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A Biomimetic Sensor for Fast Lysozyme Detection

Wentao Zhao[a], Zhenhe Chen[b], Bin Xue, Liquan Sun and Aiqin Luo[c]

School of Life Science, Beijing Institute of Technology, Beijing 100081, China

[a]yatao5099@bit.edu.cn, [b]czh@bit.edu.cn, [c]bitluo@bit.edu.cn

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Abstract. Lysozyme molecularly imprinted polymer membranes(MIMs) were prepared using polyacrylamine as matrix. Direct detection of fluorescence intensity of absorbed lysozyme(Lyz) on the MIMs was achieved for the fast detection of lysozyme. The surface morphology of polymer was characterized by SEM. Adsorption time and rebinding solution pH were optimized to improve recognition ability of MIMs for lysozyme. The results showed that the MIMs had a high selection and recognition ability for the lysozyme protein.

Introduction

Molecularly imprinted polymers(MIPs) are prepared using molecular imprinting technique, and have the same spatial structure and binding sites of a class of polymers as the template molecule[1,2]. In 1940s, Pauling[3] first proposed the theory of antibody formation, which had laid the foundation for the theory of molecular imprinting; after that, according to Pauling‘theory, Dickey[4] synthesized silica gel for recognition and adsorption of dyes.The imprinted silica can be considered as the first synthesized imprinted material. In 1970s, Wulff[5] first synthesized the organic imprinted polymer. In the following decades, molecular imprinting technology (MIT) for small molecules has been well developed [6-8]. As the development of biology science, the imprinting of bio-macromolecules has attracted more and more researchers' attention[9,10]. The current research for protein imprinting are not only confined in bulk polymerization and other conventional methods. Tai[11] used a short peptide which is epitope of anthrax protective antigen as the template molecule to synthesize the MIPs which could identiy the peptide or corresponding protein. In Gai’s research[12], polymer was grafted on the surface of superparamagnetic by the ATRP to prepare the lysozyme MIPs. Molecularly imprinted polymer membrane is also another attracing area for little template consuming (mg level), fast adsorption balance and easy transferring to optical setups [13-16]. There are two kinds of MIMs: One is composite membrane which adopts PVDF, polysulfone or other commercial films as the base film and MIPs are fixed on the surface of the base film, the prepared MIMs can obtain the high selectivity and used in membrane separation area to detect the specific target protein[17,18]; the other is to synthesize and fix the polymer membrane on the support glass[19,20], suchMIMs can be used on QCM, SPR and other high sensitivity equipments to detect trace analytes[21-23].

The purpose of this study is to prepare an sensing membrane for detection of Lyz for future using in QCM or SPR.

Experimental

Materials. Lysozyme (Lyz), Albumin egg and Bovine serum albumin (BSA) were bought from Beijing Leber Hess Biotechnology Company; MBA and APS were bought from Beijing chemical reagent company; KH570 (Qufu chenguang chemical company); AAm (Tianjin chemical reagent research institute); SDS(Xilong chemical company); TEMED(Beijing Mengyimei biotechnology company); other chemical reagents were analytical pure, water was ultra-pure.

Apparatus. HITACHI F-4500 fluorescence spectrophotometer, JEOL JSM-7500F scanning electron microscope.

Silanizing of support glass. First, clean the support glass with acetone, and then soak them in the saturated KOH solution for 1h and after that wash with water repeatedly to remove base residues. Second, soak the support glass with 15mL concentrated hydrochloric acid and 50mL water for 20min

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and then wash with water to remove acid residues. Third, soak the activited glass with 10mL concentrated hydrochloric acid, 10mL H_2O_2(30%) and 50mL water for 20 min, washed them once with aceton and several times with dry toluene. Finally, Soak the treated support glass in the solution of 2mL KH570, 0.2mL triethylamine and 50mL dry toluene, stirred 12h, and then washed them repeatedly with dried toluene and dry by N_2 and kept it sealed at 4℃.

The preparation of MIMs. Lyz(30mg), AAm(120mg) and MBA(3mg) were dissolved in 1mL PBS (pH7.0, 10mM; TEMED, 0.05vol%; APS, 2mg/mL) solution and purged with N_2 for 5min as pre-polymerization solution. Drop 15μL pre-polymer solution on the surface of support glass and cover with Mylar film to make the pre-polymer solution spread over the glass support completely. After polymerizing for 24h at room temperature, the membranes are soaked it in the PBS(10mM, pH6.0) for 2h and then the Mylar film could be easily removed from the surface of the polymer membrane.The preparation of NMIMs was the same as MIMs but without adding the Lyz.

Template Removing. The MIMs was soaked in the solution of 10vol% acetic acid mixed with 10wt% of the SDS and oscillated 12h and this step was repeated 3 times. And then washed with ethanol and oscillated for several times to fully remove SDS, finally washed with pure water for 12h several times until the washing water was neutral .

Binding Kinetics. To test the influence of adsorption time on adsorption effect, MIMs and NMIMs were put into 3mL Lyz solution(200μg/mL, PBS, pH6.2) and test the luminescent intensity of the MIMs every half hour until 2 hours. Before every test, 1mL blank PBS solution was used to remove non-specific binded proteins. In this and following parts, the exitation and emmision wavelength are: 286nm and 350nm and the slits were set as 10nm and 10nm for all analytes.

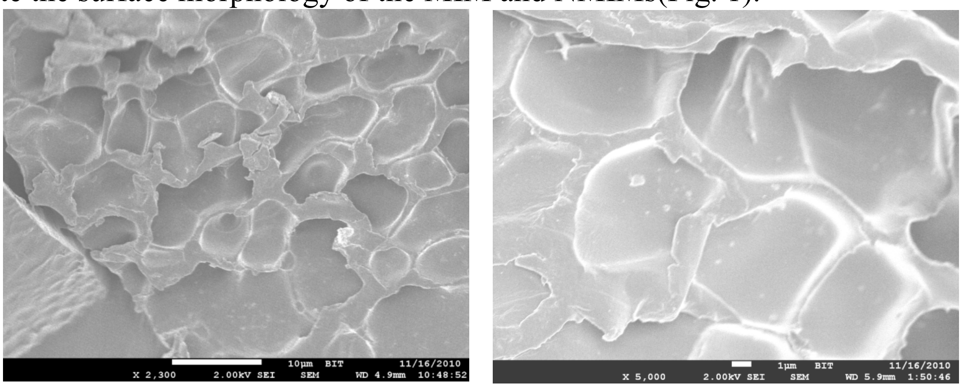
Effect of pHs on MIM adsorption. Three different PBS (pH 6.2, 7.0, 8.0) were used to evaluate the influence of pH on Lyz rebinding to MIM. The Lyz solution is in the range of 0-200μg/mL and the adsorption time is 0.5h. After rebinding, 1mL blank PBS solution was used to remove non-specific binded proteins.

Binding isotherm. At selected pH, both MIMs and NMIMs were tested using above methods to prepare the binding isotherm.

MIMS / NMIMs competitive adsorption. Both the MIMs and NMIMs were immersed into a 3mL Lyz solution and then treated and measued as above.

Results and discussion

Analysis of membrane surface morphology. Scan electron microscopy pictures were taken to investigate the surface morphology of the MIM and NMIMs(Fig. 1).

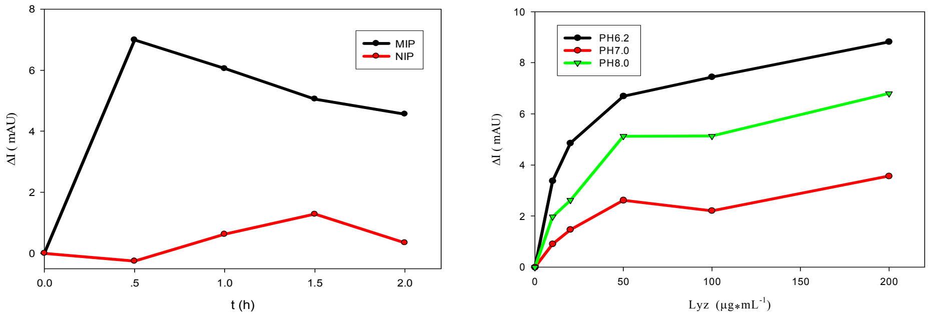


(a) (b)

Fig. 1 Surface morphology of MIM(a) and NMIM(b)

Some ridge-wrinkle structures were observed from both MIMs and NMIMs which is probably due to the freeze drying process. The MIM and NMIM are very "flat" and in the scale of micrometer, have no great difference in morphology. Thus, the NMIM is a proper reference of MIM when comparing adsorption ability. In addition, such flatness can decrease light scattering and is pretty good for the application in the optical-setups.

Adsorption kinetics of MIMs. A binding kinetic study had been carried out for MIMs and NMIMs to determine the rate of the adsorption process(Fig. 2(a)). MIMs and NMIMs are very thin and lyzozyme can easily reach the binding sites of the imprinted polymer, so the fast adsorption equilibrium was achieved within half an hour. But as the time is longer than 0.5hour, the fluorescence intensity becomes decreasing which is probably due to the binded lysozyme is denatured and the fluorescent emission efficiency is decreasing. Therefore, we chose 30min as the rebinding time in the following experiments. Moreover, in Gai's study[12], the adsorption equilibrium time of Lyz was 8h. In our case, diffusion rate of Lyz is very fast and balance can be achieved within 0.5h which is quite meaningful for fast detection of Lyz.



(a) (b)

Fig. 2 a)Lyz adsorption kinetic curves b)the influence of pHs to MIM rebinding of lysozyme

Effect of pH on the adsorption of MIM. Proteins are multi-charged species and at different pHs will carry on different charges. Different charges will influence the interaction between protein and MIMS and NMIMs. The influence of pHs to the rebinding are shown in Fig. 2(b). Three different pHs are considered and at pH6.2 the MIM has the largest fluorescence intensity (8.8 mAu) comparing with the other two pHs. While the pHs are a little acidic or basic, the protein carries different charge and the interaction becomes weaker and thus a weak fluorescent intensity was observed.

Adsorption isotherm. In order to study the imprinting effect of MIMs, the adsorption isotherms of the MIM and NMIM are prepared. Due to the limited total polymer amount of MIMs, when the lysozyme concentration is higher than 200μg/mL, the washing solution volume is not enough to remove non-retained lysozymes. Thus in the following experiments, we prepare the binding isotherms in the lysozyme range of 0-200μg/mL. The MIM and NMIM fluorescent response to different concentrations of lysozyme are well fitted by the Langmuir equation[24].

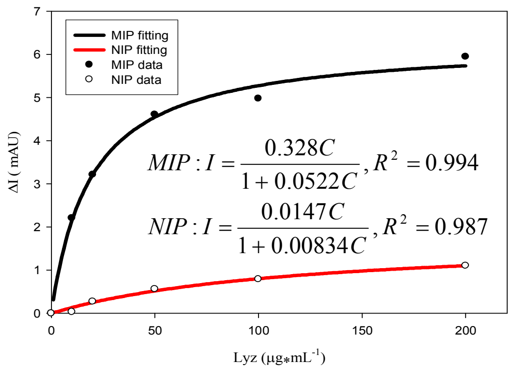


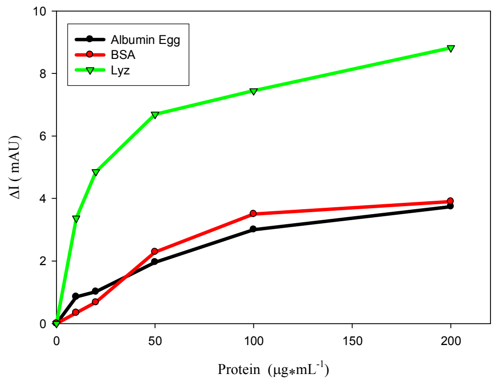
Fig. 3 Adsorption isotherm

From Fig. 3, we can see the adsorption ability of MIM are much larger than that of NMIM and as the lysozyme concentration increases, the adsorption ability reaches equilibrium for MIM . MIM has much more specific binding sites than NMIM because of imprinting effects, therefore, at low lysozyme concentration, fast increasing of the MIM fluorescent intensity was observed. While the lysozyme concentration is larger than 100μg/mL, the binding sites of MIMs become saturated and binding curve becomes flat. While the binding of NMIM is non-specific and therefore no similar binding characteristics were observed and the fluorescent intensity of NMIM are increasing slowly at all concentration range.

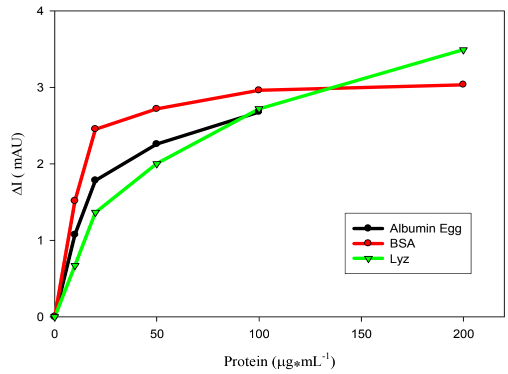
Interferent study. In order to see the selectivity of MIMs, interferents study were carried out. Albumin egg and bovine serum albumin (BSA) were chosen as the interfernts. The characteristics of these proteins are listed in Table 1.

Table 1 Protein used in the interferent study

	Mw/KDa	Isoelectric point
Lyz	14.3	11.0
Albumin Egg	45	4.7
BSA	70	4.9



(a)



(b)

Fig. 4 the adsorption of MIMs (a) and NMIMs (b) to competitive prote

During the imprinting process, the size and charge state of the imprinted pores are coinciding with lysozyme. The MIM shows much higher response to lysozyme than the other two interfering proteins(Fig.4). On the one hand, this is due to the size of albumin egg and BSA is larger than that of lysozyme (Table 1); on the other hand, the inteferent proteins carry different charges comparing with lysozyme at pH6.2. Therefore, when rebinding runs, the MIMs should recognize anlytes which has the same charge state and in our case the MIM should recognize lysozyme better than the other two proteins.

MIMS / NMIMs competitive adsorption. We aimed to use the MIM and NMIM as sensor chips in the optical sensing system. But as is known to all, non-imprinted polymer has some kind of binding ability to interferents which limits the application of MIMs. From a application point of view, if we introduced NMIM into sensing system at the same time, it's going to be a good reference. The response of MIMS and NMIMs to different concentration of lysozyme was shown in Fig.5. Within the concentration range, the imrpinting factors (the ratio of MIM response to NMIM) were calculated using the equation fitted from the experiment data(Table 2) and perfect selectivity was achieved.

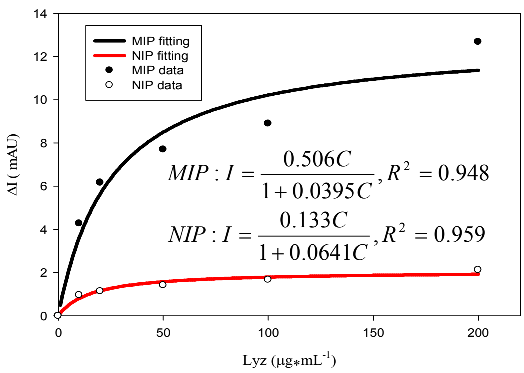


Fig. 5 the competing response of MIM and NMIM to lysozyme

Table 2 Imprinting factor

Concentration(μg/mL)	10	20	50	100	150	200
Imprinting factor	4.48	4.85	5.38	5.70	5.83	5.91

Conclusion

MIMs using lysozyme as a template was prepared under polyacrylamide system and mild reaction conditions. It avoided heating of protein and ultraviolet radiation which could make the protein denatured. The MIMs have very high specific recognition ability to the template molecule, and recognize lysozyme from other competing proteins. The MIMs could obtain adsorption equilibrium time very fast comparing with bulk polymer and response to analyte in the scale of microgram. Therefore, this sensing membrane provides a fast and simple method for detecting lysozyme in pretty low concentration and also shows potential application in well-established apparatus like QCM or SPR.

Acknowledgements

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