Tunable two dimensional protein patterns through self-assembly nanosphere template

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HIGHLIGHTS

- The samples with shape protein patterns are similar to natural biomolecules.
- Samples have alternative regions which contained separate antibodies.
- Both SERRS spectra of two kinds biomolecules can be obtained simultaneously.
- Relationship of SERRS intensity and proteins are found.

GRAPHICAL ABSTRACT

ABSTRACT

By the aim of constructing surfaces for multi-component and multifunctional bioassay, a microsphere lithography technique was employed to control the surface morphology. Two kinds of protein molecules (antibodies) were used as building blocks. As a result, dual-component biocompatible surfaces with alternate immunoglobulin micropatterns were fabricated. The employed antibodies included human Immunoglobulin G (IgG) and rabbit IgG, which composed nanometer scale surface arrays on the surfaces. The antibodies were identified specially by immunoreactions with labeled antigens of fluorescein isothiocyanate (FITC)-antihuman IgG and tetramethylrhodamine-5-(and 6)-isothiocyanate (TRITC)-antirabbit IgG. The immune responses were confirmed by confocal fluorescence (FL) microscopy. A study on the sensitivity and quantification was done by using surface-enhanced resonance Raman scattering (SERRS) spectroscopy. The obtained SERRS spectra showed satisfactory resolution in the multi-component detection objects. No interference was observed from inner- or interactions of detecting molecules. The detection limits for both of the antigens reached to as low as 1 ng/mL, which was comparable to FL method. Meanwhile, a good linear relationship between SERRS peak intensity and the logarithm of antigens' concentrations (from 1 ng/mL to 1 mg/mL) were observed. The results demonstrated that SERRS is a very promising detection technique for multi-component immunoassay, and has great potential applications in biotechnology and biochemistry.

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Introduction

Nanometer scale protein arrays have gained considerable attention nowadays due to an increasing demand on biominiaturization and bioassay. For example, uniform protein patterns were used in the research of microfluidics for living cell detection [1,2], cell adhesion [3], chemotaxis [4], cell apoptosis [5] and cell purification [6]. Multiple protein patterns were used in the study of fabricating chips of biosensor, monitoring orientations of cell growth, observing interactions of proteins [8–10]. The protein arrays have been an exciting platform for bioassays in proteomics, drug discovery, and...
diagnostics [11–15]. Although great progresses have been made in the field of protein–array-based bioassay, the detection method is mainly depends on the FL spectroscopy [16–22]. FL spectroscopy is sensitive, convenient and can be used for quantitative analysis, but also suffers from the shortage of photobleaching, which is prone to happen when increasing the complexity of the detecting system, due to an amplified inference from each component. Some supplementary methods are still needed to increase the detection performance on complex biosystems.

Surface-enhanced Raman scattering (SERS) is an emerging spectral method showing great potential applications in materials, physics, environment and chemistry [23,24]. Recently, the SERS research also demonstrates powerful applications in the detection of trace amounts of proteins [25,26,15]. The excellent features of SERS, such as high sensitivity, high selectivity, convenient, and being not affected by the presence of water, make it very suitable to investigate biomaterials, both in component analysis and in quantification [27]. For protein detections, the most important model is the immunoassay which is based on antibody–antigen immunoreactions. Dye molecules were usually used as tags on antigens or antibodies by anticipating FL detections of the obtained immunocomplex. In SERS, these dye molecules can also be probes as Raman scattering centers. Meanwhile, when the excitation lasers are selected properly to be equal to the electronic energy levels of dye molecules, additional enhancement up to $10^6$ to $10^{10}$ can be achieved due to a resonance effect. Thus, the optimal sensitivity of SERS can be on single molecule level.

Our previous work studied the assembling methods of Au or Ag nanoparticles on proteins and the corresponding SERS detections [28,29]. Herein, we aimed at combining SERS detection with nanometer scale protein arrays and inspecting the performance of sensitivity and quantification on multi-component proteins. In the present work, a polystyrene (PS) microsphere monolayer was used as template to fabricate protein micropatterns. Two kinds of IgGs were deposited on the substrate surfaces. Ordered mosaic microstructures were formed and ready for immunoassay. After immunoreactions, both FL microscopy and SERS were used to detect the labeled antigens. Due to the high sensitivity of SERS spectra, the detection limits of each labeled protein were as low as 1 ng/mL. A linear relationship between spectral intensity and logarithmic concentration (in low concentrations) was found and used for quantification. The results indicated that SERS is a very promising technique for multi-component protein detections and have great potential applications in chip-like bioassays.

**Experiments**

**Biochemicals and chemicals**

Human IgG, FITC-antihuman IgG, rabbit IgG, TRITC-antirabbit IgG, bovine serum albumin (BSA), were purchased from Sigma–Aldrich Co., Ltd., and used without further purification. AgNO₃ (99.5%) and other chemicals were obtained from Beijing Chemical Plant. Double distilled water was used throughout the present study. The phosphate buffered saline (PBS) solution (0.01 M, pH 7.2) used in this study contained 0.8% NaCl, 0.02% KH₂PO₄, 0.02% KCl, and 0.12% Na₂HPO₄·12H₂O. Human IgG and rabbit IgG in PBS solution were deployed in 0.1 mg/mL. The FITC-antihuman IgG and TRITC-antirabbit IgG were diluted with PBS solution into 1 ng/mL, 10 ng/mL, 0.1 μg/mL, 1 μg/mL and 10 μg/mL separately. A blocking buffer was prepared by dissolving BSA in the PBS solution (containing 1% BSA). A washing buffer was prepared by adding Tween 20 to the PBS solution (containing 0.05% Tween 20).

**Preparation of Ag colloids**

Colloidal Ag was prepared by the aqueous reduction of silver nitrate using the method of Lee and Meisel [30]. In a typical synthesis, 36 mg of AgNO₃ was dissolved in 200 mL of water. This aqueous solution was then heated to boil under stirring and reflux. 4 mL of trisodium citrate solution (1%, w/v) was added into the flask and allowed to react for 1.5 h. The obtained Ag nanoparticles were 50 nm in average diameter and have a maximum absorption peak at 420 nm.

**Fabrication of protein microarrays**

Glass slides were first treated in boiled piranha solution (30% HCl:98% H₂SO₄ = 3:7, v:v) for 30 min, then rinsed with water three times and dried under nitrogen stream. These hydroxized glass slides were soaked in a 2% (3-aminopropyl) trimethoxysilane ethanol solution for 30 min to undergo a salinization, then rinsed with ethanol and dried under nitrogen stream. The salinized glass slides were immersed in a 2.5% glutaraldehyde solution for 2 h for aldehyde-hydration, and then rinsed with the PBS solution three times. Ordered monolayers of PS spheres (750 nm) were deposited on the glass slides by using the self-assembly technique.

The process of protein deposition is described as follows. The glass slides covered with PS templates were initially kept in 110 °C for 5, 10, 15 and 20 min, separately. To avoid the oxidation of aldehyde groups on the surfaces, the samples were protected under nitrogen atmosphere during the heating process. The obtained samples were soaked in human IgG solution and blocking buffer solution successively. Then the samples were sonicated in PBS solution for 30 s to remove the PS templates. Finally, the glass slides were soaked in rabbit IgG solution and blocking buffer solution separately. All the biochemical processes were conducted at 37 °C for 2 h.

**Immunoreactions**

The glass slides covered with micropatterned human IgG and rabbit IgG were immersed in antigen solutions with different concentrations. They are FITC-antihuman IgG solutions with concentrations of 1 ng/mL, 10 ng/mL, 0.1 μg/mL, 1 μg/mL and 10 μg/mL, TRITC-antirabbit IgG with concentrations of 1 ng/mL, 10 ng/mL, 0.1 μg/mL, 1 μg/mL and 10 μg/mL, solutions containing both FITC-antihuman IgG and TRITC-antirabbit IgG with concentrations of 1 ng/mL, 10 ng/mL, 0.1 μg/mL, 1 μg/mL and 10 μg/mL for each of them. All the immunoreactions were taken in 37 °C for 2 h. Samples after each step were rinsed by washing buffer three times to remove the physically adsorbed proteins.

**Characterization**

The morphologies of the samples were examined on a JEOL JSM-6700 field-emission scanning electron microscope (FE-SEM) with primary electron energy of 3 kV. Atomic force microscopy (AFM) images were obtained in a tapping mode at room temperature (20 °C) with a Digital Instruments Nanoscope IIIA using Si cantilevers purchased from DI and Nanosensor Co., Ltd. Ultraviolet–visible (UV–vis) mirror reflection spectra were obtained on a Shimadzu UV–3600 spectrophotometer. FL images were taken by an Olympus Fluoview FV1000 confocal fluorescence microscope with a 100× oil objective. Two lasers were used for each fluorescent molecule. For FITC, laser wavelength was 488 nm, laser transmittance was 60%. For TRITC, laser wavelength was 543 nm, laser transmittance was 40%. SERS measurements were performed on a Renishaw 1000 model confocal microscope Raman spectrometer equipped with a CCD detector and a holographic notch filter. Be-
fore SERRS measurement the substrates covered with immuno-complexes underwent an immersion in Ag colloid solution for 30 min, then rinsed by washing buffer for three times. Radiation of 514.5 nm from an air-cooled argon-ion laser was used for the SERRS excitation. The power at the sample position was 1.0 mW. The microscope attachment is based on a Leica DMLM system and a 50× objective was used to focus the laser beam onto a spot of approximately 1 μm in diameter. The accumulation time for each SERRS measurement was 30 s.

Results and discussions

With nanosphere lithography, micropatterns with alternate nanometer scale regions can be constructed [31–33]. In this work, we fabricated protein micropatterns using nanosphere lithography after some adjustment. The preparation steps are illustrated in Fig. 1. After hydroxization, salinization, aldehydization, the antibodies can be deposited on the substrate surfaces by strong chemical bonds. The PS spheres of 750 nm were employed as templates to create micropatterned surfaces. Monolayers of PS particles existed as mask, partially covering the surfaces of glass slides by the contact of them. Human IgG was introduced to fill the expose surface areas on the glass slides firstly. After removing the PS templates, rabbit IgG was introduced to occupy new released surfaces (covered by PS mask before) secondly. Then, alternate immunoglobulin micropatterns with two kinds of antibodies were formed and ready for successive immunoreactions.

The original PS templates were spherical and tangent to the substrate surfaces. Due to the geometry consideration very small contact areas between the PS spheres and substrate surfaces could...
be formed. Since the purpose of the experiments was to design alternative regions with multi-component proteins, it would be more convenient to detection all the proteins if the alternate regions had similar areas. So a strategy of deformation by heating the PS templates to create more contact areas was taken. The partially melted PS microspheres glued more areas on the substrates.

To investigate the influence of heating on the PS templates, experiments were carried out by increasing the heating time every 5 min, from 5 to 20 min. The heating temperature was fixed at 110°C. At this temperature, the melt of PS microspheres was relatively slow and a controllable sinter could be achieved. SEM images of PS monolayers with different sinter times are showed in Fig. 2. When the heating time was 5 min (Fig. 2a), the edges of PS particles became soft slightly and allied to each other. The PS particles maintained their spherical shapes. When the heating time was increased to 10 min (Fig. 2b) and above, the top-view shapes of the PS particles were changed from circular to hexagonal. Meanwhile, the spaces between PS particles decreased. Although long sinter time made the PS microspheres melt together on top-view, there were still capillary pores at the sides of the templates. These pores were accessible for protein molecules, which proved by successive modifications in antibody solutions.

Fig. 2(e–h) show AFM images of human IgG covered substrates after washing away the PS templates with different heating time. In AFM pictures, the highlighted net-like regions are proteins of human IgG, which have a height of 5 nm (see AFM section analysis in Fig. S1 in Supporting Information). The dark circular regions represent the bare glass substrates, which have different diameters (see Table S1 in Supporting Information). Different diameters were caused by the deformation of the PS mask with different heating time. So the area ratios of dark regions and highlighted regions can be tuned by changing the heating time. By employing heating time of 5, 10, 15 and 20 min, the corresponding area ratios were 1:9, 4:6, 5:5 and 6:4, respectively. The heating time of 15 min was selected inclusively for the subsequent experiments due to the experimental purpose on multi-component protein detections.

Fig. 2i illustrates the different degrees of deformation of PS microspheres. The contact areas between the PS templates and glass slides were changed with the deformation of PS microspheres gradually. The capillary pores at the sides of the PS templates were used as the tunnels for the deposition of antibodies.

After removing PS templates, the new released surfaces on the substrates were modified with the antigen of rabbit IgG. Thus dual-component surfaces with alternate immunoglobulin micro-patterns were fabricated. The obtained protein micropatterns can be used as chip-like substrates for immunoreactions diagnostics. Herein, FITC-antihuman IgG and TRITC-antirabbit IgG were chosen as the immune response agents. FL microscopy was employed to...
testify the immunoreactions. Fig. 3 shows the FL pictures of two kinds of immunocomplexes different concentration of both labeled IgGs. The existence of the circular black regions was testified by the immunoreactions of TRITC-antirabbit IgG and rabbit IgG. As shown in Fig. 3, ordered circular red regions were observed. The red regions were generated due to the illumination of the red FL of TRITC. In Fig. 3, the label molecules of FITC on antihuman IgG generated a green FL, which was supposed to cover the platforms constituted by human IgG because of the specific immune recognition. According to the precursor structures shown in the AFM image (Fig. 2g), the FL image should have ordered circular black regions due to the un-immune response areas of rabbit IgG. On the contrary to TRITC-antirabbit IgG composed pattern, the FITC-antihuman IgG fabricate a continuous green background, it’s difficult to distinguish circular black regions in FITC-antihuman IgG covered area. Another reason of such effect is the limited optical resolution of the FL microscopy; this is also the shortcoming of FL detection method. According to Fig. 3, when concentration went lower than 1 μg/mL, FL images were unable to distinguish these proteins. This FL picture precisely proved the success on constructing dual-component alternative immunoglobulin micropatterns and specific immune responses on these proteins.

Before SERRS measurement, a silver nanoparticle staining experiment was done by referring a similar method to our former published work [29]. UV–vis spectra were employed to prove the success of silver staining (see Fig. S2 in Supporting Information). The absorbance peak at 430 nm corresponds to the local surface plasma resonance of Ag nanoparticles, which have a great contribution to SERS signals [29]. The excitation line for Raman spectra was selected to be 514.5 nm, which match the allowed electronic transition of the dye molecule for both FITC and TRITC, and was also attribute to enhancement signals [28,29]. Fig. 4 demonstrates the SERS spectra of FITC-antihuman IgG (Fig. 4a) and TRITC-antirabbit IgG (Fig. 4b) when 100 μg/mL solutions were employed for each of them in the immunoreactions. A very good spectral resolution was obtained in the present experiments. It demonstrated that the SERS measurement had a great potential of in the quantification application.

In order to explore the sensitivity and quantification of SERS spectra, a series of mixture solutions, consisting with FITC-antihuman IgG and TRITC-antirabbit IgG, with the concentrations of 10 μg/mL, 1 μg/mL, 0.1 μg/mL, 10 ng/mL, and 1 ng/mL for each of them, were prepared and used for SERS measurements. Fig. 5 shows the resulting SERS spectra. The protein arrays prepared here have nanoscale size. However, microscope assembled on Raman spectrometer can focus the laser beam onto a spot of 1 μm in diameter, which means no matter the point laser irradiated, will cover both FITC-antihuman IgGs and TRITC-antirabbit IgG regions. Thus, SERS spectra contained FITC and TRITC signals simultaneously. The spectra included the signals from both FITC and TRITC. An observable detection limit was as low as 1 ng/mL, which was comparable to the FL method [7].

The SERS peaks at 1621 and 1649 cm\(^{-1}\), which were characteristic for FITC and TRITC, were selected to investigate the quantification application. The correlations of peak intensities and logarithmic concentrations were plotted in Fig. 6. Error bars was calculated by the intensities of 10 different spectral measurements. After curve fitting it can be seen that there was exponential increase for SERS signals when the concentration was higher than 1 μg/mL. Meanwhile, good linear relationships were observed in the concentration range where the fluorescence method was still able to distinguish these proteins. This FL picture precisely proved the success on constructing dual-component alternative immunoglobulin micropatterns and specific immune responses on these proteins.
low concentrations (from 1 ng/mL to 1 mg/mL) for both immuno-complexes (insets in Fig. 6). No interference was observed from inner- or interactions of different components. It is very important to obtain robust signals for biochemistry and biology because most of the bioassay systems have complex components and structures, which usually induce big interference on the detecting signals. Thus, it is hard to obtain quantification assays in situ in complex biomaterials. For example, the FL spectra usually suffer from the instability of spectral intensities when increasing the complexity of the components. However, SERRS spectra are seldom affected by components since it is a vibration spectroscopy. So the SERRS-based method is very suitable for measuring a large number of proteins. SERRS has great potentials in the bioanalysis of multi-component proteins.

Conclusions

A method for preparing nanometer scale protein micropatterns was introduced in this paper. These patterns contained alternate regions of two kinds of antibodies. The ratios of coverage areas on these two antibodies were controllable by changing the heating time of the PS templates. Immune responses were carried out as well as FL microscopy was done to testify the immunoreactions. A sensitive and quantitative analysis was done using SERRS spectroscopy. The detection limit was as low as 1 ng/mL, and good linear relationships were observed in low concentrations (from 1 ng/mL to 1 mg/mL). The results indicated that SERRS is a promising tool for micropattern-based protein detections, which had a wide range of applications in biosensors, clinical medicine and biochemistry.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.saa.2012.05.023.