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Highly sensitive colorimetric immunosensor for influenza virus H5N1 based on enzyme-encapsulated liposome

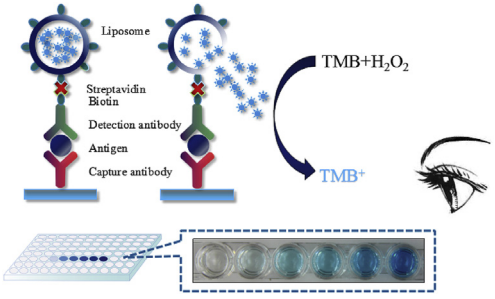
Cuiying Lin [a], Yajuan Guo [a], Mengmeng Zhao [a], Mi Sun [a], Fang Luo [b, *], Longhua Guo [a], Bin Qiu [a], Zhenyu Lin [a], Guonan Chen [a]

[a] College of Chemistry, Fuzhou University, Fuzhou, Fujian 350116, China
[b] College of Biological Science and Technology, Fuzhou University, Fuzhou, Fujian 350116, China

HIGHLIGHTS

- We have proposed a simple but sensitive colorimetric immunosensor for influenza detection.
- The biosensor combines the advantages of high selectivity of immunoassay and simplicity of colorimetric detection.
- The sensitivity of the biosensor is much higher than that of conventional ELISA method.
- Such immunosensor has been successfully demonstrated its practical application for the detection of H5N1 in human serum.

GRAPHICAL ABSTRACT



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ABSTRACT

Development of simple but sensitive biosensor for influenza detection is highly important in immediate and effective clinical treatment. In this study, a sensitive colorimetric immunosensor which combines the advantages of high selectivity of immunoassay and simplicity of colorimetric detection has been developed to detect influenza virus H5N1 based on enzyme-encapsulated liposome. Biotin-tagged liposome encapsulated with large amount of horseradish peroxidase (HRP) was firstly synthesized. In the presence of H5N1, H5N1 co-bound with the capture antibody and the biotinylated detection antibody to form sandwich immunocomplex. Subsequently, the HRP-encapsulated liposome was introduced to conjugate with the detection antibody through biotin-avidin-biotin linkage. Upon the addition of substrate (mixture of 3,3′,5,5′-tetramethylbenzidine (TMB) and H_2O_2), the liposome was directly lysed to release large amount of HRP by TMB. The released HRP catalyzed the H_2O_2-mediated oxidation of TMB, resulting in color change of the system, which was observed by naked eyes or UV–vis spectra. The result showed that the absorption intensity enhanced with the increase of H5N1 concentration ranging from 0.1 to 4.0 ng/mL, and the detection limit was calculated to be 0.04 ng/mL. The sensitivity of the proposed biosensor is much higher than that of conventional enzyme-linked immunosorbent assay method. The proposed immunosensor is relatively simple, low-cost, sensitive, and selective without using any sophisticated instruments, therefore it may have a promising prospect for detecting targets in clinical medicine, food safety analysis, and environmental monitoring.

* Corresponding author.
E-mail address: luofang0812@163.com (F. Luo).

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1. Introduction

Liposomes are spherical vesicles that are formed by amphiphilic molecules dispersed in aqueous and consist of an aqueous core and a lipid bilayer, the hydrophilic substances can be dissolved in the aqueous cores of the liposomes while the hydrophobic compounds can be embedded into the bilayers. Liposomes have been used as the multi-functional carriers [1–3] in the immunoassay owing to their ability to encapsulate large amounts of small molecules [4], therapeutic drugs [5], nucleic acids [6], and enzymes [7], which can realize the signal amplification because a large quantity of molecules are released from liposomes. For example, an ultrasensitive immunoassay for the biotoxins detection has been developed on the basis of DNA-encapsulated liposomes [8], the accuracy has also been quantified by real-time PCR. Furthermore, DNA-encapsulated liposomes have been coupled with rolling circle amplification (RCA) to develop an ultrasensitive immunosensor for protein detection; primary amplification via releasing numerous DNA primers from a liposome is followed by a secondary RCA amplification to realize high sensitivity [6]. On the basis of small molecules-encapsulated liposome, an electrochemiluminescence (ECL) immunoassay strategy has been proposed to detect heart failure biomarker N-terminal pro-brain natriuretic peptide with high sensitivity and specificity [9]. A dual amplification strategy has been developed for highly sensitive fluorescence detection of DNA based on fluorescein-encapsulated liposomes [3]. All these studies have demonstrated the successful applications of liposomes in the development of highly sensitive detection platform. However, most of these methods require relative sophisticated equipments, and the assay procedures are relative complicated and time-consuming. To address these issues, it is necessary to develop a facile strategy for target detection.

Colorimetric assay normally based on the comparison or measurement of the color change of the solution to determine the content of the component, which has attracted increasing attention in different areas primarily because it enables the detection of targets by naked eyes without any sophisticated instruments [10]. Enzyme-linked immunosorbent assay (ELISA) is a simple, rapid, and highly selective tool that is used to detect particular biomarker through the specific interaction between the antigen and antibody and signal via color change. In conventional ELISA method, the immunocomplex is conjugated to an enzyme that catalyzes chromogenic substrate; because a small amount of enzyme is modified on the immunocomplex, its sensitivity is limited. To improve the sensitivity, one way is to combine the immunocomplex with many enzymes. One of the best candidatures enzyme-encapsulated liposomes have been employed to couple with ELISA to develop highly sensitive biosensors because each immunocomplex contains a large quantity of enzymes. For example, a highly sensitive chemiluminescence immunosensor has been constructed for the detection of prostate-specific antigen (PSA) based on enzyme-encapsulated liposome [11], the result verified that the enzyme-encapsulated liposome can be used to amplify the detected signal. But to the best of our knowledge, little attention has been paid to develop colorimetric biosensor based on the enzyme-encapsulated liposome.

In the above mentioned liposomes-based biosensor, the liposomes need to be lysed with the addition of surfactants such as Triton X-100, making the procedure become complex; furthermore, the surfactants may have adverse effect on enzymes. 3,3′,5,5′-tetramethylbenzidine (TMB) has been widely used as a chromogenic substrate for colorimetric immunoassays, which causes little adverse effect to the enzyme activity. In this study, liposomes were found to be lysed by TMB easily. This character was applied to develop a sensitive colorimetric ELISA biosensor based on horseradish peroxidase (HRP)-encapsulated liposome. H5N1 poses a public health threat and has high morbidity and mortality, so it was chosen as the example target in this study.

2. Experimental section

2.1. Reagents and materials

L-α-phosphotidylcholine (PC) and HRP were purchased from Sigma-Aldrich (USA). Tris-buffered saline Tween (TBST, referring to 10 mM Tris buffer, 0.15 M sodium chloride, 1% Tween-20, pH 7.5), 3% bovine serum albumin in Tris buffer (BSA-TBS), and sodium phosphate saline buffer (PBS, 10 mM phosphate buffer, 2 mM potassium phosphate, 137 mM sodium chloride, and 2.7 mM potassium chloride) were provided by Sangon Inc. (Shanghai, China). H5N1 ELISA kits (containing H5N1 capture antibody, H5N1 antigen and biotinylated detection antibody), H7N9 antigen were supplied by Sino Biological Inc. (Beijing, China). H9N2 antigen was provided by Dingguo Biotechnol. Inc. (Beijing, China). Alpha fetoprotein (AFP) was supplied by Wuhan Boster Biological Technology Co., Ltd. (Wuhan, China). Phosphoethanolamine-conjugated biotin (DSPE-PEG2000-biotin) and cholesterol were obtained from RiBio Co., Ltd. (Fuzhou, China). TMB and H_2O_2 were purchased from Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). All reagents and solvents were analytical grade or better and used directly without further purification. Millipore purification system-based ultrapure water was used in this study (18.2MΩ·cm, Milli-Q, Millipore).

2.2. Preparation of the HRP-encapsulated liposomes

HRP-encapsulated liposomes were prepared by the ethanol injection method [12,13]. Normally, the hydrophobic compounds can be embedded into the bilayers of the liposomes and the present of polyethylene glycol (PEG) can improve the stability of liposomes [14], however PEG is not hydrophobic compound. In order to insert PEG into the membrane of liposome, PEG was initially conjugated with hydrophobic phosphoethanolamine. To successful combine liposomes with biotin, the best way is to conjugate biotin with the end of PEG [15]. DSPE-PEG2000-biotin [16] can embedded into the bilayers via the hydrophobic interaction. Briefly, PC(10 mg), cholesterol (2.04 mg), andDSPE-PEG2000-biotin (0.75 mg) with a molar ratio of 70:28:2 were dissolved in chloroform solution (1 mL). To form liposomes, the chloroform was firstly removed under nitrogen to form a thin film, the thin film was subsequently dissolved in ethanol (250 μL), and the resulting ethanol solution was then injected into phosphate buffer (10 mL, 10 mM, pH 7.4) containing 0.2 mg/mL HRP for 1 h at room temperature under vigorous stirring. The liposomes were sonicated using a probe-type sonicator in ice bath to reduce the average size (5–10 min, 40 W), and the solution was later passed through a sephadex G-100 column to remove the unencapsulated HRP. The obtained biotin-tagged HRP-encapsulated liposomes (~1.0 mg/mL) were stored at 4 °C until use.

2.3. Characterization of liposome

To clearly understand the morphology of the as-synthesized liposome, characterization techniques including dynamic light scattering system (DLS, Malvern nano ZS, UK), atomic force microscope (AFM, Bruker Multimode III SPM, USA), and transmission electron microscope (TEM, HITACHI H-7650, Japan) were employed. The average size of liposome was measured with 1:50 dilution of liposomes in PBS buffer (pH 7.4), while a 1:10 dilution of liposomes in PBS (pH 7.4) was used for AFM measurement. Droplets