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ORIGINAL PAPER

Determination of salbutamol using R-phycoerythrin immobilized on eggshell membrane surface as a fluorescence probe

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Abstract A fluorescence sensor was fabricated using R-phycoerythrin (R-PE) immobilized on eggshell membrane as the fluorescence probe, and salbutamol was determined based on the decrease in fluorescence intensity of R-phycoerythrin. The scanning electron and fluorescence micrographs showed the microstructure of the eggshell membrane and indicated that the R-PE was successfully immobilized on the eggshell membrane surface. The effects of some experimental parameters on the response of the biosensor were investigated in detail. The fluorescence sensor has a linear response to salbutamol concentrations ranging from 5.00 to 100 ng mL^{-1}. The detection limit for the salbutamol is 3.50 ng mL^{-1} (S/N=3). The reproducibility of fabricating the biosensors using six different membranes was good with a relative standard deviation (RSD) of 3.28%. The fluorescence sensor showed extremely good stability with a shelf life of at least 50 days and reversible response to salbutamol. Some common potential interferents showed little effect on the response of the salbutamol fluorescence sensor. The proposed method was successfully applied to the determination of the salbutamol in urine samples.

Keywords R-phycoerythrin · Eggshell membrane · Salbutamol · Fluorescence quenching

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Introduction

Phycobiliproteins found in cyanobacteria and red algae are unique photosynthetic pigment–protein complexes, which play a crucial role in efficiently harvesting sunlight that chlorophylls poorly absorb and then transferring this energy via a series of energy transfer mechanisms to chlorophyll in the membranes of these organisms [1–12]. Based on their absorption spectra, phycobiliproteins can be divided into three main categories: phycoerythrin (PE), phycocyanin (PC), and allophyxoxyanin (APC). Phycobiliproteins possess several unique characteristics that make them attractive for use as fluorescence labels in the analysis of biomolecules and cells. Some of the desirable properties of phycobiliproteins include high absorbance coefficients over a wide region of the visible spectra due to their multiple bilin chromophores; high fluorescence quantum yields that are remarkably constant over a broad pH range; large Stokes shift that minimizes interferences from Rayleigh-scattering, Raman-scattering, and other fluorescing species; and high solubility in aqueous solutions. In this paper, R-phycoerythrin (R-PE), which is a 240-kDa fluorescent protein, was used as fluorescent probe. R-PE comprises three polypeptide subunits forming a $(\alpha\beta)_6\gamma$ aggregate, and these subunits contain phycobilin chromophores based on a linear tetrapyrrole skeleton. There are two kinds of chromophores in R-PE molecule: phycoerythrobilin (PEB) and phycourobilin (PUB) (Fig. 1a), which connect with the cysteine residues in apoprotein by a thioether bond [5, 13–16]. R-PE has strong absorption bands that start at 440 nm and strong emission bands that begin at 550 nm and extend well into the red region of the visible spectrum where there is minimal interference from biological molecules. R-PE is very stable

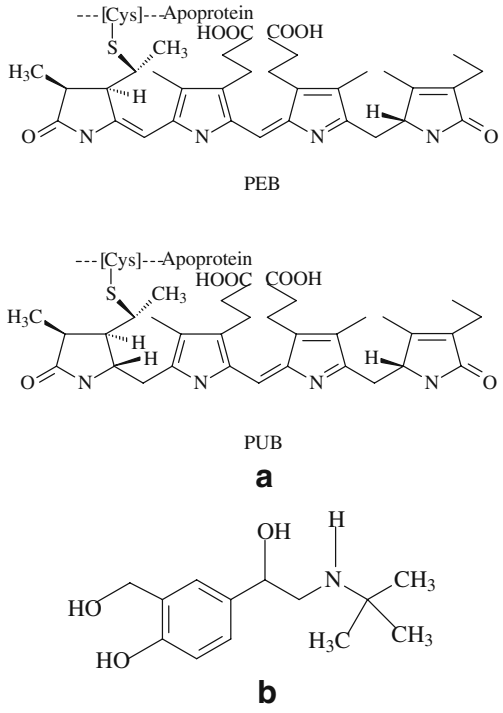

Fig. 1 Structures of **a** two chromophores of R-PE: PEB and PUB; **b** SAL

not only in solution but also in the solid phase and thus can be stored for long periods on the shelf [2, 3, 5, 11].

Sensors are devices that provide information about the concentrations and chemical states of the species present in a sample [17]. Recently, research into the development of chemosensors and biosensors has been very fast growing. They represent an active, exciting, and innovative research area in analytical chemistry with more than 1,000 papers being published annually [18]. Optical sensors for a wide variety of chemical species have drawn considerable attention in the past few decades because they possess certain advantages over conventional electrochemical sensors or devices. The promising features of optical sensors are often related to simple sensor design and ease of operation, free from electric and magnetic interference, and suitable for in situ or online remote monitoring. For a reusable biosensor, a protein must be immobilized on the surface of the biosensor. As a result, strategies for protein immobilization are of paramount importance to preserve their biological activity [19]. Over the years, many methods for protein immobilization on solid supports have been attempted, which fall into three categories: physisorption, covalent attachment, and entrapment. Long-term operational and storage stability often hinder the development of sensors. And the retention of these qualities is critical for a reusable sensor. It has been reported that some biomaterials, such as silk [20–22], collagen [23, 24], bamboo inner shell membrane [25], and eggshell membrane [26–30], were employed as platforms for the immobilization of proteins and the life-times of the immobilized proteins could be prolonged. Eggshell membrane, of which the thickness is about 65–96 μm [31], is a light pink double-layered membrane inside the eggshell.

Eggshell membrane is mainly composed of biological molecules and highly cross-linked protein fibers, and possesses excellent gas and water permeability [32]. The special performance of eggshell membrane is due to its special composition and structure. Eggshell membrane is semipermeable, allowing small molecules to selectively permeate through, but not larger ones such as glucose [33]. Eggshell membrane is a nontoxic and natural product that it is facile to obtain and inexpensive. The amino groups of the eggshell membrane make it is easy to combine with many biomolecules, such as enzymes, proteins, and antibodies. Therefore, eggshell membrane can be used as an ideal bioplatform for protein immobilization, which is helpful in maintaining the protein activity. In previous works, we studied the application of eggshell membrane as an immobilization platform in sandwich immunoassays [34]. In this study, we explore the possibility of fabricating a stable fluorescence sensor based on R-PE immobilized on eggshell membrane. The strong fluorescence of R-PE based on the sensor is quenched considerably in the presence of salbutamol.

Salbutamol (SAL, see Fig. 1b) is one of the β_2-agonists used in human and veterinary medicine for the treatment of pulmonary disorders. It is also extensively misused in farm animals, where high doses give rise to a preferential muscle to fat ratio, resulting in financial gain for the farmer. This abundant misuse raised serious concerns about a toxicological risk for the consumer [35, 36], leading the USA, the European Union, and most other countries to forbid the use of some or all of these substances in animals raised for human consumption [37, 38]. Therefore, a rapid, simple, convenient, and effective method to monitor therapeutic use as well as to control the illegal use of salbutamol is essential. The determination of salbutamol can be done by many analytical procedures such as spectrophotometry, immunoassay, capillary electrophoresis, gas chromatography, high-performance liquid chromatography (HPLC), and mass spectrometry [39–49]. Among these methods, HPLC is the most commonly used method for the quantification of salbutamol. However, these methods usually require time-consuming pretreatment of the sample prior to the chromatographic separation. There is still demand for an inexpensive, quick, and sensitive method for the determination of salbutamol.

The interactions between R-PE and some drugs, including barbituric acid, phenacetin, and salbutamol, were investigated and it was found that salbutamol can significantly quench the fluorescence of R-PE. When SAL was introduced

into the R-PE solution, the absorption intensity of R-PE decreased, but the position of absorption peak was not changed. It is supposed that the hydrogen bonds among chromophores and those between chromophore and apoprotein are partly destroyed in the presence of the hydroxy group of SAL, the microenvironment of the R-PE chromophore is altered, and the conformation of R-PE is not changed. Therefore, the absorption ability of R-PE decreases, which results in the decrease of fluorescence quantum yield.

In this work we report the development of a salbutamol fluorescence sensor that shows promising features: low cost, simple design, ease of operation, and multiple regenerations. R-PE was initially covalently immobilized on a fresh eggshell membrane using glutaraldehyde as cross-linking regent. It is observed that the fluorescence of R-PE is quenched in the presence of salbutamol. The proposed method has been successfully applied to the analysis of urine samples.

Experimental

Apparatus

The absorption spectra were recorded on a Cintra 10w UV–visible spectrometer. Fluorescence spectrum measurements were performed on a fluorospectrophotometer (Shimadzu RF-5301PC, Shimadzu, Japan). The fluorescence emission intensity at 572 nm was collected under an excitation wavelength of 495 nm. Fluorescence micrographs were recorded on a fluorescence microscope (Olympus inverted research microscope IX71, Olympus, Japan). Scanning electron micrographs were obtained on a scanning electron microscope (JEOL JSM-6700F, JEOL, Japan). The pH value was measured with a Delta 320 pH meter. Unless otherwise stated, all fluorescence measurements were performed at room temperature and atmospheric pressure. The eggshell membrane with immobilized R-PE was placed on the surface of a quartz plate, subsequently laid on black cardboard, and then fixed firmly on the solid sample holder for fluorescence measurements at certain reaction times. The diagram of the experimental arrangement is shown in Fig. 2.

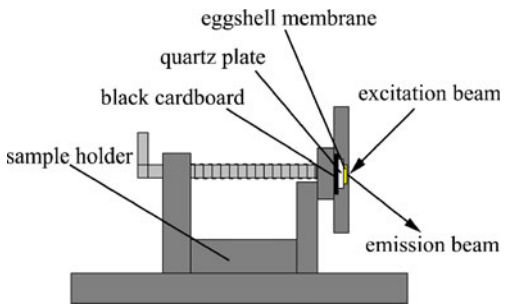

Fig. 2 Experimental setup

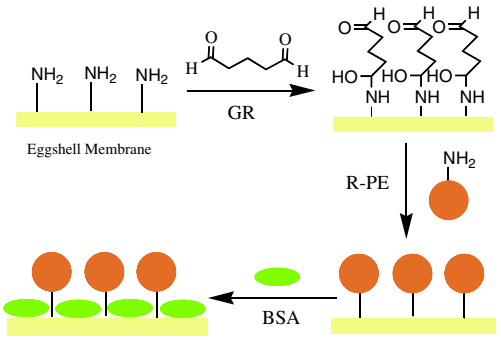

Fig. 3 Scheme of immobilization of R-PE on the eggshell membrane

Chemicals and materials

R-phycoerythrin (R-PE) was acquired from H&R Bioscience CO., Ltd. Salbutamol (SAL) was acquired from Sigma-

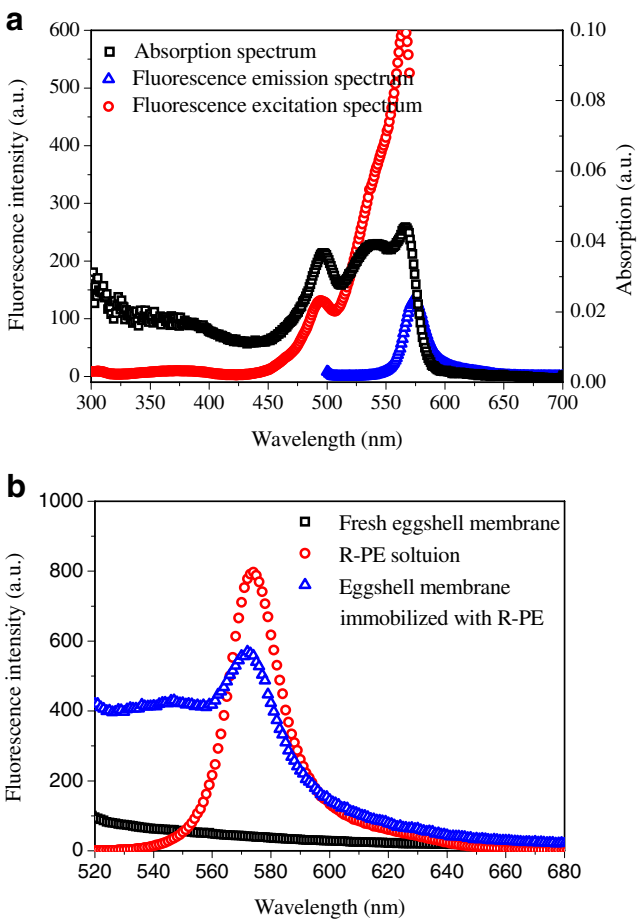

Fig. 4 **a** Absorption and fluorescence spectrum of R-PE solution. **b** Fluorescence spectra of R-PE solution, fresh eggshell membrane, and eggshell membrane with immobilized R-PE

Aldrich. Glutaraldehyde solution (GA, 25%, w/w) in water was obtained from Beijing Chemical Reagent Corporation. Ethanolamine (EA) was purchased from Tianjin Guangfu Fine Chemical Industry Institution. Bovine serum albumin (BSA) was purchased from Beijing DingGuo Biotechnology Co., Ltd. All the other chemicals were of analytical reagent grade. A 10 mmol L^{-1} phosphate buffer solution (PBS) was used for the preparation of solutions. All water used was deionized water. Chicken eggs were purchased from local markets.

Immobilization of R-PE on eggshell membrane

Eggs were dipped in glacial acetic acid (17.4 mol L^{-1}) at 4 °C for 6 h to obtain whole eggshell membranes. The obtained eggshell membranes were cut into four equal parts and cleaned with a large amount of deionized water after removing the albumen and yolk. Further cleaning steps of the eggshell membrane were necessary to completely remove the albumen from the eggshell membrane [50]. The cleaned eggshell membrane was finally stored in PBS at 4 °C until further use.

R-PE was purified by centrifugation, dialysis desalting, and then dissolved in PBS prior to use. The fresh eggshell membrane (10×20 mm) was placed on a clean small watch glass. The R-PE solution was dropped onto the surface of the eggshell membrane at 4 °C for adsorption. After approximately 20 min, 10 μL of GA was added as a cross-linking agent and spread evenly on the membrane surface with a small glass rod. The immobilized membrane was kept for 2 h at 4 °C, then rinsed with deionized water and immersed in PBS three times alternately to remove the excess protein and cross-linking reagent. A 10-μL aliquot of BSA, which was used as a blocking reagent, was added onto the membrane which was then incubated at 37 °C. Finally, the membrane with immobilized R-PE was rinsed with deionized water again, and stored in PBS at 4 °C until further use. The experimental procedure for R-PE immobilization on the eggshell membrane is summarized in Fig. 3.

Analysis of samples

The urine samples were centrifuged at 15,000 rpm for 10 min at 4 °C; the supernatants were obtained and stored at 4 °C until being analyzed. The immobilized eggshell membrane was positioned on the surface of the quartz plate, and kept in a steady position by the solid sample holder. The SAL standard solutions or urine samples (10 μL) were injected onto the membrane. It is observed that the fluorescence of R-PE is quenched in the presence of SAL.

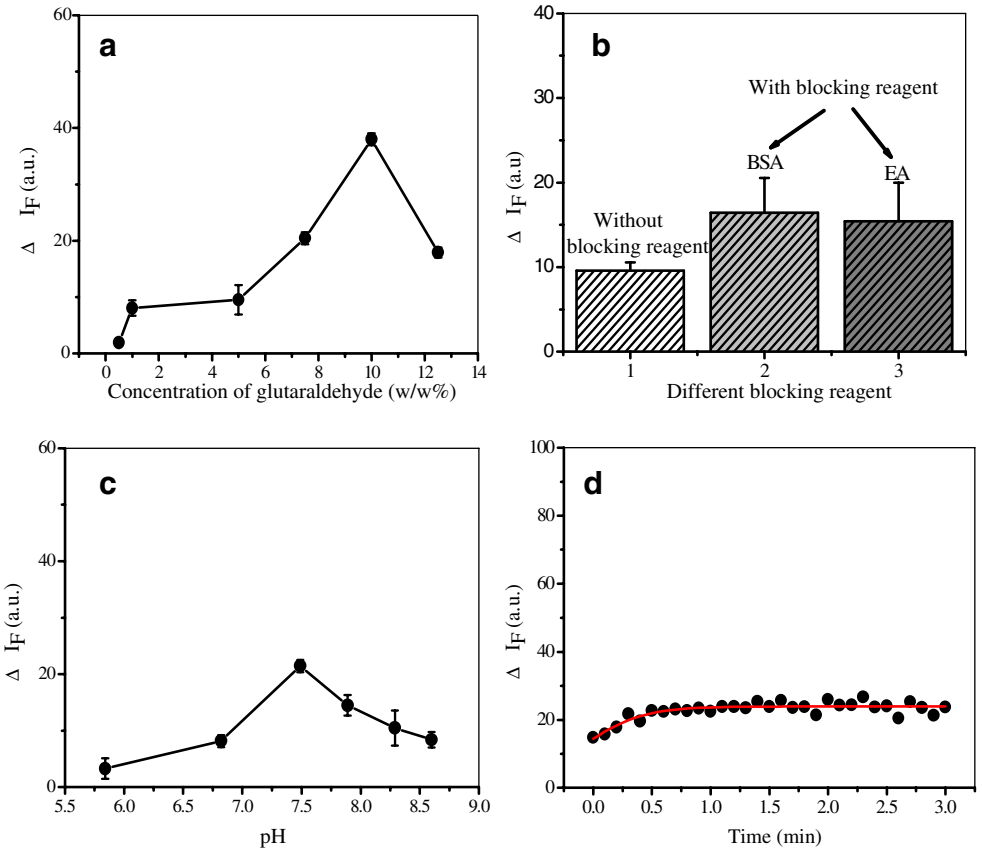

Fig. 5 Effect of different experimental conditions. **a** Effect of the GA concentration. Experimental conditions: R-PE, 100 μg mL^{-1}; SAL, 100 ng mL^{-1}, pH=7.5. **b** Effect of different blocking reagents. Experimental conditions: R-PE, 100 μg mL^{-1}; GA, 10% (w/w); SAL, 100 ng mL^{-1}, pH=7.5; concentration of blocking reagents: BSA, 2.5 mg mL^{-1}; EA, 1 mol L^{-1}. **c** Effect on pH value of SAL. Experimental conditions: R-PE, 100 μg mL^{-1}; GA, 5% (w/w); SAL, 100 ng mL^{-1}. **d** Effect of reaction time. Experimental conditions: R-PE, 100 μg mL^{-1}; GA, 5% (w/w); SAL, 100 ng mL^{-1}, pH=7.5. The *error bars* denote the standard deviation of the values obtained with the three same assays

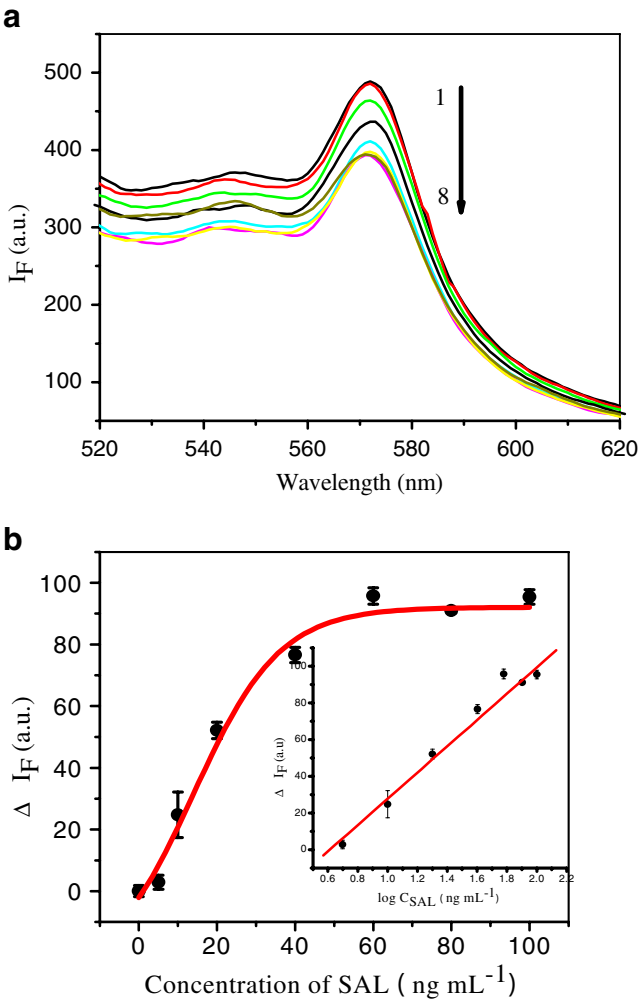

Fig. 6 **a** Fluorescence spectrum of the SAL sensor: R-PE, 80 μg mL^{-1}; GA, 10% (w/w); BSA, 2.5 mg mL^{-1}; concentration of SAL: (*1*) 0, (*2*) 5, (*3*) 10, (*4*) 20, (*5*) 40, (*6*) 60, (*7*) 80, (*8*) 100 ng mL^{-1}. **b** Calibration curve for determining SAL; the *inset* displays the linear calibration curve. The *error bars* denote the standard deviation of the values obtained with the three same assays

Results and discussion

Absorption and fluorescence spectrum of the sensor

The absorption and fluorescence spectra of R-PE solution are shown in Fig. 4a. The excitation and emission maxima are at 495 and 572 nm, respectively. The fluorescence spectra of R-PE solution, fresh eggshell membrane, and eggshell membrane with immobilized R-PE are displayed in Fig. 4b. The fluorescence intensity of fresh eggshell membrane is very low under the same conditions. When R-PE was immobilized on eggshell membrane, the fluorescence spectrum was observed with emission peak at 572 nm, which was essentially consistent with that of R-PE. It is also proved that the immobilization of R-PE onto eggshell membrane was successful. When SAL was added onto the membrane with immobilized R-PE, the fluorescence intensity of the membrane was decreased. Therefore, the SAL could be determined as a quencher. The decrease of fluorescence intensity of R-PE (ΔI_F) was measured at 572 nm and represented as

$$\Delta I_F = I_{F0} - I_F$$

where I_{F0} is the fluorescence intensity of the R-PE immobilized on eggshell membrane in the absence of SAL, and I_F is the fluorescence intensity in the presence of SAL.

FM and SEM images of the eggshell membrane

Scanning electron microscopy (SEM) and fluorescence microscopy (FM) were used to examine the microstructure of fresh eggshell membrane and eggshell membrane with immobilized R-PE, respectively. The membranes were placed on a clean glass slide, and examined with the fluorescence microscope under ×400 magnification. The fluorescence micrographs of eggshell membranes are shown in Fig. S1 in the "Electronic supplementary material", which provide different visualizations of eggshell membrane. Experimental results demonstrated that a little green fluorescence was observed on fresh eggshell membrane when the excitation wavelength is 490 nm (Fig. S1a, "Electronic supplementary material"). However, it is seen from Fig. S1b ("Electronic supplementary material") that a red fluorescence was markedly observed on eggshell

Table 1 Effect of foreign substances

Foreign substances	Concentration of foreign substances (μgmL^{-1})	Relative error (%)
K^+, Cl^-	78	−1.81
Na^+, Cl^-	69	0.21
Cu^{2+}, Cl^-	63	3.54
Ca^{2+}, Cl^-	40	−0.72
Fe^{2+}, Cl^-	56	−0.28
Zn^{2+}, Cl^-	33	3.54
Mn^{2+}, SO_4^{2-}	55	−0.32
Mg^{2+}, SO_4^{2-}	24	0.64
Al^{3+}, SO_4^{2-}	54	−4.24
Cr^{3+}, NO_3^-	52	1.71
Glucose	50	1.86
Urea	50	0.27
DL-β-Phenylalanine	25	−2.75
L-Tryptophan	50	1.85
L-Aspartic acid	50	−1.21
L-Cysteine	50	2.07
L-Tyrosine	50	3.33

The concentration of SAL is 100 ng mL^{-1}

membrane with immobilized R-PE, which is also consistent with the characteristic spectrum of R-PE.

The surface morphologies of eggshell membrane with and without the immobilized R-PE were investigated by using SEM to visualize the R-PE immobilized onto the surface of eggshell membrane. The membranes were placed on a clean silicon wafer, coated with a thin layer of gold by using a spray gun, and then observed on the scanning electron microscope. Figure S2 ("Electronic supplementary material") shows the SEM images of fresh eggshell membrane, eggshell membrane just treated with GA, and eggshell membrane with immobilized R-PE. As mentioned above, the fresh eggshell membrane is mainly composed of highly cross-linked protein fibers and cavities. Because the SEM images cannot visualize the individual size of R-PE, the immobilized R-PE can only be observed as some condensed spots or clusters on the fiber. All of these experimental results clearly show that R-PE was successfully immobilized on the eggshell membrane.

Optimization of experimental conditions

Effect of cross-linker reagents

Since the immobilization of R-PE strongly depends on the amounts of cross-linker, the eggshell membranes with immobilized R-PE were prepared by using GA as the cross-linker, and the effect of GA concentration was studied. It is seen from Fig. 5a that when the concentration of GA is lower than 10% (w/w), the amount of the cross-linker is not enough to bind all R-PE molecules onto the membrane; however, when the concentration of GA is higher than 10% (w/w), the cross-linker may be in excess and can induce the R-PE to denaturalize. As a result, 10 μL of solution containing 10% (w/w) GA was chosen for the R-PE immobilization on membrane.

Effect of blocking reagents

The effect of blocking reagent was investigated (see Fig. 5b). The results show that sensor response obtained with BSA and EA is higher than that without blocking reagent. Both EA and BSA can block the excess binding sites. However, considering the effect of pH on fluorescence quenching, the optimum blocking reagent was BSA.

Effect of pH value

It is well known that the analytical performance of fluorescence quenching is highly sensitive to the variation of pH. The effects on pH value of sample solution were investigated. The decrease of fluorescence intensity (ΔI_F) was measured in the presence of 100 ng mL^{-1} SAL at various pH (5.8–8.6) phosphate buffer solutions (10 mmol L^{-1}). The results in Fig. 5c show that the ΔI_F value increases when the pH increases from 5.8 to 7.5, and then decreases when the pH values are higher than 7.5. The optimum pH of sample solution chosen was 7.5.

Table 2 Recovery of SAL from urine samples

Sample number	Added (ngmL^{-1})	Found (ngmL^{-1})	Recovery (%)
1	10	10.18±1.31	101.8
2	15	13.68±0.64	91.22
3	20	16.91±1.12	84.56
4	25	24.40±0.20	97.60
5	30	29.53±1.99	98.44

Effect of reaction time

The effect of reaction time of fluorescence quenching was studied. As depicted in Fig. 5d, the ΔI_F value increases in the first 30 s; however, the response of SAL sensor was more or less constant after 30 s. The result shows that the fluorescence quenching of the SAL–R-PE–membrane system remained stable after 30 s. Therefore, the optimum testing time of fluorescence quenching was determined to be 30 s.

Linearity, sensitivity, and precision

Typical fluorescence quenching spectra of the SAL sensor, obtained under the optimal conditions described above, are displayed in Fig. 6a; and the relationship between ΔI_F and the concentration of SAL is shown in Fig. 6b. There is a good linear relationship between ΔI_F and the concentration of SAL in the range 5.00–100 ng mL^{-1}. With a signal to noise ratio of 3, the detection limit was found to be

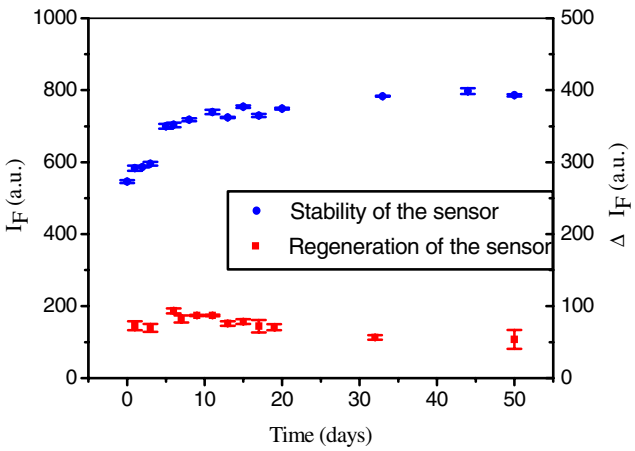

Fig. 7 The stability and regeneration of the SAL fluorescence sensor. Experimental conditions: R-PE, 80 μg mL^{-1}; GA, 10% (w/w); BSA, 2.5 mg mL^{-1}; SAL, 100 ng mL^{-1}, pH=7.5. The *error bars* denote the standard deviation of the values obtained with the three same assays

3.50 ng mL^{-1}. The linear regression equation is $\Delta I_F = -43.819 + 71.562 \log C$, where C is the SAL concentration (ng mL^{-1}), and the correlation coefficient is 0.988. The repeatability of the method was tested by the analysis of a standard SAL solution. The relative standard deviation (n=4) is 3.22% at 100 ng mL^{-1} SAL solution.

Interference study

In order to examine the effect of foreign substances (common ions and amino acids) on the determination of the proposed method, the influence of a number of ions and amino acids was investigated according to the recommended procedure at a concentration of SAL (100 ng mL^{-1}, in PBS). The tolerance limit of an ion was taken as the maximum amount causing an error of ±5%. The results of the interference studies are summarized in Table 1. It can be seen that most of the ions and amino acids examined do not interfere with determination, whereas some can produce slight interference on the determination. Because most biomolecules do not quench the fluorescence of R-PE [2–5, 11], and common components in human urine hardly influenced the determination (Table 1), it is considered that the proposed method exhibits a relative selectivity.

Analysis of samples

The SAL fluorescence sensor was applied to the determination of SAL in urine samples obtained from a healthy adult volunteer. The urine samples were centrifuged at 15,000 rpm for 10 min at 4 °C; the supernatants were obtained and diluted 10 times with PBS to eliminate the matrix interference. Because the concentration of SAL in the urine from the healthy human is below the detection limit of the proposed method, five parallel urine samples were analyzed, and the results indicated that the SAL in urine was not detectable. Recovery of SAL was investigated by adding known amounts of SAL standard into the urine samples. The spiked samples were analyzed by the proposed method and the results are listed in Table 2. As shown, the proposed method can be applied for the determination of SAL in urine. In addition, a comparison of the proposed method and the enzyme immunoassay (EIA) using the Ridascreen beta-agonist test kit was also performed. The SAL in the urine from the healthy human was not detectable when the EIA was applied. The urine sample spiked with 30 ng mL^{-1} SAL was analyzed by EIA, and the obtained concentration of SAL was 37.92 ng mL^{-1} (SD=6.15, n=4).

Analytical performance of the SAL sensor

In this study, the SAL sensor response was completely reversible after a running PBS buffer had flowed through for 1 min. Figure 7 depicts the repeatability and reversibility of this SAL sensor when it was used to determine 100 ng mL^{-1} SAL for many times. The results demonstrated that the sensor had a highly reproducibility and reversible response to SAL. In addition, it was found that the membrane sensor could be stored in PBS at 4 °C without any remarkable change in its fluorescent intensity for at least 50 days, which implies that the R-PE immobilized on eggshell membrane is quite stable. Thus, the membrane sensor was immersed in PBS when not in use. The reproducibility of the fabrication of the R-PE immobilized eggshell membrane was also investigated by preparing six membranes under the same conditions. The fluorescence intensity of these six membranes was determined and the relative standard deviation was 3.28%, which indicated that the fabrication process for the fluorescence sensor was reproducible.

Conclusions

The developed SAL sensor has a very simple principle and it is cost-effective. It has been successfully constructed and applied to the determination of SAL in human urine. The fluorescence micrographs and scanning electron micrographs reveal the microstructure of the eggshell membrane and show that R-PE is successfully immobilized onto the eggshell membrane. The proposed SAL sensor exhibits good stability with a shelf life of at least 50 days. It also displays a highly reproducibility and reversible response to SAL. This work proves that eggshell membrane material is a promising protein immobilization platform for the fabrication of biosensors which have practical applications in various analytical methods, in this case to monitor SAL in blood and meat samples for evaluation of salbutamol misuse in farm animals.

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