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

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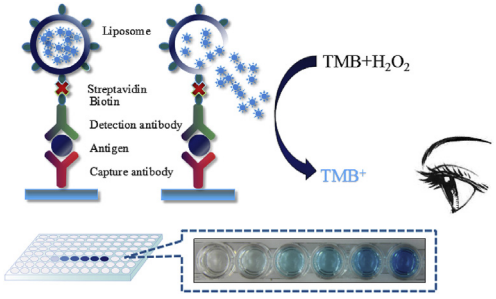
HIGHLIGHTS

- 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.

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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

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of liposomes (8 μL) were deposited on a small mica plate with a diameter of 1 cm. Then the liposomes were dried under nitrogen. For TEM measurement, a 1:10 dilution of liposomes in PBS (pH 7.4) was used. A carbon-coated copper gird was covered with droplets of liposomes (10 μL) for 2 min. After being removed the excess liposomes, the liposomes left on the TEM-grid were negatively stained with phosphotungstic acid aqueous solution (10 μL, 2%, pH 7.4) for 30 s. The excess staining solution was removed with filter paper.

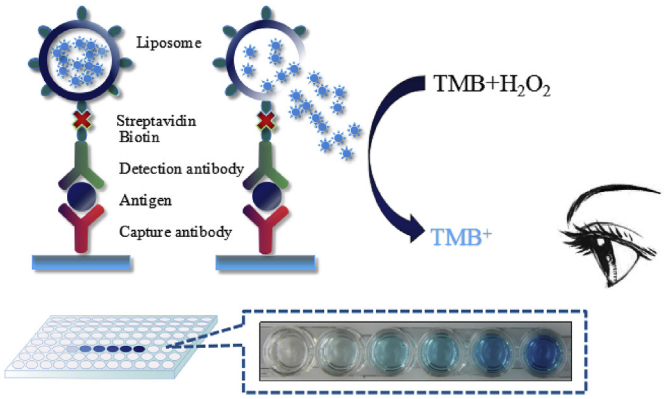
2.4. Detection of H5N1

A 96-well microplate was coated with 100 μL of capture antibody diluted in carbonate buffer (pH 9.6) and stored overnight at 4 °C. The plate was then washed with washing buffer (300 μL, 10 mM TBST), followed by adding blocking buffer (300 μL, 3% BSA-TBS, pH 7.4) for another 2 h at 37 °C. Keep repeating the washing step three times. To the plates was added 100 μL per well of H5N1 standard or sample for incubation (2 h, 37 °C). After washing three times, 100 μL of biotinylated detection antibody diluted in 0.5% BSA-TBST buffer was added to the microplate for incubation (1 h, 37 °C). Keep repeating the washing process three times, and then adding 100 μL of streptavidin solution (1.25 μg/mL) diluted in 0.5% BSA-TBST buffer to the incubated mixture for another 1 h at 37 °C. The plates were washed twice with washing buffer and twice with PBS (10 mM, pH 7.4) to remove remaining Tween 20 in the plates. To the plates was then added 50 μL of HRP-encapsulated liposome diluted in PBS buffer (10 mM, pH 7.4) for incubation (30 min, 37 °C). The plates were gently washed by PBS buffer (300 μL, 10 mM, pH 7.4) to remove the non-bound liposomes. Upon the addition of mixed solution of TMB and H_2O_2 (200 μL, v:v = 1:1), the liposome was lysed to release the HRP [17]. The released HRP catalyzed the H_2O_2-mediated oxidation of TMB, leading to the color change from colorless to blue. The optical absorbance of TMB oxidation products was measured with a 96-well UV-STAR plate (Greiner) and the representative photos of the solution were recorded with a DSLR camera (Canon EOS 600D).

3. Results and discussion

3.1. Principle of HRP-encapsulated liposomes linked immunosorbent assay for H5N1

Scheme 1 shows the principle of the proposed immunosensor based on HRP-encapsulated liposomes. The capture antibody was firstly fixed in the wells of the microplate by physical adsorption. H5N1 as the target was captured by capture antibody and then recognized by biotinylated detection antibody to form a sandwich immunocomplex through the specific antigen and antibody interaction. Streptavidin has an extraordinarily high affinity for biotin [18]. The biotinylated detection antibody is able to combine with biotin-tagged liposome through biotin-avidin-biotin linkage. Unlike the conventional ELISA method, in which only limited HRP is modified on the detection antibody, more HRP can be modified on the detection antibody when the liposome is modified on the detection antibody because large amount of HRP is encapsulated in single liposome. The addition of the mixed solution of TMB and H_2O_2 caused the direct lysis of liposome (TMB and H_2O_2 could permeate through the membrane of liposomes [17], HRP encapsulated in liposomes catalyze H_2O_2 to generate ·OH, leading the lipid preoxidation and lysis of the liposomes [19]) to release a large amount of HRP, which catalyzed the H_2O_2-mediated oxidation of TMB and hence resulted in the color change of the system. As a consequence, the concentration of H5N1 (0.1–4 ng/mL) can be observed by naked eyes easily through this colorimetric method.



Scheme 1. The principle of the immunosensor for H5N1 based on HRP-encapsulated liposome.

3.2. Characterization of the HRP-encapsulated liposomes

The DLS measurement in Fig. 1A shows that the biotin-tagged HRP-encapsulated liposomes had an average diameter of ~100 nm, and the polydispersity index (PdI) of 0.247 demonstrates the uniform distribution. Images of liposomes were further obtained by AFM in tapping mode. AFM image clearly shows the liposome had a height of ~3.0 nm (Fig. 1B), indicating the successful encapsulation of liposome. Herein, the deviations from the perfectly spherical shape of liposomes are likely caused by the drying process on the mica surface. TEM image (Fig. 1C) shows the liposomes were well distributed and there were no ruptured liposomes, further demonstrates the successful formation of the liposomes. These results are similar with those in previously reported studies in which thin-film dispersion and reverse phase evaporation methods are used to synthesize liposomes [3,20].

To confirm HRP was successfully encapsulated in the liposomes, different concentrations of streptavidin modified magnetic beads were utilized to combine with biotin-tagged liposomes. The absorbance value increased with the rise of magnetic beads concentration, and there was a good linear relationship between the absorbance value and the concentration of magnetic beads. The linear equation was as follows:

$$A = 13.67C\ (\mathrm{mg/mL}) + 0.08\ \left(R^2 = 0.9926\right)$$

where A, C, and R^2 are the absorbance value, concentration of magnetic beads, and correlation coefficient, respectively. As shown in Fig. 1D (corresponding photographs), the color became more blue with the enhanced concentration of magnetic beads, and this phenomenon can be observed by naked eyes easily. These results indicate that (1) the biotin-tagged liposome can effectively combine with streptavidin modified magnetic beads; and (2) HRP has been successfully encapsulated in the liposomes. Therefore, the biotin-tagged HRP-encapsulated liposome can be used for the following study.

3.3. Optimization of the experimental conditions

To achieve a better sensing performance, two factors influencing the formation of HRP-encapsulated liposome and the detection of H5N1 were investigated. The cholesterol is one of the components in liposomes [21–24], so the density of cholesterol in liposome is one of the most important parameters affecting the stability of liposome. More specifically, a low density of cholesterol cannot prevent the HRP encapsulated in liposome from leakage, hence

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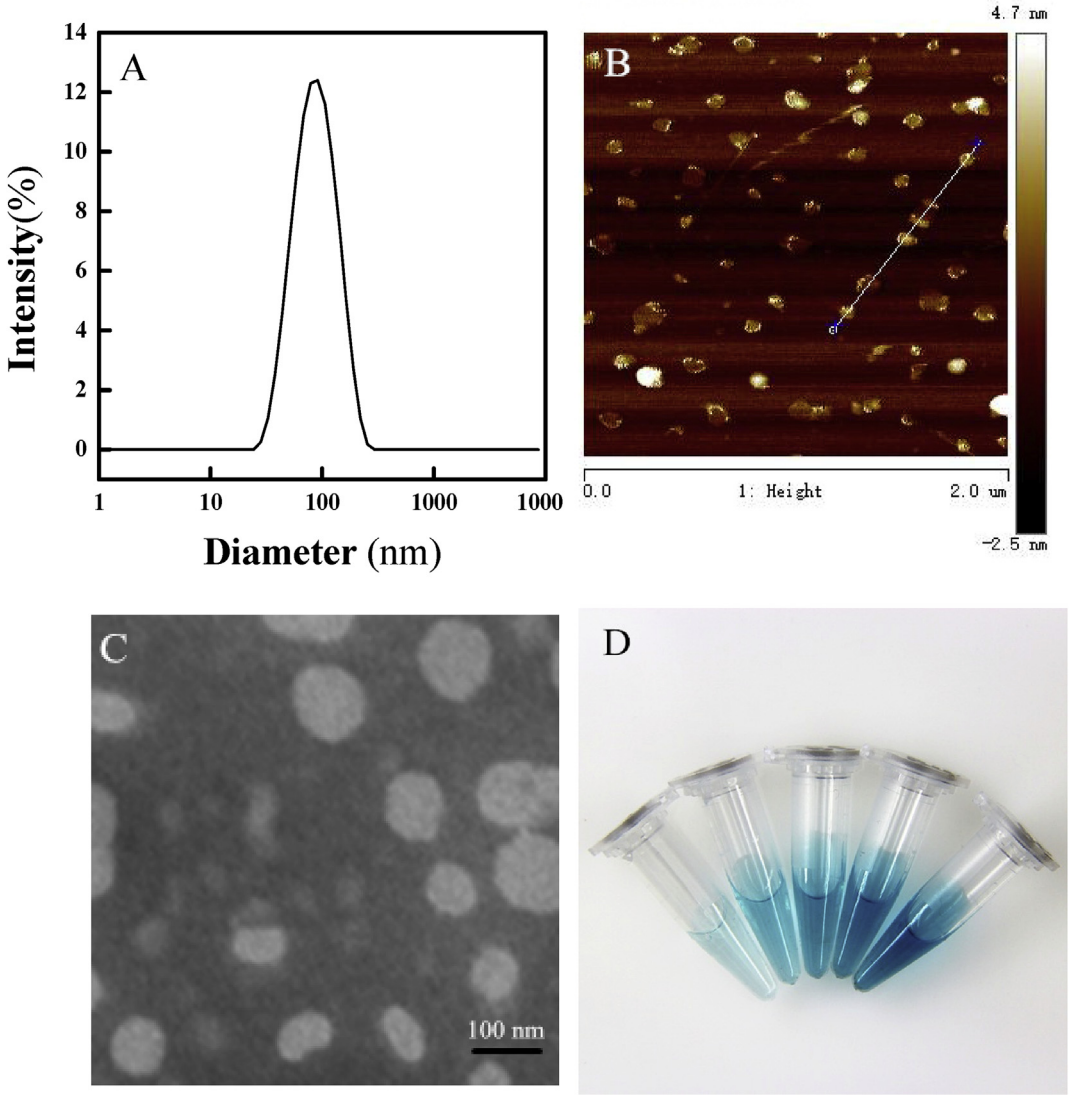

Fig. 1. (A) Diameter distribution of HRP-encapsulated liposome determined by DLS; (B) AFM image of liposomes; (C) TEM image of liposomes; (D) the corresponding photographs.

decreasing the sensitivity of the immunosensor; whereas a high density of cholesterol enables the lysis of liposome, however, it also decreases the sensitivity of the immunosensor. Therefore, it is prerequisite to control the density of cholesterol in liposome by adjusting the mole ratio of cholesterol and PC. The concentration of H5N1 was set at 2 ng/mL, and the density of cholesterol in liposome was from 30% to 90%. Fig. 2A reveals the absorbance value initially increased as the density of cholesterol in liposomes increased from 30% to 40%, and then declined beyond 40% (red line). And the background signal had a little increase when the density of cholesterol was beyond 40% (black line). Thus, 40% was selected as the optimal density of cholesterol in liposome. The catalytic time for TMB oxidation is another key factor in signal generation. As shown in Fig. 2B, the absorbance value increased with the increment of the catalytic time and reached a plateau at 30 min. Therefore, 30 min was selected for further investigation.

3.4. Quantitative analysis of H5N1

Under the optimum conditions, different concentrations of H5N1 were added to evaluate the performance of the proposed immunosensor. Fig. 3A depicts the obtained UV–vis spectra, where the absorption peak at 652 nm increased with the increase of H5N1 concentration in the range from 0.1 to 4 ng/mL. The detection limit was calculated to be 0.04 ng/mL (S/N = 3). There was a good relationship between the enhanced absorbance value and the concentration of H5N1 (Fig. 3B). The inset figure in Fig. 3B also shows that the color became more blue with the enhanced H5N1 concentration, and the color at 0.1 ng/mL was observed by naked eyes easily. To further assess the performance of the proposed biosensor, the result was compared with that from the conventional ELISA. As shown in Fig. 3C, almost no color change appeared in the conventional ELISA when the concentration of the target was lower than 4.0 ng/mL, which is difficult to distinguish via naked eyes. The reason lies in that the immunocomplex of this approach combines more enzymes than the conventional ELISA, resulting in the signal amplification. Thus, the proposed method is more sensitive than the conventional ELISA.

Three parallel experiments were performed at concentration of 2 ng/mL, and the relative standard deviation (RSD) was calculated to be 6.9% (n = 9). This result indicates the proposed immunosensor has good repeatability. The prepared HRP-encapsulated liposomes were stored in 4 °C for four weeks and then used for studies. Furthermore, as shown in Fig. 3D, a slight decline of absorbance

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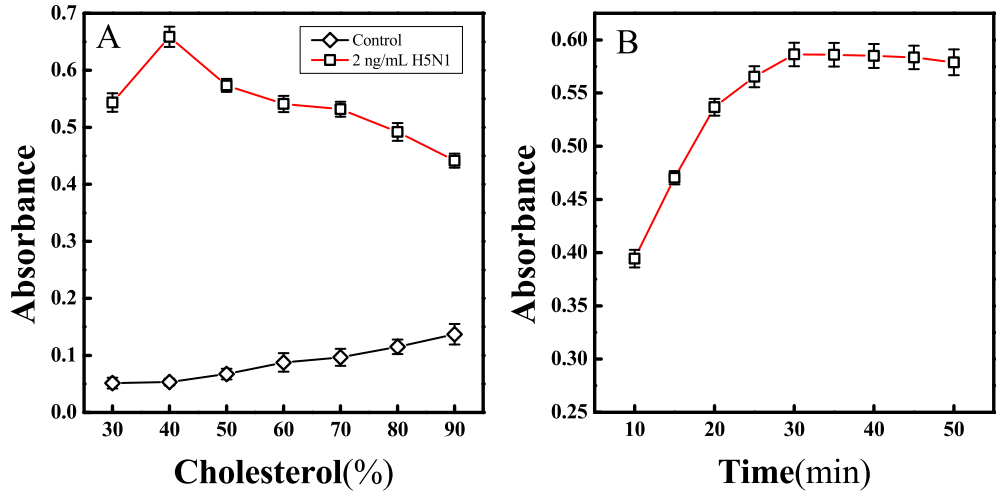

Fig. 2. Optimization of experimental conditions: (A) the effect of the density of the cholesterol on the absorbance of the system and background signal; (B) the effect of enzymatic reaction time on the absorbance of the system. The concentration of H5N1 was 2.0 ng/mL (n = 5). Error bars on graphs were used to indicate the uncertainty of the proposed approach and calculated by standard deviation.

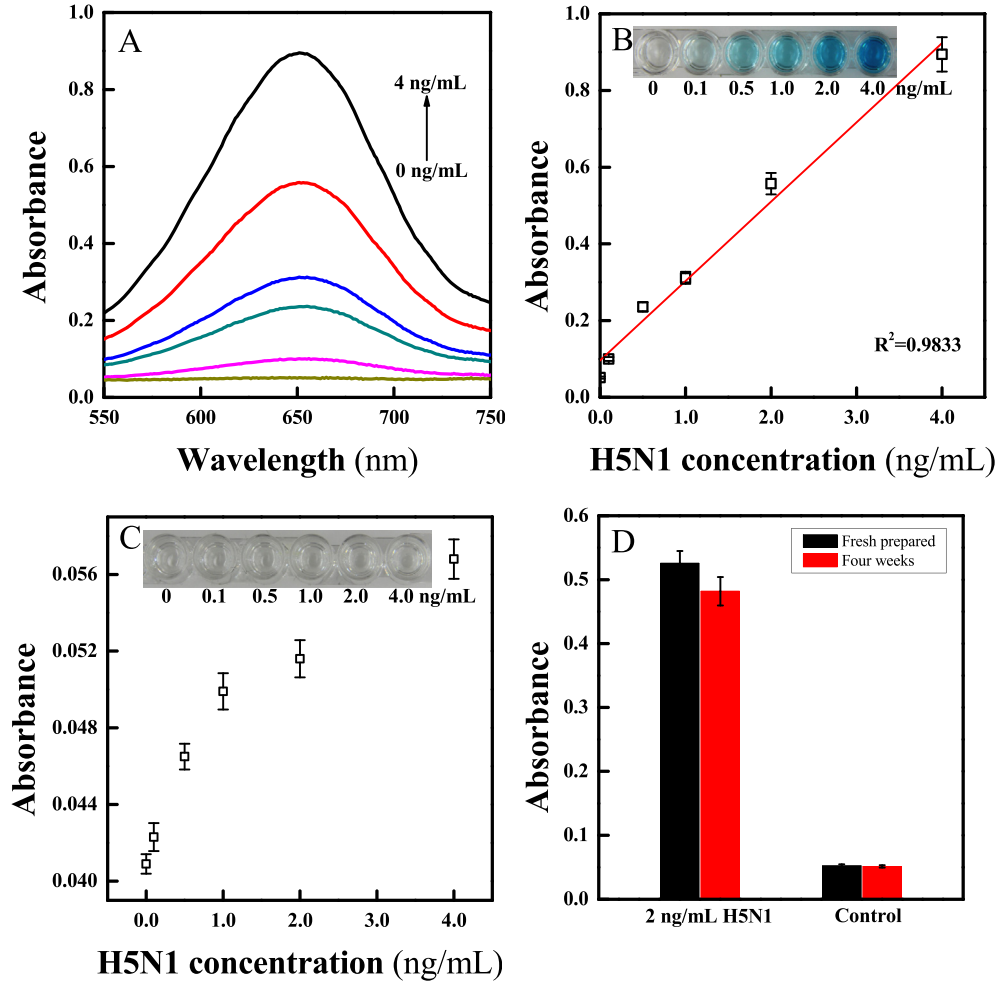

Fig. 3. (A) UV–vis spectra of the system in the presence of different concentrations of H5N1; (B) the relationship between the absorbance intensity and the H5N1 concentration (the inset figure is the corresponding photographs). The concentrations of H5N1 were 0, 0.1, 0.5, 1.0, 2.0, and 4.0 ng/mL. The linear equation was $A = 0.207C + 0.096$ ($R^2 = 0.9833$), where A, C, and R^2 are the absorbance value, concentration of H5N1, and correlation coefficient, respectively. (C) The performance of H5N1 immunoassay based on the conventional ELISA (the inset figure is the corresponding photographs). (D) The compared stability of liposomes.

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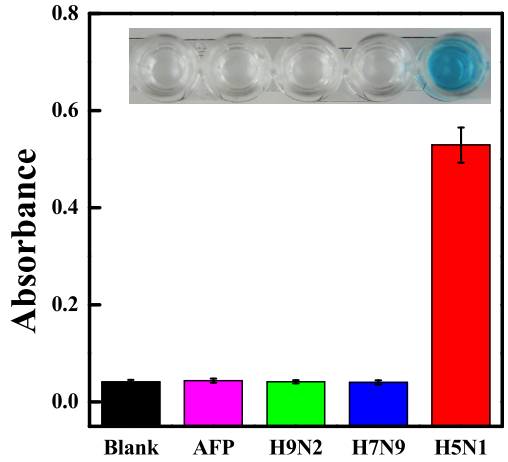

Fig. 4. Specificity of the proposed immunosensor (the inset figure is the corresponding photographs, n = 5).

on HRP-encapsulated liposome for the detection of H5N1 with high selectivity and sensitivity. The proposed immunosensor exhibits high performance and possesses some advantages such as simple, low-cost, and unnecessary for using any sophisticated instruments. Such immunosensor has been successfully demonstrated its practical application for the detection of H5N1 in human serum. By taking advantage of the proposed immunosensor, a potential application may appear in clinical medicine, food safety analysis, and environmental monitoring.

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Table 1
Detection of H5N1 in 20% human serum samples using the proposed biosensor (n = 3).

Sample number	Added (ng/mL)	Naked eyes observation	Total found (ng/mL)	Recovery (%)	RSD (%)
1	0.0		0.02		4.1
2	1.0		1.05	105.0	5.3
3	1.5		1.54	102.7	3.9
4	3.5		3.47	99.1	5.2

intensity was observed for the HRP-encapsulated liposomes stored for four weeks, indicating that the prepared HRP-encapsulated liposomes have good stability, which is the same as the previous study [3].

3.5. Selectivity of the proposed immunosensor and sample analysis

To verify the selectivity of the proposed sensor, several substances were chosen as interferents, including AFP, H9N2, and H7N9. The concentrations of interferents and H5N1 were 10 and 2 ng/mL, respectively. As shown in Fig. 4, the signals upon the addition of the interferent were nearly the same as that of the blank and only H5N1 could induce an apparent signal enhancement along with an obvious color variation due to specific antigen-antibody interaction. These results clearly demonstrate that the proposed method has good selectivity.

The proposed sensor was eventually applied to detect H5N1 in the human serum. Different H5N1 standard solutions were added to the serum samples to test the reliability of the proposed sensor. As shown in Table 1, if no H5N1 was added to the sample, no color change was observed; the color of the solution became more blue with the enhanced H5N1 concentration added. The standard addition recovery rates were in the range of 99.1%–105.0% and the RSD was in the range of 3.9%–5.3%. These results confirm the reliable of the proposed biosensor.

4. Conclusion

In summary, we have developed a novel immunosensorbased

References

[1] R. Genc, D. Murphy, A. Fragoso, M. Ortiz, C.K. O'Sullivan, Signal-enhancing thermosensitive liposomes for highly sensitive immunosensor development, Anal. Chem. 83 (2011) 563.
[2] K. Koide, M. Karel, Encapsulation and stimulated release of enzymes using lecithin vesicles, Int. J. Food Sci. Technol. 22 (1987) 707.
[3] F. Zhou, B. Li, Exonuclease III-assisted target recycling amplification coupled with liposome-assisted amplification: one-step and dual-amplification strategy for highly sensitive fluorescence detection of DNA, Anal. Chem. 87 (2015) 7156.
[4] J.A. Ho, S.C. Zeng, W.H. Tseng, Y.J. Lin, C.H. Chen, Liposome-based immunostrip for the rapid detection of Salmonella, Anal. Bioanal. Chem. 391 (2008) 479.
[5] C. Caviglia, K. Zór, L. Montini, V. Tilli, S. Canepa, F. Melander, H.B. Muhammad, M. Carminati, G. Ferrari, R. Raiteri, A. Heiskanen, T.L. Andresen, J. Emnéus, Anal. Chem. Vol. 4 (2015) 2204. American Chemical Society.
[6] L.J. Ou, S.J. Liu, X. Chu, G.L. Shen, R.Q. Yu, DNA encapsulating liposome based rolling circle amplification immunoassay as a versatile platform for ultrasensitive detection of protein, Anal. Chem. 81 (2009) 9664.
[7] B. Lin, D. Liu, J. Yan, Z. Qiao, Y. Zhong, J. Yan, Z. Zhu, T. Ji, C.J. Yang, Enzyme-encapsulated liposome-linked immunosorbent assay enabling sensitive personal glucose meter readout for portable detection of disease biomarkers, ACS Appl. Mater Interfaces 8 (2016) 6890.
[8] J.T. Mason, L. Xu, Z.M. Sheng, T.J. O'Leary, A liposome-PCR assay for the ultrasensitive detection of biological toxins, Nat. Biotechnol. 24 (2006) 555.
[9] L. Mao, R. Yuan, Y.Q. Chai, Y. Zhuo, Y. Xiang, Signal-enhancer molecules encapsulated liposome as a valuable sensing and amplification platform combining the aptasensor for ultrasensitive ECL immunoassay, Biosens. Bioelectron. 26 (2011) 4204.
[10] R. de la Rica, M.M. Stevens, Plasmonic ELISA for the ultrasensitive detection of disease biomarkers with the naked eye, Nat. Nanotechnol. 7 (2012) 821.
[11] Y. Zheng, H. Chen, X.P. Liu, J.H. Jiang, Y. Luo, G.L. Shen, R.Q. Yu, An ultrasensitive chemiluminescence immunosensor for PSA based on the enzyme encapsulated liposome, Talanta 77 (2008) 809.
[12] M. F, J. E, M. Pons, Liposomes obtained by the ethanol injection method, Int. J. Pharm. 95 (1993) 51.
[13] S. Batzri, E.D. Korn, Single bilayer liposomes prepared without sonication,

Please cite this article in press as: C. Lin, et al., Highly sensitive colorimetric immunosensor for influenza virus H5N1 based on enzyme-encapsulated liposome, Analytica Chimica Acta (2017), http://dx.doi.org/10.1016/j.aca.2017.01.031

Biochim. Biophys. Acta 298 (1973) 1015.
[14] A.B.T. Ghisaidoobe, W.S. Yun, S.J. Chung, Development of stable non-ionic lipid nanoparticles, J. Nanosci. Nanotechnol. 16 (2016) 11873.
[15] J.Y. Wong, T.L. Kuhl, J.N. Israelachvili, N. Mullah, S. Zalipsky, Direct measurement of a tethered ligand-receptor interaction potential, Science 275 (1997) 820.
[16] R. Chattaraj, P. Mohan, C.M. Livingston, J.D. Besmer, K. Kumar, A.P. Goodwin, Mutually-reactive, fluorogenic hydrocyanine/quinone reporter pairs for in-solution biosensing via nanodroplet association, ACS Appl. Mater Interfaces 8 (2016) 802.
[17] M.F. Arcan Güven, B. Hauer, U. Schwaneberg, Molecular understanding of sterically controlled compound release through an engineered channel protein (FhuA), J. Nanobiotechnol. 8 (2010).
[18] P.C. Weber, D.H. Ohlendorf, J.J. Wendoloski, F.R. Salemme, Structural origins of high-affinity biotin binding to streptavidin, Science 243 (1989) 85.
[19] D.X. Zhang, T. Yasuda, S. Okada, A carboxyfluorescein-enveloping liposome as a physicochemical damage model of the biomembrane for the study of lipid peroxidation, J. Clin. Biochem. Nutr. 14 (1993) 83.
[20] M.P. Bui, S. Ahmed, A. Abbas, Single-digit pathogen and attomolar detection with the naked eye using liposome-amplified plasmonic immunoassay, Nano Lett. 15 (2015) 6239.
[21] S. Shrivastava, A. Chattopadhyay, Influence of cholesterol and ergosterol on membrane dynamics using different fluorescent reporter probes, Biochem. Biophys. Res. Commun. 356 (2007) 705.
[22] S. Shrivastava, Y.D. Paila, A. Dutta, A. Chattopadhyay, Differential effects of cholesterol and its immediate biosynthetic precursors on membrane organization, Biochemistry 47 (2008) 5668.
[23] T. Róg, M. Pasenkiewicz-Gierula, I. Vattulainen, M. Karttunen, Ordering effects of cholesterol and its analogues, Biochim. Biophys. Acta (BBA) - Biomembr. 1788 (2009) 97.
[24] F. de Meyer, B. Smit, Effect of cholesterol on the structure of a phospholipid bilayer, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 3654.

Please cite this article in press as: C. Lin, et al., Highly sensitive colorimetric immunosensor for influenza virus H5N1 based on enzyme-encapsulated liposome, Analytica Chimica Acta (2017), http://dx.doi.org/10.1016/j.aca.2017.01.031