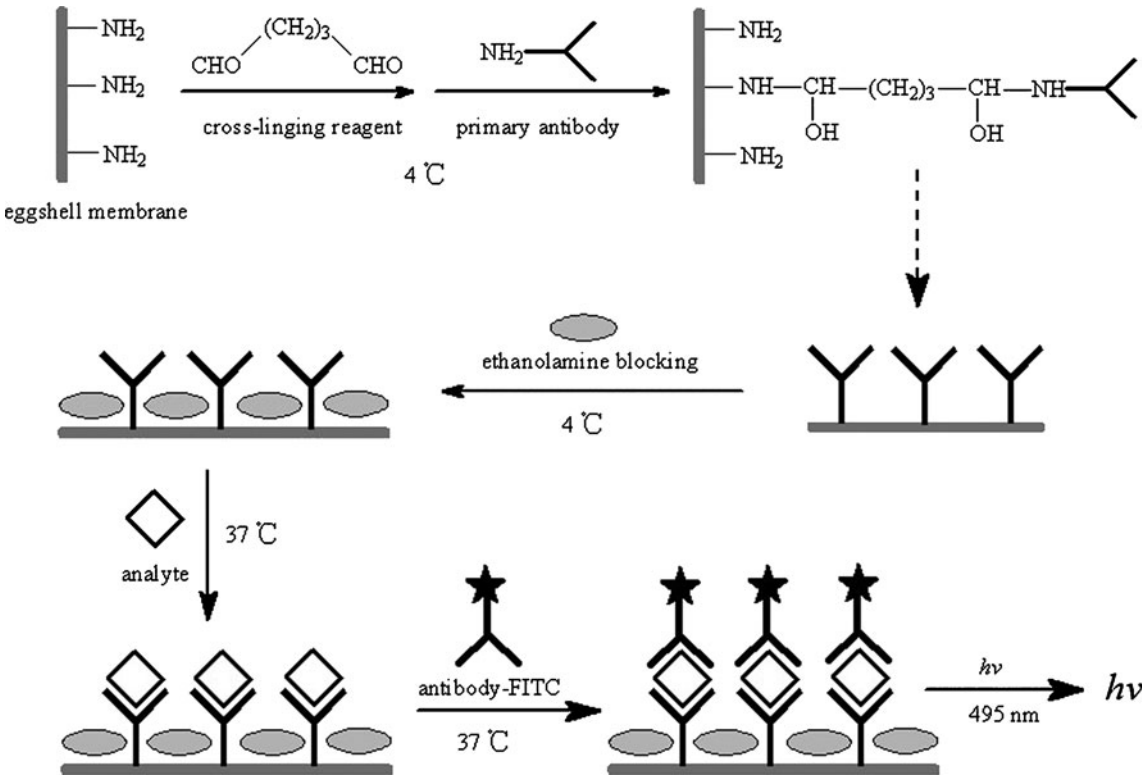
Fluorescence Immunoassay for HIgM

Forty microlitres of the HIgM solution was dropped onto the eggshell membrane and incubated at 37°C for 40 min. Next, 40 μL goat anti-human IgM/FITC was added and incubated successively for 40 min, followed by rinsing with distilled water three times. The fluorescence intensity of as-prepared sample was recorded at the excitation wavelength of 495 nm.

Results and Discussion

Fluorescence Spectrum of the Eggshell Membrane

Scheme 1 shows the protocol for the detection of antigen using eggshell membrane as matrix. After first chemically immobilizing primary antibody on the surface of eggshell membrane and making the immunoreactions occur with antigen, the labeled secondary antibody was bond to the antigen so that the fluorescence immunosensor was fabricated. Figure 1 shows the fluorescence spectra of fresh eggshell membrane, sandwich complex on membrane and solution containing the goat anti-human IgM/FITC. It is found that the background interference of eggshell membrane is very low. As shown in Fig. 1, the emission peak of the sandwich complex on membrane is essentially consistent with that of FITC, which displays that the sandwich complex is coupled with the eggshell membrane, and it is feasible to determine the target antigen using FITC as the probe.



Scheme 1 Schematic representation of sandwich immunoassay using eggshell membrane as matrix

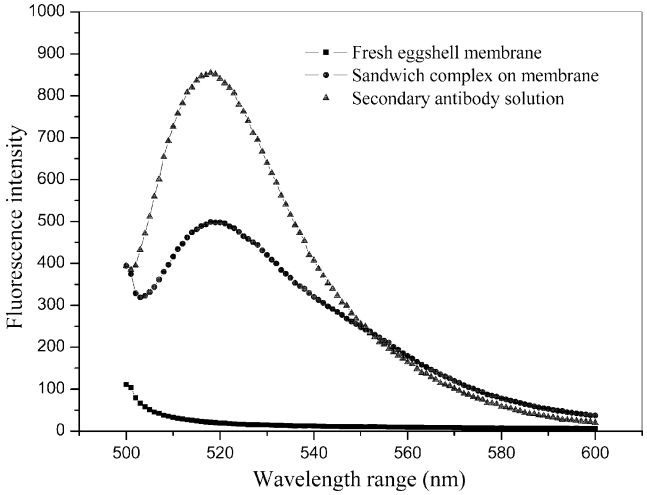


Fig. 1 Fluorescence spectra of fresh eggshell membrane, membrane with sandwich complex and the solution containing goat anti-human IgM/FITC

SEM Images of the Eggshell Membrane

The surface morphologies of eggshell membrane with and without the immobilized antibody are displayed in Fig. 2. It is observed from Fig. 2a that a network-like structure without any aggregation exists in the cleaned eggshell membrane, which indicates that the eggshell membrane consists of highly cross-linked protein fibers and cavities, and this special structure makes the membrane possess excellent gas- and water-permeability. However, in Fig. 2b, some specks or clusters of sandwich complex can be clearly seen on the protein fibers, but Fig. 2a does not show the attachment of sandwich complex on the protein fibers. Since the individual size of antibody cannot be observed from the SEM images, the immobilized antibodies can be observed as some condensed spots or clusters on fibers. Hence, it is confident that the antibodies were successfully immobilized on the eggshell membrane.

The Effect of Temperature on Fresh Eggshell Membrane

The fresh eggshell membrane was incubated at different temperatures, and then the intrinsic fluorescence intensity of the eggshell membrane was measured using 280 nm as the excitation wavelength. The experimental results show that fluorescence intensity of the eggshell membrane does not obviously change at the different temperature: 4, 17, 25, 40 and 60°C, and just slight higher at 25°C. Therefore, it is suitable for the experiment measurement at room temperature.

The Effect of pH Value on Fresh Eggshell Membrane

Seven fresh eggshell membranes were dipped in PBS of different pH value for 1 h, 24 h and the effect of pH value

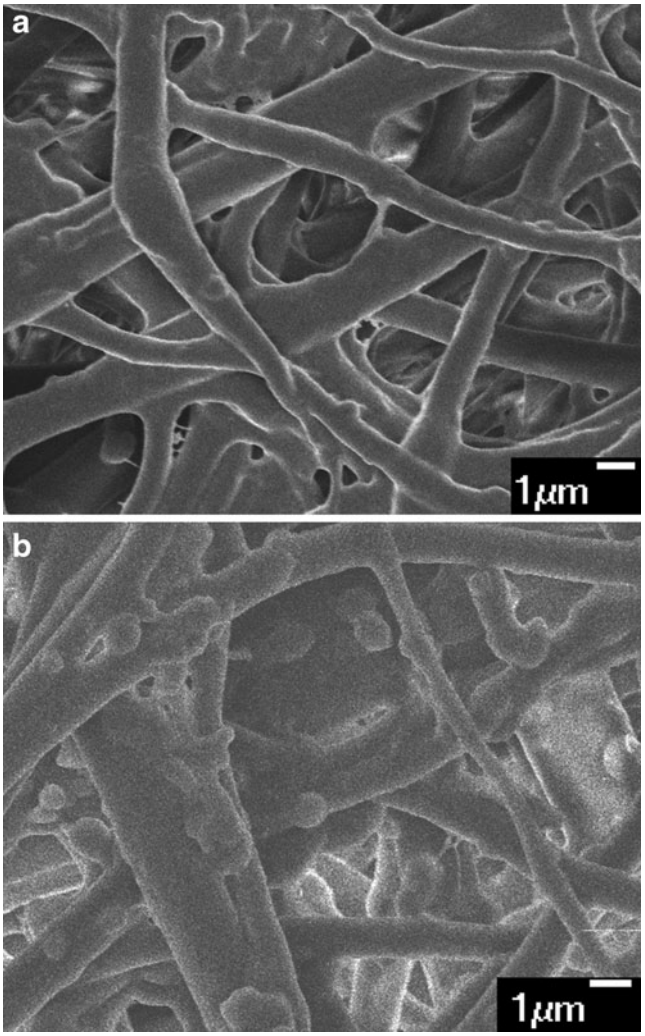


Fig. 2 SEM images of fresh eggshell membrane (**a**) and eggshell membrane with sandwich complex (**b**). SEI 5.0 kV, ×3000, WD 7.7 nm

on fresh eggshell membrane was investigated. It can be seen that the change rate of intrinsic fluorescence intensity of different eggshell membranes was little, which indicated that eggshell membrane was stable themselves with wide pH range from 3.5 to 10.5.

Stability of the HIgM Immunosensor

The fluorescence intensity of the membrane was measured using 280 nm and 495 nm as the excitation wavelength in order to evaluate the performance of eggshell membrane obtained from different eggs. The comparison of six different eggshell membranes is displayed in Fig. 3. It can be seen that the intrinsic fluorescence intensity of different eggshell membranes are basically same and the relative standard deviation is 0.072, so the repeatability of the membranes is satisfactory. On the other hand, it can also be seen that the background value of membrane is very low and the

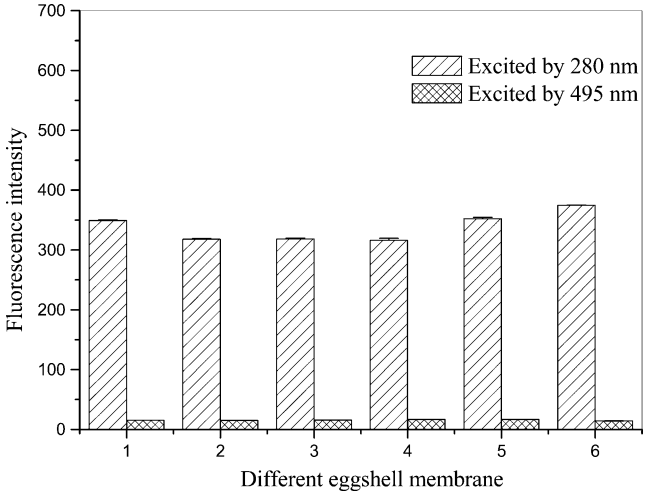


Fig. 3 Comparison of different eggshell membranes

background interference can be ignored. The performances of the membrane were periodically tested and the shelf-life is over two months at 4°C. It is shown in Fig. 4 that the fluorescence intensity does not change obviously in the storage period of 30 days. The remarkable stability of the HIgM immunosensor is related to the biological compatibility of the eggshell membrane with the antibodies. Eggshell membrane has the net-veined structure and its gas-permeable hydrophilic property of eggshell membrane can provide an excellent biological micro-environment for the antibody to survive and maintain its activity.

Optimized Conditions of the HIgM Immunosensor

Effect of Concentration of Glutaraldehyde

The covalent bonding can be used to achieve the immobilization of antibodies on the membrane matrix, is based on the cross-linking reaction between the same terminal functional groups of the biological molecules and reactive groups on the solid surface. In this system, the amino groups of the eggshell membrane and the antibody can react with glutaraldehyde. The concentration of glutaraldehyde is an important factor effect the covalent binding. The effect of glutaraldehyde concentration to cross-linked primary antibody onto the membrane was investigated. The results are displayed in Fig. 5 and it can be seen that the fluorescence intensity of the immunosandwich membrane increases with the increase of glutaraldehyde concentration when glutaraldehyde is in the range of 0.5–2.5% (*w*/*w*). But the fluorescence intensity decreases appreciably in the range of 2.5–7.5% (*w*/*w*). Therefore, 10 μL glutaraldehyde solution of 2.5% (*w*/*w*) was chosen as the optimum amount of cross-linking agent for the primary antibody immobilization on the membrane.

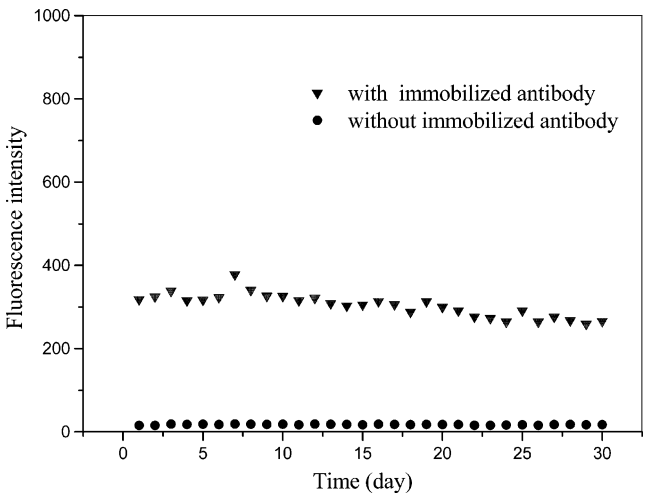


Fig. 4 The effect of storage period of eggshell membrane on fluorescence intensity

Effect of Antibody Dilution Ratio

The concentrations of primary and secondary antibody strongly affected the performance of the immunosensor. As shown in Fig. 6, the fluorescence intensity of the biosensor has undergone dramatic change with the change of dilution ratio. The amount of antigen-antibody complex gradually increases with the increase in the amount of primary antibody, and the fluorescence intensity should increase accordingly. However, the fluorescence intensity of the probe gradually decreases when the amount of the primary antibody is too large. Since antigen-antibody reactions occur via non-covalent bonds, including hydrogen bonds, electrostatic bonds and Van der Waals forces, they are by their nature reversible. The complex of antigen-antibody

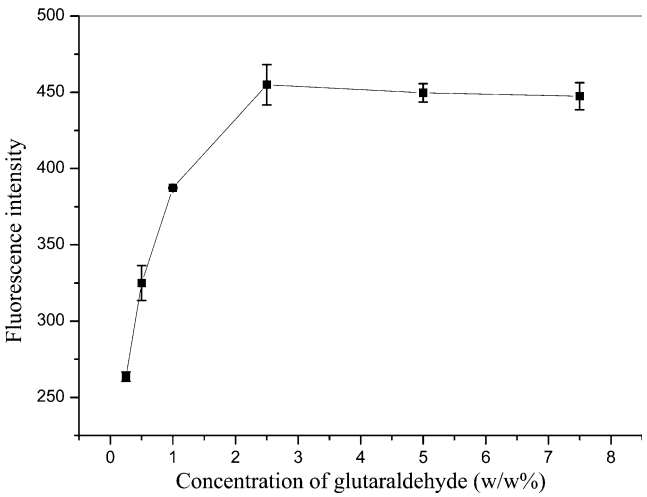


Fig. 5 Effect of glutaraldehyde concentration on the fluorescence intensity. Primary antibody, 1:16; HIgM, 100 ng mL^{-1}; secondary antibody, 1:8. The error bars denote the standard deviation of the values with the three same assays

dissociates to free antigen and antibody. In brief, the ratio between the antigen and antibody influences the detection of antigen-antibody complexes. From Fig. 6a it is displayed that the fluorescence intensity increases considerably when the dilution ratio is higher than 1:16, and then decreases with the decrease of the amount of primary antibody. An optimal dilution ratio of primary antibody at 1:16 was chosen in sandwich immunoassay. The effect of dilution ratio for the secondary antibody was also studied and the results are shown in Fig. 6b. It can be found that the fluorescence intensity is related to the dilution ratio of secondary antibody. The fluorescence intensity could be enhanced as the increase of amount of secondary antibody, which means that the amount of formed sandwich complex increases. However, the fluorescence intensity decreased when the dilution ratio of secondary antibody was lower than 1:8. It was the result that the dissolution of sandwich complex with further increases of amount of the secondary antibody. So the dilution ratio of secondary antibody 1:8 was chosen for the further study.

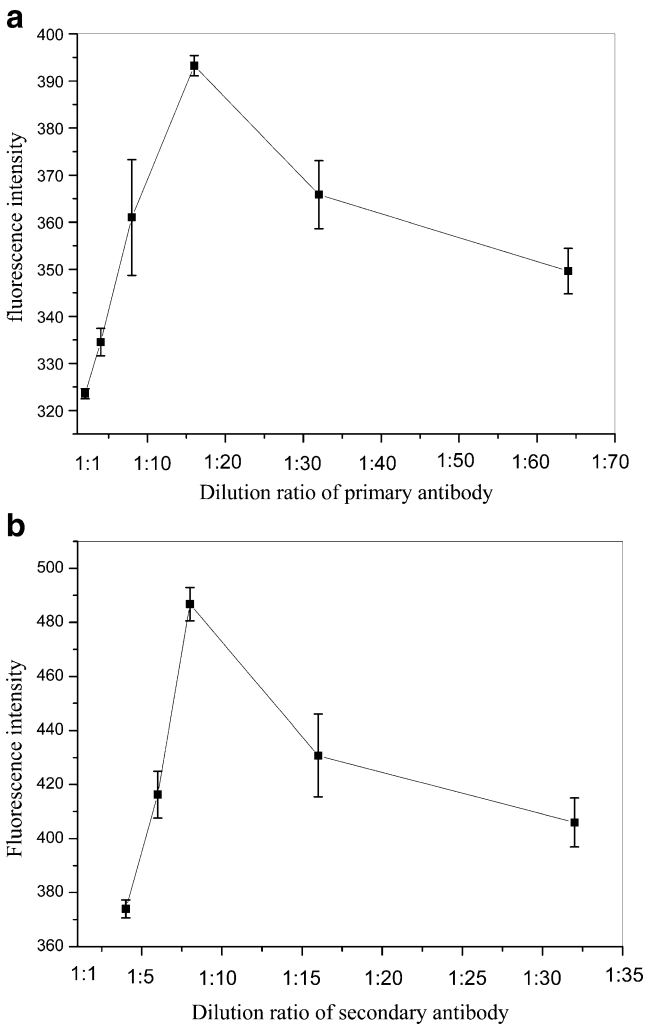


Fig. 6 Effect of dilution ratio of primary antibody (**a**) and secondary antibody (**b**). Glutaraldehyde, 2.5% (*w*/*w*); HIgM, 100 ng mL^{-1}; Initial concentrations of primary and secondary antibodies are 1 mg mL^{-1} and 0.6 mg mL^{-1}, respectively

Calibration Curve for the Determination of HIgM

The relationship between the relative fluorescence intensity and the HIgM concentration was obtained under the selected conditions mentioned above. It is clearly shown in Fig. 7 that there is good linear relationship in the range of 5–60 ng mL^{-1} with the correlation coefficient of 0.9918. The regression equation is $F=180.811 + 1.426\ C$ (where F is fluorescence intensity and C is the concentration of HIgM, ng mL^{-1}). The narrow working range may be due to the low amount of primary antibody immobilized on the eggshell membrane. The detection limit (DL) is 4.3 ng mL^{-1} and the DL=3 S_{bk} / κ (the S_{bk} was standard deviation of eight blank samples; the κ was the slope of the calibration curve). The detection limit was improved compared with that obtained by immunonephelometric method and the serum samples need dilute to achieve effective detection when the HIgM concentration is higher than 60 ng mL^{-1}.

Analysis of the Sample Serum

The quality control serum sample was analyzed in order to test the feasibility of the proposed method. The sample was diluted with PBS prior to analysis of the serum sample. It is seen from Fig. 7 that the resulting sample solutions were directly analyzed by the proposed method and the obtained concentration of HIgM are 0.92±0.03, 0.88±0.03, 1.11±0.04 and 1.05±0.03 g L^{-1}, respectively. The sample was also detected by the immunonephelometric method, which offered by the Second Hospital of Jilin University, and the

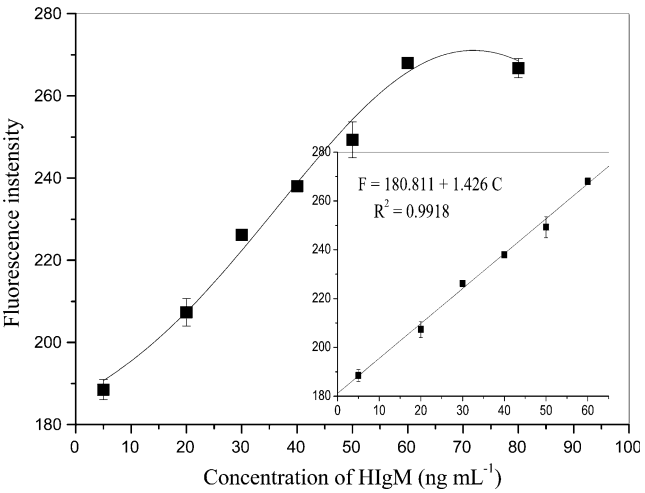


Fig. 7 Standard curve for the determination of HIgM, the inset displays the linear calibration curve

obtained HIgM concentration is 0.84±0.20 g L^{-1}. So it is clear that the results obtained by the proposed methods and immunonephelometric methods are similar. On the other hand, the sensitivity of proposed methods was improved significantly compared with the immunonephelometric method which the sensitivity was 4.2 mg dL^{-1} at 95% confidence level.

Conclusions

In this study, a sensitive fluorescence immunosensor using eggshell membrane as matrix has been successfully fabricated and used to determine the HIgM in serum sample. It is demonstrated that the cheap and easily obtained eggshell membrane exhibited high biocompatibility, remarkable permeability for gas and water. The experimental results show that eggshell membrane can provide a simple, high-specific immobilization matrix to construct a friendly environment for keeping the activity of antibody. Furthermore, the sensitivity of immunoassay and the detection limit were highly improved. We believe that the eggshell membrane can provide an immobilization platform to fabricate a new type of immunosensor and find further applications in other antigens and bioactive molecules.

Acknowledgements The work described in this paper was partially supported by a grant from the Key Lab for Supramolecular Structure and Materials, College of Chemistry, Jilin University, Changchun 130012, P.R. China. The authors acknowledge the Second Hospital of Jilin University for the technical support.

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