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Chitosan cushioned phospholipid membrane and its application in imaging ellipsometry based-biosensor

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ABSTRACT

Chitosan cushion can support the air stability of phospholipid membrane, but the problem of serum solubility of phospholipid membrane prevents it from use in serum detection applications. Poly (ethylene glycol) (PEG) shielding promises both stability and non-specific adsorption resistance for phospholipid membrane. An air stable phospholipid membrane microarray has been successfully fabricated on chitosan modified silicon wafer. We have demonstrated the potential application of PEGylated phospholipid membrane in imaging ellipsometry-based protein biosensor. Because of the strong resistance against non-specific adsorption of serum, antigens are immobilized onto the membrane surface through chemical activation and further bind their antibodies without using blocking agent. Taking advantage of the multiple and parallel reaction capabilities of microfluidic reactor system, we have assayed the binding by varying both the density of antigen on the membrane surface and the concentration of antibody in solution.

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1. Introduction

Imaging ellipsometry-based protein biosensor [1,2] is a newly developed nonlabeling technique with many advantages, such as phase sensitive and rapid detection of proteins in liquid media. Proteins immobilized directly on silicon surface have been successfully used to capture biomarkers [3,4], phage [5] or virus [6,7]. Aiming at protein detection in complex biological samples, much attention should be paid to avoid non-specific protein adsorption, which is the main source of noise in immunoassays and has become the major obstacle in developing more sensitive and higher throughput biosensor assays [8–11].

A variety of non-fouling materials have been synthesized and researched during the past decades [12–15]. Among those strategies, supported phospholipid membrane has shown good validity in preventing protein adsorption [16–18] and has the potential to develop biosensors into highly selective devices for a variety of biological analytes [19]. However, the instability of lipid membrane in air is one challenge for imaging ellipsometry based-biosensor. Recently, a few published research works have focused on creating air stable phospholipid membranes, nevertheless, there are still some problems, such as reduced fluidity [20], high density protein coverage [21] or the need for careful handling [22], which make these lipid membranes unsuitable for biosensor applications [23]. In our previous work chitosan cushion and PEG shielding could achieve air stability of the phospholipid membrane and the density of PEGylated lipids had influence on the final structure of dried lipid membrane [24]. However, during recent experiments, we have found model phospholipid bilayers, without PEG polymers protection, can also maintain air stability on chitosan modified silicon surface.

In this article, model phospholipid membranes with or without polymers shielding are formed on chitosan modified silicon surfaces. After comparing the interaction with serum, PEGylated lipid membrane is finally employed for protein immobilization in imaging ellipsometry based-biosensor. Albumin is one of the abundant proteins in serum. Human serum albumin and its antibody are used as model proteins to demonstrate the potential application of the air and serum stable phospholipid membrane in imaging ellipsometry based-biosensors.

2. Experimental

2.1. Materials

1,2-Dimyristoyl-sn-glycero-3-phophatidylcholine (DMPC), 1,2-dilauroyl-sn-glycero-3-phosphatidylethanolamine

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(DLPE), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[carboxy(polyethylene glycol)2000] (DSPE–PEG2000–COOH) and Mini-Extruder were purchased from AvantiPolar Lipid, Inc. (Alabaster, AL). PLL and cholesterol were purchased from Sigma. All lipids were stored at −20 °C. Human serum albumin (HSA), goat anti-HSA and glutaraldehyde were obtained from Sigma. γ-Aminopropyl-triethoxysilane (APTES), N-hydroxysuccinimide (NHS) and N-ethyl-N′-(3-diethyl-aminopropyl) carbodiimide (EDC) were from Acros. LMW chitosan (Mr: ∼10,000, ∼85% deacetylation) was from Jinan Haidebei Marine Bioengineering Co. (Jinan, Shandong, China). Hyaluronic acid (HA) (MW: 60,000) was provided by Freda Biochem. Co. Ltd. (Jinan, Shandong, China). Milli-Q ultrapure water with a resistivity ∼18 MΩ cm (Millipore, Molsheim, France) was used for all the solutions. Polished silicon wafers were purchased from General Research Institute for Nonferrous Metals (China). Other chemicals used were analytical grade or better.

2.2. Surface modification of silicon wafers

Polished silicon wafers were cleaned with piranha solution and further modified with APTES and glutaraldehyde according to the protocols described before [25]. The aldehydated silicon surface was then reacted with chitosan (10 mg/ml in ultrapure water) solution for at least 1 h. The modified silicon wafers were kept in clean Petri dish and stored at 4 °C before usage.

2.3. Phospholipid membrane formation and polymer covering

A microfluidic system [26] developed in our laboratory was used to fabricate lipid membrane microarray. Phospholipid membrane was formed by small unilamellar vesicles (SUVs) adsorption and rupture on the chitosan modified silicon surface. Preparation of SUVs was carried out via the extrusion method [24]. Vesicles solution was stored at 4 °C until use. 10 μl of vesicles was introduced into each tiny chamber at 1 μl/min followed by water rinsing (10 μl/min, 10 min) to remove excess vesicles.

For covering membranes with polymers, amino groups of chitosan or PLL were linked with DLPE, which also has amino groups, through glutaraldehyde as crosslinker. In brief, after dotting SUVs on chitosan modified silicon wafer to form lipid membranes, the membranes were treated with glutaraldehyde solution (2.5% in PBS) for 1 h. After being rinsed with ultrapure water, the wafers were further reacted with chitosan (10 mg/ml in ultrapure water) or PLL (5 mg/ml in ultrapure water) solution for at least 1 h. Residual free aldehydes were quenched by 5 min sodium borohydride treating. For HA protection strategy, carboxyl groups of HA were firstly activated with NHS/EDC (50 mM/200 mM) mixture for 5 min and then bound to DLPE. The residual NHS sites were deactivated by ethanolamine (1 M, pH 8.5).

2.4. Nonspecific protein adsorption of phospholipid membrane

Here, we used human serum from a healthy donor to examine the biomolecular resistance ability of silicon surface after being coated with phospholipid membrane. Firstly, chitosan cushioned phospholipid membrane was rinsed with pure water to remove redundant vesicles. After that, 20 μl serum was delivered across the membrane surface at 1 μl/min and followed by PBS washing at 10 μl/min for 10 min. The silicon wafer was finally rinsed with deionized water before analysis.

2.5. Proteins coupling onto phospholipid membrane

Amino groups of proteins were coupled with carboxyl groups floating on lipid membrane surface through NHS/EDC methods. Equal volumes of NHS and EDC (50 mM/200 mM) were mixed together, and then 25 μl of this mixture was pumped at 5 μl/min across membrane coating surface, followed immediately by an injection of 10 μl HSA (0.1 mg/ml, pH 4.5) at 1 μl/min. PBS was used to remove those non-specific adsorbed proteins. Residual NHS esters were inactivated by further injection of 10 μl ethanolamine chloride (1 M, pH 8.5) at 5 μl/min. Finally 10 μl anti-HSA (20 μg/ml, pH 7.4) was added with a flow rate of 1 μl/min followed by PBS and pure water washing.

2.6. Imaging ellipsometry

The detection of microfluidic protein/phospholipid membrane array was carried out on imaging ellipsometer, which was detailed elsewhere [1,2,27]. The ellipsometer used is enhanced, combined the power of ellipsometry with microscopy and worked in the off-null mode. The thickness of biomolecular layers was calibrated with a rotating analyzer-type ellipsometer (SE 400, Sentech) equipped with a He–Ne laser (λ = 632.8 nm), of which incidence angle is 75°. The gray scale was associated with the amount of proteins binding on the phospholipid membrane.

2.7. Quartz crystal microbalance (QCM)

The home-made QCM was used to monitor the formation of phospholipid membrane and its interaction with serum or proteins. The measured signals from the sensor crystal are the change in frequency as functions of time. Chitosan (10 mg/ml) was pre-absorbed onto the gold surface through electrostatic attraction. After the adsorption and fusion of SUVs onto the sensor chip the system was washed with ultrapure water until the frequency reached a stead state, then whole serum was injected into the reaction cell, followed by PBS washing for at least 30 min (flow rate of 100 μl/min). For HSA assembly, NHS/EDC mixture was pumped across chitosan cushioned lipid membrane, followed immediately by an injection of HSA (0.1 mg/ml, pH 4.5). Residual NHS esters were inactivated by further injection of ethanolamine chloride (1 M, pH 8.5). Anti-HSA (20 μg/ml, pH 7.4) was added to examine the activity of HSA assembled onto lipid membrane. The stability of the lipid–protein complex in long time washing procedure is also evaluated at the same time.

2.8. Atom force microscopy (AFM)

All AFM images were taken using a commercially available instrument (Veeco; MultiMode III). A tapping mode cantilever with an oscillation frequency of 200–300 kHz was used during the procedure. AFM imaging was performed in air with no environmental controls.

3. Results and discussion

3.1. Model phospholipid membrane

3.1.1. Stability in air

Stable and uniform lipid membranes are strongly related to the miscibility of lipids, which is predictable from the phase transition temperatures (Tm) of the lipids involved. Here, DMPC and DLPE (DLPE is introduced to covalently immobilize proteins) are selected as SUVs forming lipids because of their similarity in phase condition at room temperature (Tm of DMPC is 23 °C and DLPE is 29 °C). The close phase transition temperatures of DMPC and DLPE promise better miscibility [28]. Dotting SUVs comprised of DMPC and DLPE with different mixing ratios on the chitosan modified silicon surface can form visible and uniform lipid membranes under the imaging

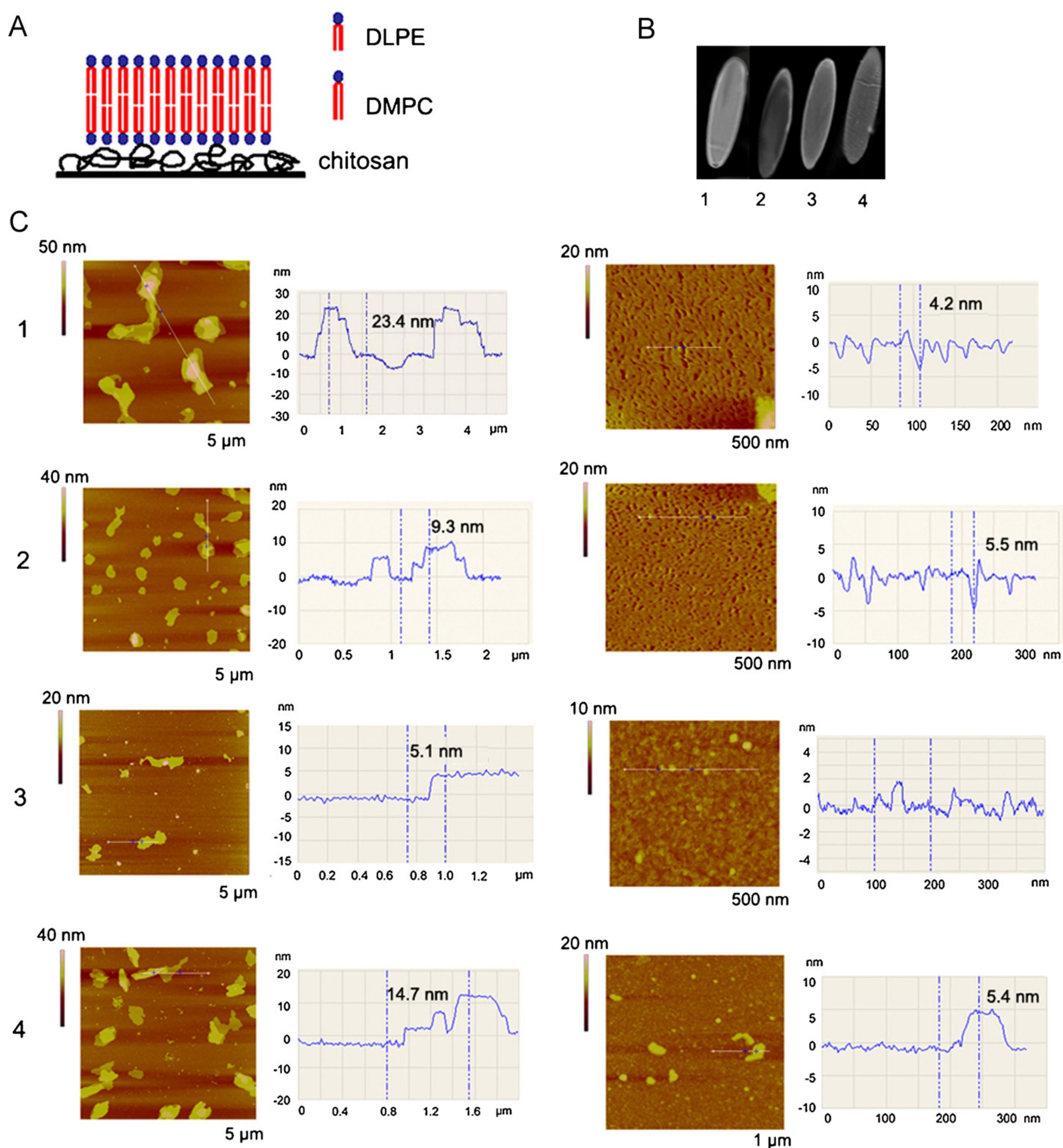

Fig. 1. Ellipsometric and AFM characterization of chitosan supported lipid membranes. (A) Scheme of chitosan cushioned phospholipid bilayers; (B) ellipsometric image of chitosan supported phospholipid membrane; (C) AFM analysis of dried phospholipid membrane; SUVs: (1) DMPC; (2) DMPC:DLPE = 3:1; (3) DMPC:DLPE = 1:1; (4)DMPC:DLPE = 3:1.

ellipsometer (Fig. 1A and B). This is an interesting phenomenon compared with our former discovery [24]. Spectroscopy ellipsometer (Woollam M2000DI) analysis indicates their thicknesses are within 4–5.5 nm range, in close agreement with the estimate thickness of model phospholipids bilayers, although the grayscales of lipid membrane formed by DMPC is only a bit higher than the others. It demonstrates that chitosan cushion only can support the air stability of phospholipid membrane without PEG protection.

AFM characterization confirms the existence and the details of these lipid membranes, as is illustrated in Fig. 1C. Floating debris on the lipid membrane is a common phenomenon under AFM tips, which is caused by the van der Walls attraction between vesicles and formed membranes. It is still not clear why stacking pieces on DMPC formed bilayers can pile up as much as four or five bilayers, according to the line analysis in Fig. 1C. Scanning photos with higher resolution reveal numerous cracks within the dried membranes

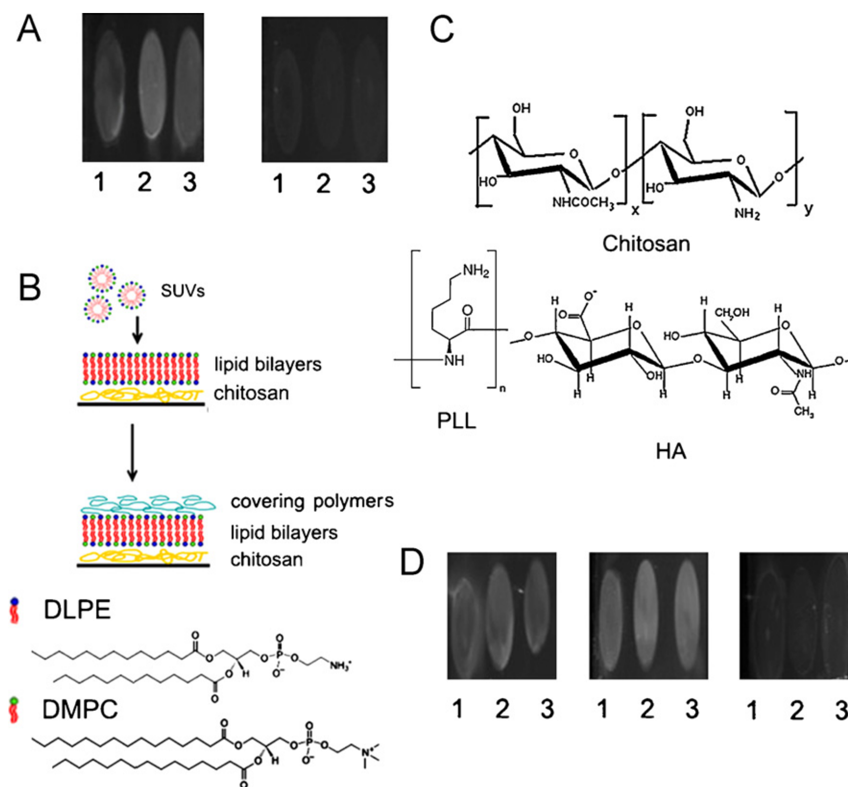

Fig. 2. Polymer covering strategy for serum stability of phospholipid membrane. (A) Ellipsometric images of chitosan supported phospholipid membrane before (the left one) and after (the right one) reaction with serum. SUVs: (1) DMPC:DLPE = 3:1; (2) DMPC:DLPE = 1:1; (3) DMPC:DLPE = 1:3; (B) illustration of polymer covering phospholipid membrane; (C) chemical structure of shielding polymers, including chitosan, poly-L-lysine and hyaluronic acid; (D) ellipsometric images of polymers shielding phospholipid membrane after reaction with serum (chitosan (left), PLL (middle), HA (right)).

(Fig. 1C-1), which is caused by dehydration. But more interestingly, cracks within the DMPC membrane can be healed by further DLPE mixing. One possible reason may be their difference in critical packing parameter [29,30]. DMPC prefers to be a spherical micelle. For DMPC forming membrane, the hydrophobic tails have to draw close to each other in water, but their exposition in air induces separation of the inner of lipid bilayers. Thanks to the underlying chitosan cushion, the lipid sheets did not delaminate from the supporting substrate upon passage through an air–water interface. DLPE itself is prone to form planar bilayers in water phase. The presence of DLPE helps drive the lateral condensing in DMPC bilayers and also helps bilayers survive the air–water interface.

3.1.2. Stability in serum

When trying to evaluate the function of model lipid membrane coating biosensor in clinical serum assay, serum resistance ability should be examined in advance. The developed air stable lipid membranes and sensitive imaging ellipsometer provide convenient tools to study the interaction between lipid bilayers and serum. SUVs with different DMPC/DLPE mixing ratios were dotted onto two parallel chitosan modified silicon surfaces. One of them was further treated with whole serum solution. The left image of Fig. 2A stands for membranes themselves. The right image of Fig. 2A was taken after serum treatment. The grayscales of DMPC/DLPE assembled membranes are reduced nearly to the background, which indicate the lipid membranes are removed by serum. Early work has reported serum can induce content leakage of liposomes [31]. In that case, we believe there is something existing in serum that can destroy the interaction among the densely packed lipids. However, the lipid membranes can endure the diluted serum. There are almost no signal changes for membranes after treating with 10% serum (data not shown), which is in accordance with the results in published papers [32,33]. As a result, it is proposed that the lipid membrane soluble effect of serum is a function of concentration.

3.1.3. Serum insoluble lipid membrane with polymer shielding

Due to the importance of serum stability for biosensor application in clinical detection, the above phospholipid membrane cannot be used directly, although proteins can be assembled on the lipid membrane and show good vitality (Data not shown here). Looking into the extracellular surface of biological membrane, it is often decorated with carbohydrate moieties. Besides well-known roles in cell recognition, bacterial attachment and so on, those carbohydrates might help lipid bilayers surviving the body fluid in vivo. In a similar way, to mimic the outside dressing of cell membrane, three different kinds of polymers, chitosan, PLL and HA, are used to cover the supported phospholipid bilayers. These natural or biocompatible polymers have amino or carboxyl groups which can be easily chemically activated and linked with the amino groups of DLPE. Fig. 2B shows the main steps. After forming phospholipid membranes on chitosan modified silicon surfaces, polymers were covalently linked with DLPE floating on the upper layer. The chemical structures of polymers can be seen in Fig. 2C. The results by imaging ellipsometry analysis in Fig. 2D show that membranes covered with chitosan or PLL, but not HA, can be maintained on the surface after serum treatment. It demonstrates the usefulness of polymer protecting strategy. However, there is an obvious grayscales increase for the polymer covering membranes after serum reaction, which is caused by nonspecific adsorption of molecules in serum. For the future application in serum biomarker detection, non-fouling ability of protecting polymers should be considered.

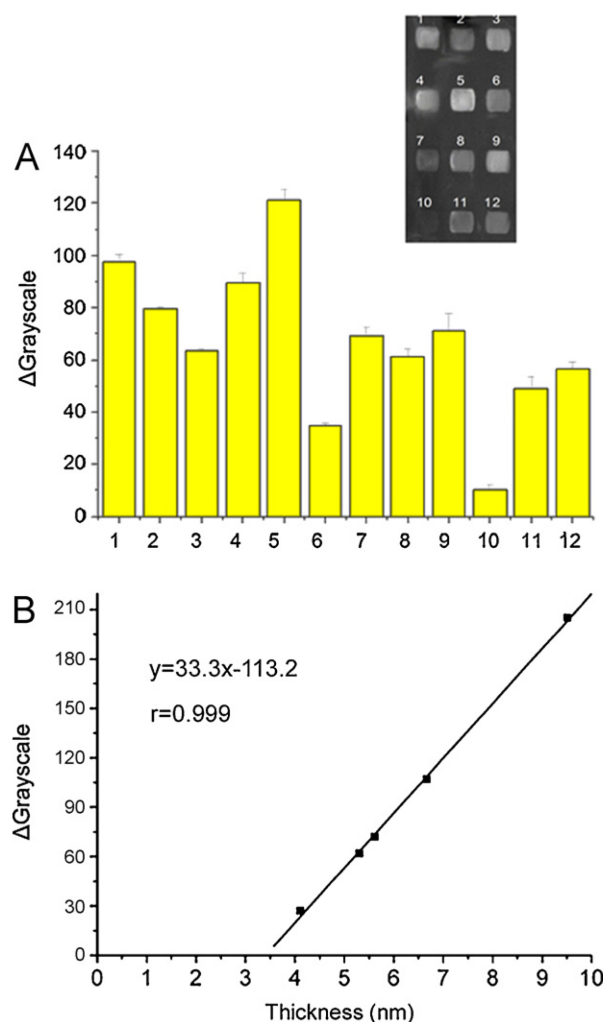

Fig. 3. Air stable phospholipid membrane microarray by imaging ellipsometry. (A) ΔGrayscales comparison of different phospholipid membranes. (B) Relationship between Δgrayscales and thicknesses. SUVs used for forming lipid membranes: (1) DMPC/0.5 mol% PEG; (2) DMPC/1.5 mol%PEG; (3) DMPC/1.5 mol% PEG/5 mol% cholesterol; (4) DMPC/1.5 mol% PEG/10 mol% cholesterol; (5) DMPC/1.5 mol% PEG/30 mol% cholesterol; (6) DMPC/3 mol% PEG; (7) DMPC/3 mol% PEG/5 mol% cholesterol; (8) DMPC/3 mol% PEG/10 mol% cholesterol; (9) DMPC/3 mol% PEG/30 mol% cholesterol; (10) DMPC/5 mol% PEG; (11) DMPC/5 mol% PEG/5 mol% cholesterol; (12) DMPC/5 mol% PEG/30 mol% cholesterol.

3.2. PEGylated phospholipid membrane

3.2.1. Air stable lipid membrane microarray

PEG is one kind of well-used anti-fouling material in surface modification [34–37]. Commercial PEGylated lipids can self-assemble into membranes through hydrophobic force without any surface activation. A microfluidic array reactor has been developed for auto-patterning PEGylated phospholipid membranes. Fig. 3 shows an air stable phospholipid membrane microarray by imaging ellipsometry. Membranes with low PEG density or high cholesterol loading make higher signals, the possible reason for which have been discussed [24]. The terminal methoxy group on the PEGylated lipid is replaced by a carboxyl terminal group in order to immobilize proteins. The ellipsometric images indicate the terminal groups of PEGylated lipid head have little influence on the phospholipid membranes formed on chitosan modified silicon surface.

Otherwise, different from popular microarray fabrication methods, including dotting and spraying solutions onto the substrate, microarray in this article are produced through automated and independent microfluidic channels. This improvement can easily realize high throughput protein–protein interactions research without contaminant.

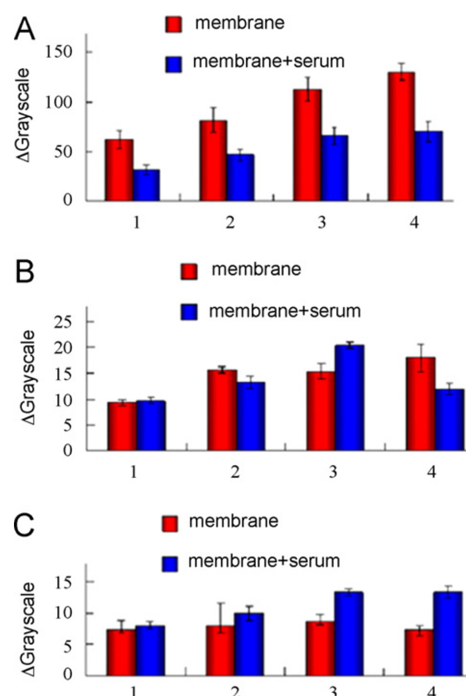

Fig. 4. Ellipsometric analysis of PEGylated phospholipid membranes before (red bar) and after (blue bar) reaction with serum. (A) DMPC/1.5 mol% DSPE–PEG_{2000}; (B) DMPC/3 mol% DSPE–PEG_{2000}; (C) DMPC/5 mol% DSPE–PEG_{2000}. The numbers on the *x*-axis stands for the molar ratios of cholesterol: (1) 0 mol%; (2) 5 mol%; (3) 10 mol%; (4) 30 mol%.

3.2.2. Serum resistance of lipid membrane

After coating PEGylated phospholipid membrane on the silicon surface, healthy human serum is used to evaluate nonspecific adsorption. As cholesterol has a condensing and stability enhancement effect on lipid membrane [24,38–40], we compare the roles of different amounts of cholesterol in lipid membranes when reacted with serum. The bar chart in Fig. 4 shows grayscale changes of PEGylated lipid membranes before and after serum treatment. Considering the limit of signal noise (~5), most of the membranes can maintain their stability in serum and keep strong resistance against adsorption except the membranes with 1.5 mol% PEG, which show an obvious signal drop after serum treatment. The floating debris upon phospholipid membranes with low content PEG may be easily removed by serum, while the underlying lipid bilayers may survive the serum under PEG protection. Although the coverage density of PEG is relatively low, the underlying phospholipid membrane may still serve as a second barrier against serum proteins approaching and adsorption. Anyway, our results suggest phospholipid membranes with appropriate PEG shielding as a more appropriate platform for serum assay.

We also used QCM, which can monitor the mass change on the quartz chip, to confirm the serum resistance of PEGylated lipid membrane. The response after each sample injection is recorded in real time. From the dynamic curve showed in Fig. 5, when serum passes through the membrane, the frequency level drops greatly due to the adsorption of biomacromolecules from serum. However, the frequency can recover to the original level of lipid membrane after PBS rinsing (the adsorbed molecules can easily be washed off using PBS buffer). This can be considered as strong proof of serum resistance by polymer shielding lipid membrane in dynamic fluid condition, and also confirms the conclusion from the above results of dried membrane by imaging ellipsometry.

3.2.3. Ligands assembly on the lipid membrane

In order to illustrate the potential application of PEGylated lipid membrane coating silicon substrate in imaging ellipsometry based-biosensor, human serum albumin and its antibody are chose as model proteins to be bound onto lipids membranes with 1.5 mol%, 3 mol%, and 5 mol% of DSPE–PEG2000–COOH. By coupling amino groups with free carboxyl termini of PEG through NHS/EDC method [41], proteins are covalently immobilized onto the lipid membrane surface. We find that the proteins cannot be bind onto lipid membrane before the membrane is chemically activated. The results are not shown here. The relationship between coupling protein and PEG density is examined after binding antibodies (the same antibodies are used). Original grayscales of membranes subtracted from the final grayscales gives data shown in Fig. 6A.

It is easy to understand a low level of coupling sites giving low signal enhancement after protein binding and vice versa. But we find protein binding is inhibited when DSPE–PEG2000–COOH is overloaded. It may be caused by the spatial hindrance of large PEG moieties in fluid condition. As one PEG2000 molecule can bind more than 138 water molecules [23], a strong hydration repulsion can pose an obstacle to large molecules approaching. We believe that it is hard for ligands to attach to the activated carboxyl groups, which might be hided in PEG pockets. A previous report from Whitesides et al. [41], where thiols containing oligo ethylene glycol were used to form self-assembled monolayers (SAMs) on gold, said the binding amount of proteins increased with molar fraction of carboxylic acid-terminated thiol, but 2 mol% was sufficient for immobilization of almost a complete monolayer of protein. Our study also suggests a proper density of active sites for protein assembly and further protein–protein interaction.

Cholesterol insertion could improve the quality of lipid membrane through its ordering and condensing effect [42,39,43]. It was reported that 50 mol% cholesterol mixed lipid layer would greatly activate protein adsorption, whereas the 10–30 mol% cholesterol strongly suppressed protein adsorption due to highly packed phosphocholine groups [44]. Interestingly in our experiments, we find cholesterol can promote the protein assembly on the membrane according to the data in Fig. 6A. The membrane with 3 mol% PEG and 30 mol% cholesterol makes the highest grayscales increase after the same proteins binding. Based on this membrane, the relationship between the laddered concentration of anti-HSA and the net increase of grayscale values are shown in Fig. 6B, which could be used as a calibration.

We finally apply QCM to show the dynamic process of lipid membrane formation and protein binding. From the curve shown in Fig. 7, the frequency drops obviously after membrane formation and protein binding, which is caused by the addition of mass density on the QCM chip. As there is no obvious fluctuation in frequency during a long time rising, it is believed that the complex of proteins and phospholipids is stable enough for realizing the detection goal.

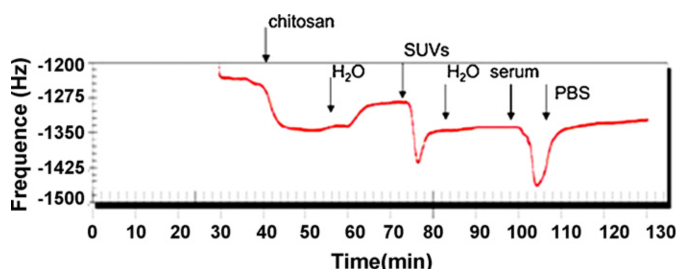

Fig. 5. QCM real time response during the phospholipid membrane (DMPC/3 mol% DSPE–PEG_{2000}) formation on chitosan and its reaction with serum.

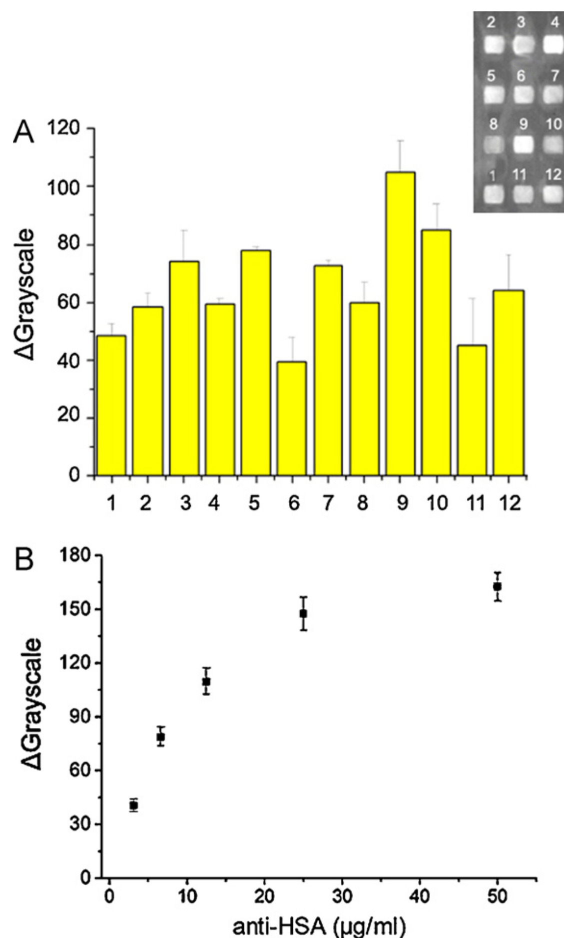

Fig. 6. Air stable phospholipid membrane microarray and its potential application in imaging ellipsometry-based protein biosensor. (A) Comparison of proteins binding efficiency on different phospholipid membranes. SUVs for forming lipid membranes: (1) DMPC/0.5 mol% PEG; (2) DMPC/1.5 mol% PEG; (3) DMPC/1.5 mol% PEG/5 mol% cholesterol; (4) DMPC/1.5 mol% PEG/10 mol% cholesterol; (5) DMPC/1.5 mol% PEG/30 mol% cholesterol; (6) DMPC/3 mol% PEG; (7) DMPC/3 mol% PEG/5 mol% cholesterol; (8) DMPC/3 mol% PEG/10 mol% cholesterol; (9) DMPC/3 mol% PEG/30 mol% cholesterol; (10) DMPC/5 mol% PEG; (11) DMPC/5 mol% PEG/5 mol% cholesterol; (12) DMPC/5 mol% PEG/30 mol% cholesterol; (B) biosensor output value in grayscale with the concentration of anti-HSA (error bar stands for the standard deviation calculated from at least three separate measurements).

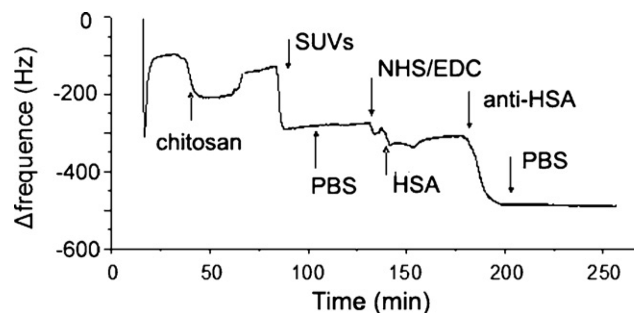

Fig. 7. QCM real time analysis of protein binding on PEGylated lipid membrane. SUVs: DMPC/3 mol% DSPE–PEG_{2000}–COOH/30 mol% cholesterol.

4. Conclusion

In this work, we find chitosan cushion can support the air stability of common phospholipid membranes without PEG protection,

but their instability in serum causes their failure as surface modification of biosensor in serum biomarker detection. With a relatively low density of PEG, self-assembled phospholipid membrane shows good serum stability and also strong resistance against serum adsorption. Air stable PEGylated phospholipid membrane microarray on silicon surface is successfully fabricated using our home-made microfluidic system. After chemical activation, proteins can be immobilized on membrane surface and further binding with antibodies without the use of conventional blocking agent. Proper cholesterol insertion may help protein–protein binding occur at the upper layer of lipid membrane. Nevertheless, considering the differences in the proteins, such as molecular weight, structure and charge, for the other biosensor application, molar fraction of PEG and cholesterol should be screened carefully. In conclusion, chitosan cushioned air stable phospholipid membrane has a considerable potential use in biosensor assay, especially in serum related applications.

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