Strategy of Dimercaptothiol as Self-assembled Monolayers Enhance the Sensitivity of SPR Immunosensor for Detection of Salbutamol

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The formation of self-assembled monolayers (SAMs) is a normal method for the immobilization of biorecognition elements immobilized on SPR sensors. With this method, mercaptopropionic acid (MPA) with carboxylic and thiol group is the most commonly used. Dimercaptosuccinic acid (DMSA) having two carboxyls and two thiol groups is a classical antidote for heavy metal ions. In this paper, DMSA was first used to form SAMs to connect the antigen on the chip of a surface plasmon resonance (SPR) immunosensor for detection of salbutamol (SAL), and the results were compared with a traditional (MPA)-SPR sensor. Dihydrolipoic acid (DHLA)-SPR showed that the recognition efficiency of antigen and antibody of DMSA-SPR immunosensor was 170.1% at room temperature with the linear range of 5 – 150 ng/mL. The recovery rate of this sensor applied to SAL detection in pork reached 94.9 – 108.0% and the limit of quantification (LOQ) was 5 ng/mL. The results were in good correlation with the analysis results of ultra-high phase liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) analysis. This novel DMSA-SPR immunosensor provides insight into a new idea and method for improving the sensitivity of SPR immunosensors and can be widely used in the detection of other small molecules.

Keywords Surface plasmon resonance, thiol, self-assembled monolayers, salbutamol

(Received January 11, 2021; Accepted February 24, 2021; Advance Publication Released Online by J-STAGE March 5, 2021)

Introduction

As an optical detection tool, a surface plasmon resonance (SPR) biosensor offers real-time and label-free analysis of molecular interaction through the detection of a refractive index change in the metal-solution interface.1-2 To date, the SPR biosensor has been widely applied in the fields of clinical diagnosis, environmental monitoring, and food safety.3-4 However, the SPR biosensor exhibits poor sensitivity to small changes in refractive index, such as binding of small molecules to the metal surface.1 The immobilization of large biomolecules, such as antibodies or antigens, on the chip of an SPR immunosensor is a crucial step for analytical applications because the direct adsorption of proteins on the metal surface can lead to denaturation. Although organic polymers and inorganic carbon materials are commonly used as bonding layers,5-9 thiol molecules are still the most frequently used compounds. The specific surface area of a single gold-alkyl sulfate unit in the thiol monolayer is much smaller than that of protein. The high density of thiol monolayers inevitably leads to excessive immobilization of bioactive molecules on the chip surface. Such excessive immobilization results in steric hindrance in the bioactive molecule layer on the chip surface, hindering the binding of bioactive sites to target molecules, and inhibiting the improvement of detection sensitivity of SPR biosensors.10 Conventionally, a mixed thiol molecule is used to solve the above-mentioned issue of the thiol monolayer.11-15 However, it fails to alter the regular and uniform arrangement of mixed thiol molecule on the chip surface. Therefore, the effect of the immobilization of biologically active molecules on the sensitivity of the immunosensor via thiol molecules with a uniform and suitable density remains largely unexplored.

In the present study, in order to prevent overlapping of the antibody binding sites, DMSA-based SAMs were incorporated to immobilize antigen to create a spacious environment. Three types of thiol with different ratios of carboxyl group to mercapto group, including MPA, DMSA and DHLA, were examined in our current study. The fixed density of thiol on gold sheet was examined by UV-Vis spectrophotometry and contact angle test. The activity of salbutamol hydrochloride-bovine serum albumin conjugate (SAL-BSA) was analyzed by an SPR biosensor, and the morphology of the immobilized enzyme was assessed by atomic force microscopy (AFM) and scanning electron microscopy (SEM). Moreover, we also investigated the sensitivity of the immunosensor chip based on the immobilization of SAL-BSA by DMSA molecule layer. Collectively, the DMSA-based SAMs strategy provided a combined platform consisting of oriented and ordered macromolecules, facilitating its application for a high-sensitivity immunosensor.

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Experimental

Reagents and chemicals

MPA, DMSA and DHLA were obtained from Shanghai Sigma-Aldrich. Aalbutamol, nortepamine, clenbuterol, mapenterol hydrochloride, cimaterol and salbutamol were purchased from BePure. 1-Ethyl-(3-dimethylaminopropyl) carbonyldiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) were supplied by Bioengineering (Shanghai) Co., Ltd. Ethanolamine hydrochloride was purchased from Aladdin Reagent. K₃[Fe(CN)₆]₃, K₅[Fe(CN)₆]·3H₂O, KNO₃, KCl and sodium acetate trihydrate were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Anhydrous acetic acid was provided by Merck, Germany. SAL-BSA and salbutamol monoclonal antibody (SAL-Ab) were supplied by Shenzhen Baoankang Bio Company.

Apparatus

The YC-SPR-A1 biomolecular interaction instrument was equipped with a sensor chip of 18 × 18 mm in size, which was coated with 45-nm nanogold (Beijing ZhonglongYicheng Technology Co., Ltd.). The FE 28 pH meter used was provided by Mettler Toledo, USA.

Immobilization analysis of thiols

The thiol molecule is a type of bifunctional molecule containing thiol and carboxyl groups. By covalent coupling method, the basement gold film and protein can be linked together. It forms Au-S bond with gold through thiol group, generating a monolayer on the gold film surface. Carboxyl group covalently bonds with protein molecule through carbodiimide method, thus fixing the protein on the chip surface. The thiol monolayer can effectively reduce the non-specific adsorption of protein on the gold chip. Therefore, the homogeneity and compactness of the monolayer are very important for the immobilization of proteins. Researchers have reduced the density of carboxyl group in the monolayer by mixing the monolayer to improve the quality of the monolayer. However, the molecular arrangement is disordered and irregular. It is necessary to make the monolayer with not only a reduced density of the carboxyl group in the monolayer, but also with an ordered arrangement.

A piece of bare gold chip was added respectively to 6 mL of 0.5 mM MPA, DMSA and DHLA solutions. After 24 h, the gold chip was removed, and the absorbance of the remaining solution was determined by an ultraviolet (UV)-visible double-beam spectrophotometer within a wavelength range of 275 - 500 nm. The contact angle was determined by droplets of deionized water.

Fabrication of bare chip

The bare gold chips were cleaned in piranha solution (H₂SO₄/35% H₂O₂, 7:3, v/v) at room temperature for 10 min. The piranha-treated bare Au chips were then rinsed with anhydrous ethanol and deionized water, followed by drying process using a stream of nitrogen gas. The cleaned Au chips were immediately immersed in 10 mM ethanol/water (2:1, v/v) solution of MPA, followed by incubation at room temperature overnight.

The modification of Au chips with the other compounds was carried out using a similar method. Upon removal from the ethanol solution, the slides were sequentially rinsed with ethanol, deionized water and ethanol, followed by drying process using a stream of nitrogen gas.

The COOH-terminated gold slides were immersed in a freshly prepared solution of 400 mM EDC and 100 mM NHS in deionized water at room temperature for 30 min. Subsequently, the slides were rinsed with deionized water, dried with nitrogen gas, and immediately used in the next step. SAL-BSA was covalently immobilized by covering the EDC/NHS-treated slides with a small volume (200 μL) of 1 mg/mL SAL-BSA solution at room temperature for 1 h. The SAL-BSA solution was prepared in sodium acetate buffer (10 mM, pH 4.5). Finally, the unreacted active site was blocked with ethanolamine solution (1.0 M, pH 8.5). The enzyme-coated slides were rinsed several times with PBS and called MPA-SAL-BSA chip, DMSA-SAL-BSA chip or DHLA-SAL-BSA chip, which were then stored in a sealed container at 4°C prior to further analysis.

Electrochemical characterization of modified chips

A standard three-electrode electrochemical cell was used for electrochemical experiments with a bare chip or modified chips as working electrode, and a platinum (Pt) wire and an Ag/AgCl electrode were used as auxiliary electrode and reference electrode, respectively. In the range of 0.5 - 1 V, the electrochemical behaviors of different chips were studied by CV (cyclic voltammetry) in the probe solution (5.0 mM [Fe(CN)₆]³⁻, 0.1 M KCl and 0.1 M KNO₃) with a scanning speed of 100 mV s⁻¹.

Assay of SAL-BSA activity

To determine the activity of the immobilized enzyme, SPR measurements were performed using gold slides with covalently attached SAL-BSA. SAL-Ab enzyme analyte stock solutions of different concentrations were prepared to flow them on the sensor surface (prediluted in PBS). PBS was used as the running buffer, and the flow rate was 100 μL/min. In addition, 100 mM NaOH was used as the regeneration solution, and the flow rate was 400 μL/min.

Detection of salbutamol in pork sample

The pretreatment of pork samples was carried out as follows. Briefly, the pork sample (5.0 g) was mixed with a certain amount of saliva, followed by addition of 10 mL acetonitrile. The mixture was shaken for 1 min, and then 9 mL supernatant was taken, dried with nitrogen gas and dissolved in 2 mL of n-hexane. Subsequently, 1 mL PBS was added, and the mixture was vortexed for 2 min and incubated in a water bath at 85°C for 3 min. The lower liquid was taken as the test liquid. After the liquid was passed through a 0.22-μm filter, 250 μL was used for biosensor detection, and each concentration was repeated prepared.

Salbutamol standard solution of different concentrations and 12.5 μg/mL of salbutamol antibody was mixed at 37°C. When the actual sample was tested, the standard solution was replaced and mixed with salbutamol antibody. The SPR measurements were carried out in PBS, and all analyte stock solutions were prepared and then diluted in PBS. Experiments were performed by monitoring the refractive index changes as a function of time under flow conditions. The surface was washed with NaOH (running buffer) between each concentration. The film was reusable because the change in signal was the same when the same concentration was applied. The experiments were carried out at room temperature with a flow rate of 20 μL/min.

Results and Discussion

Formation of SAMs

Three thiol molecules, MPA, DMSA and DHLA, were
selected to prepare SAMs for the chip. Since the amount of thiol was difficult to determine after it was fixed on the chip surface, we could indirectly measure the amount of mercaptan fixed on the chip by assessing the difference of the ultraviolet (Fig. 1) absorption value of –COOH in the solution before and after SAM construction. Secondly, when thiol molecules were fixed on the chip, the free carboxyl groups became hydrophilic. The more carboxyl groups, the stronger the hydrophilicity. We could determine the hydrophilicity of the three chips by measuring the contact angle (Fig. 1). Figure 1a shows that the difference of ultraviolet absorption of MPA before and after SAM construction was about 0.1. The difference of absorption of DHLA was about 0.5 (Fig. 1e), while there was no obvious difference for DMSA (Fig. 1c). The amount of thiol fixed on the MPA chip was the most, while the least was found on the DMSA chip. The contact angle test also proved the above-mentioned results. The MPA chip had an average contact angle of 25.6° (Fig. 1b), the DMSA chip had an average contact angle of 55.9° (Fig. 1f), and the DHLA chip had an average contact angle of 61.8° (Fig. 1d). The bare gold chip only showed slight hydrophilicity due to the influence of carboxyl group, which was positively related to the number of the carboxyl groups. The enhanced hydrophilicity of SAM-modified gold chip could significantly affect protein adsorption. 

Chip characterization

Three types of chips were tested by SEM and AFM in order to understand the specific morphology of SAL-BSA immobilization on the chip surface. Figure 2 shows that SAL-BSA was fixed on the chip surface. The surface of the MPA chip was not uniform, which was confirmed by AFM and roughness test. The antigen distribution on the surface of the DHLA chip was relatively uniform, and there were some gaps. Moreover, the most even distribution of antigen was found on the DHLA chip, making full use of the surface space of the chip, which was also illustrated by AFM and roughness test. SEM was used to characterize the bare chip and modified chips. As shown in Fig. 2, SAL-BSA was fixed on the chip surface, and the roughness of the bare chip surface was obviously lower compared with the modified chips. The surface roughness \( R_q \) and \( R_a \) values of the bare gold chip were 0.836 and 0.684 nm, respectively, and the surface roughness \( R_q \) and \( R_a \) values of the modified gold chips DMSA-SAL-BSA were 1.86 nm and 1.49 nm, respectively. In addition, the surface roughness \( R_q \) and \( R_a \) values of the MPA-SAL-BSA chip were 2.09 and 1.49 nm, respectively, and the \( R_q \) and \( R_a \) values of the DHLA-SAL-BSA chip were 0.788 and 0.606 nm for the DHLA-SAL-BSA chip, respectively.

Figure 3 shows that three modified SAL-BSA chips and a bare chip were tested for CV. The conductivity of the bare chip was the best, and the conductivity of the three modified antigens was lower compared with the bare chip, which might be attributed to the poor conductivity of the protein having a hindering effect on the transmission of electrons on the chip surface. Among the three modified chips, the conductivity of the MPA chip was stronger compared with the other two chips, which could be attributed to the fact that its surface protein distribution was the most uneven and there were more exposed gold sites, resulting in its good conductivity. Moreover, the conductivity of the DHLA chip was better compared with the DMSA chip. CV test also proved that the protein distribution on the DMSA chip was uniform, making full use of the space on the chip surface, which was consistent with the results of
SEM and AFM. Taken together, the DMSA monolayer modified chip had the best performance.

Detection of SAL-BSA activity on chip surface

In order to evaluate the performance of the chip, different gradient concentrations (12.5, 25, 50, 100 μg/mL) of salbutamol antibody were introduced into MPA, DMSA and DHLA chips on the SPR instrument, and the relative response values were recorded. Figure 4 shows that when the SAL-Ab concentration is 50 μg/mL, the MPA chip is close to saturation, and its response value is 59.7 (Fig. 4a). Under the same conditions, the response values of the DHLA and DMSA chips are 80.6 (Fig. 4c) and 101.6 (Fig. 4b), respectively, and the response value of the DMSA chip is 170.1% times that of the MPA chip, indicating that the DMSA chip could combine more SAL-Ab under the same construction conditions. According to the above-mentioned experimental results, the more organic thiol, the more fixed SAL-BSA, while the least amount of SAL-Ab was found on the MPA chip. In contrast, the DMSA chip exhibited the least organic thiol and the most SAL-Ab binding. The amount of SAL-Ab binding on the chip was enough to reflect the number of active sites of SAL-BSA. Therefore, we speculated that the poor performance of the MPA chip might be attributed to the fact that the SAL-BSA on the chip was unevenly distributed, and the active sites binding with SAL-Ab were blocked by each other, resulting in the decrease of SAL-Ab binding.

SPR immunoassay of salbutamol

Selection of the best antibody concentration. DMSA was chosen as the single molecular layer, 1.0 mg/mL SAL-BSA was modified on the chip, PBS was used as the running buffer, and 100 mM NaOH was used as the regeneration solution. In addition, 12.5, 25 and 50 μg/mL of antibody and different concentrations (5, 10, 20, 30, 40, 60, 80, 100, 150 ng/mL) of salbutamol standard solution of were added into the sample disk in a random order to determine the response value. Under the optimized experimental conditions, the optimum concentration of SAL-Ab in the detection of salbutamol by DMSA-SAL-BSA chip was investigated. As can be seen from Fig. 5, within the range of salbutamol concentrations (5 – 150 ng/mL), when the concentration of SAL increases, the inhibition rate of SAL to SAL-Ab increases. The linear equation of Fig. 5a is $y = -0.0086x^2 + 1.7883x + 18.524$ ($R^2 = 0.912$), the linear equation of Fig. 5b is $y = -0.0054x^2 + 1.471x - 1.7194$ ($R^2 = 0.987$), and the linear equation of Fig. 5c is $y = 0.0006x^2 + 0.1862x + 2.5692$ ($R^2 = 0.961$). When the antibody concentration is 25 μg/mL, the detection sensitivity is the best. Considering the experimental cost and detection sensitivity, the SAL-Ab concentration of 25 μg/mL was taken as the reaction concentration. The relative standard deviation (RSD) of the data point is less than 4% and is shown as an error line in the diagram (Fig. 5b).

The calibration curve for the detection of salbutamol by SPR immunosensor. Under the above optimal conditions, the SPR immunosensor based on DMSA as linker was applied to the detection of SAL. As shown in Fig. 6, taking the logarithm of SAL molecular concentration $c$ as abscissa and the inhibition rate as ordinate, the standard curve was drawn by software curve fitting, and the regression equation $y = 0.008c^2 - 1.95c + 114.29$ ($R^2 = 0.982$) was obtained. The lowest amount of salbutamol detected by the sensor was taken as the LOQ, the value of LOQ is 5 ng/mL and the value of IC50 is 39.1 ng/mL.

Detection of salbutamol in pork sample

In order to verify the precision and accuracy of the SPR method, pork was used as the sample matrix with the addition levels of 5, 10 and 20 μg/kg. Each addition level was repeated three times. According to the above-described sample processing method, SPR was detected. The recovery of salbutamol ranged from 94.9 to 108%, and RSD was 1.3 - 5.58%. In addition, compared with the results of the HPLC-MS/MS method, the two methods exhibited good consistency. Therefore, the method established in this experiment was suitable for quantitative analysis of salbutamol in a pork sample (Table 1).
In order to investigate the recognition specificity of this method, ractopamine, clenbuterol, mambuterol and simaterol at the same concentration (120 ng/mL) were selected as structural analogues of SAL. As shown in Fig. 7, the inhibition rate of SAL is significantly higher than that of other compounds, and is not significantly different from that of the mixture (120 ng/mL). Therefore, this method has good specific recognition performance. At the same time, the proposed competitive DMSA-SPR immunosensor method was compared with the previously reported method for the detection of salbutamol. As shown in Table 2, the detection range and minimum detection value of the SPR sensor method are lower than those listed in the table, indicating that the competitive DMSA-SPR immunosensor method is a sensitive method for the detection of salbutamol in food.

In this experiment, the concentration and arrangement of DMSA and MPA as carboxyl functional groups on the surface of SAMs were compared for the first time. The experimental results show that the sensitivity of DMSA with special structure of dimercapto and dicarboxylic acid is higher than that of the SPR chip constructed by MPA traditional connecting molecules of mono-mercapto and monocarboxylic acid. It was verified that the density and distribution of carboxyl groups in thiol would affect the binding of antigen/antibody on the surface of the SPR chip. In this paper, using DMSA as SAMs to improve the detection efficiency of the SPR immunosensor is an effective new idea, which can be applied to the convenient, fast and highly sensitive detection of other small molecules.

**Conclusions**

In this experiment, the concentration and arrangement of DMSA and MPA as carboxyl functional groups on the surface of SAMs were compared for the first time. The experimental results show that the sensitivity of DMSA with special structure of dimercapto and dicarboxylic acid is higher than that of the SPR chip constructed by the MPA traditional method of connecting molecules of mono-mercapto and monocarboxylic acid. It was verified that the density and distribution of carboxyl groups in thiol would affect the binding of antigen/antibody on the surface of the SPR chip. In this paper, using DMSA as SAMs to improve the detection efficiency of the SPR immunosensor proved to be an effective new idea, which can be applied to the convenient, fast and highly sensitive detection of other small molecules.

**Acknowledgements**

This research was supported by the Agricultural Science and Technology Innovation Project of Shandong Academy of Agricultural Sciences (CXGC2018E05). We would like to thank Zhaozhen Cao and Fang Tian from the Analytical Center.
for Structural Constituent and Physical Property of Core Facilities Sharing Platform, Shandong University, for expert technical assistance and helpful discussions with the experiment.

References

1. Y. V. Stebunov, O. A. Aftenieva, A. V. Arsenin, and V. S. Volkov, ACS Appl. Mater. Interfaces, 2015, 7, 21727.
2. Y. Mei, C. Zhong, L. Li, and J. Nong, Anal. Bioanal. Chem., 2019, 411, 4577.
3. W. Wang, X. Zhou, S. Wu, and S. Li, Sens. Actuators, A, 2019, 286, 59.
4. S. Miyake, Y. Hirakawa, and T. Yamasaki, Anal. Sci., 2020, 36, 335.
5. Y. Cao, B. Griffith, P. Bhomkar, and D. S. Wishart, Analyst, 2017, 143, 289.
6. L. Wu, J. Guo, Q. Wang, and S. Lu, Sens. Actuators, B, 2017, 249, 542.
7. X. Liu, R. Huang, R. Su, and W. Qi, ACS Appl. Mater. Interfaces, 2014, 6, 13034.
8. M. Singh, M. Holzinger, M. Tabrizian, and S. Winters, J. Am. Chem. Soc., 2015, 137, 2800.
9. M. Toma, and K. Tawa, ACS Appl. Mater. Interfaces, 2016, 8, 22032.
10. S. Peeters, T. Stakenborg, G. Reekmans, and W. Laureyn, Biosens. Bioelectron., 2008, 24, 72.
11. W. C. Tsai and P. J. R. Pai, Microchim. Acta, 2009, 166, 115.
12. A. J. Guiomar, J. T. Guthrie, and S. D. Evans, Langmuir, 1999, 15, 1198.
13. K. V. Gobi, K. Matsumoto, K. Toko, and H. Ikezaki, Anal. Bioanal. Chem., 2007, 387, 2727.
14. O. V. Shynkarenko and S. A. Kravchenko, Theor. Exp. Chem., 2015, 51, 273.
15. K. Isozaki, T. Shimoaka, S. Oshiro, and A. Yamaguchi, ACS Omega, 2018, 3, 7483.
16. L. Zou, Y Li, S. Cao, and B. Ye, Electroanalysis, 2014, 26, 1051.
17. K. Chang, Y. T. Chang, and C. E. Tsai, J. Food Drug Anal., 2018, 26, 725.
18. A. C. Servais, P. Chiap, P. Hubert, and J. Crommen, Electrophoresis, 2004, 25, 1632.
19. E. Ekiert, C. Garcia-Ruiz, M. A. Garcia, and M. L. Marina, Electrophoresis, 2003, 24, 2680.