Surface-enhanced fluorescent immunoassay on 2D silver nanotriangles array

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HIGHLIGHTS

- Silver nanotriangles array with long ranged periodicity was fabricated on the surface of glass wafer.
- High enhancement of the fluorescent intensity of the target antibodies was obtained by the silver nanotriangles array.
- High sensitivity and good reproducibility for antibodies detection was obtained by the 2D silver nanotriangles array.

GRAPHICAL ABSTRACT

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ABSTRACT

Long range ordered silver nanotriangles array was fabricated for surface-enhanced fluorescent immunoassay in this paper. By polystyrene (PS) microspheres based LB template method, the silver nanotriangle array with about 100 nm in height was constructed on the surface of glass slide. On the surface of Ag nanotriangles array, the immune reaction of antigens and labeled antibodies was carried out. Based on the interaction of fluorophores from antibodies with the plasmon resonance from Ag nanotriangles and the enrichment effect of this patterned array, 3.11 times enhancement of the fluorescent intensity of the target antibodies was obtained. According to the fitting curve of fluorescent intensities and logarithmic concentrations of labeled antibodies from 100 pg/mL to 10 µg/mL, it concludes that the limit of detection by this Ag nanotriangles array for immune complex is 100 pg/mL. Due to the advantages of high sensitivity, good reproducibility, and convenient fabrication, the 2D silver nanotriangles array could be an exciting platform for bioassays in proteomics, drug discovery and diagnostics.

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phenomenon. One important method to achieve fluorescence enhancement is to utilize metallic nanoparticles, which are known to change the emission of vicinal fluorophores [6,7]. Metallic nanoparticles can influence the fluorescent emission from the nearby molecules by the interaction of fluorophores with the plasmon resonance from metal particles, resulting in an increase in the radiative decay rate and stronger fluorescence emission [7–9]. Exactly control of the enhanced-fluorescent is essential for the successful achievement of high quality fluorescence spectrum, especially when fluorescence was used as a detection tool in analytical chemistry field [10].

The metal nanoparticle-enhanced fluorescence process can be influenced by many factors, such as the distance between molecule and nanoparticle, the material of nanoparticle, the size of nanoparticle and the shape of nanoparticle. While the fluorescence from molecules directly adsorbed on the surface of metallic nanoparticles would be strongly quenched [11,12], the intensity of fluorescence could be enhanced if the distance between fluorescent molecule and metal substrate satisfied the requirement of 5 nm < d < 20 nm [13–28]. Precisely keep the distance between fluorescent molecules and metallic nanoparticles is the key point for getting a maximum-enhanced fluorescence spectrum. Moreover, the feature of the substrate used in surface enhanced fluorescence (SEF) is also very important for the enhancement behavior. The material, size and shape of the nanoparticles (NPs) determine the properties of the localized surface plasmons it supports, which are collective coherent oscillations of the conduction band electrons in the NPs [19–23]. When the plasmon from NPs interacts with the fluorophores from surface molecule, its character will affect the fluorescence enhancement capability. Colloidal particles assemblies are easy and economical to fabricate and can feature locations of giant E-field enhancement [24,25]. However, by this method, it is difficult to control their resonance wavelengths, precise spatial locations, and field intensity values, which are crucial to quantitative analysis result when SEF was applied in analytical chemistry. If long range ordered nanostructured metal surfaces that combine high E-field enhancement with low on-chip and chip-to-chip variability could be fabricated, it would contribute a lot to the sensing fields of SEF. The need for reliable and easy-to-fabricate planar plasmonic substrates is well recognized and is reflected in significant theoretical and experimental activities aimed at developing rational design criteria and fabrication methods for NP-based structures and arrays [26–31].

In this manuscript long range ordered silver nanotriangles array was fabricated for surface-enhanced fluorescent immunoassay. Polystyrene (PS) microspheres based LB template method was applied to fabricate the silver nanotriangles array. Each unit of the array is a silver nanotriangle with about 100 nm in height. Due to the long range ordered periodicity of this array, when it works as a surface-enhanced fluorescent substrate, the reproducibility of the fluorescent spectrum observed in this ordered nanostructures is significantly better than those obtained from colloidal particles assemblies. Furthermore, this substrate combined with the primary antigen–antibody immune reaction was applied as an SEF based immunoassay platform. According to the size of rabbit IgG and TRITC-antirabbit IgG, the distance between fluorescent signal molecule and metallic surface was controlled at 4 nm to 12 nm, which is an ideal distance for SEF, in accordance with the optimal condition of maximum enhancement for the fluorophore is at 8 nm from the metal surface [32,33]. Based on the plasmon coupling effect and the enrichment effect of this substrate, 3.11 times of the fluorescent intensity of the target antibody was obtained, and the detection limit of it was 100 pg/mL. Due to the advantages of high sensitivity, good reproducibility, and convenient fabrication, the 2D silver nanotriangles array could be an exciting platform for bioassays in proteomics, drug discovery and diagnostics.

Experiments

Chemicals

Rabbit IgG, TRITC-antirabbit IgG and bovine serum albumin (BSA) were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO) and used without any further purification. Ag (99.99%) was purchased from Sinopharm Chemical Reagent Co. Ltd. All other chemicals were obtained from Beijing Chemical Reagents Co. (Beijing, China). The phosphate buffered saline (PBS) solution (0.01 M, pH 7.2) used to dilute rabbit IgG and TRITC-antirabbit IgG into different concentration containing 0.8% NaCl, 0.02% KH2PO4, 0.02% KCl and 0.12% Na2HPO4. 1% BSA in PBS was used as the blocking buffer, and the washing buffer was prepared by adding Tween 20 to the PBS solution (containing 0.05% Tween 20). All aqueous solutions were prepared using deionized water (DIW, 18 MQ) obtained from a Milli-Q system (Millipore S.A., Bedford, USA).

Fabrication of silver nanotriangles array

The method used here is according to a previous work reported by R.P. Van Duyne [34] with a little modification. Glass slide with 1 cm in length and 1 cm in width were pretreated by boiling piranha solution (30% H2O2: 98% H2SO4= 3:7; V: V) for 30 min to let the surface have a hydroxyl group layer. By LB method, 2 μm PS microspheres are self-assembled on the surface of the glass slide substrate to form a periodic array of it, working as a template for later experiment. Then, Ag was deposited on those substrates by a ZHD-300M2 high vacuum resistive evaporation coating machine. After Ag deposition, the substrates were sonicated in chloroform for 30 s to remove PS microspheres. After the PS templates were washed out, only ordered Ag nanotriangles were left on the surface of glass slide.

Construction of immune complex on the silver nanotriangle array

The as prepared ordered Ag nanotriangles on glass slides were submerged in 1 mL 100 μg/mL rabbit IgGs solution and then soaked in BSA solution to block the uncovered Ag surface. For immune complex, samples are separately immersed in 1 mL TRITC-antirabbit IgGs solution with concentration of 10 μg/mL, 1 μg/mL, 100 ng/mL, 10 ng/mL, 1 ng/mL, 100 pg/mL, 10 pg/mL and 1 pg/mL. All the immunoreactions have been taken at 37 °C for 2 h. After each step substrates were rinsed by washing buffer three times to remove the physically adsorbed proteins.

Instruments

The morphologies of the silver nanotriangle array are examined by a JEOL JSM-6700 field-emission scanning electron microscope (FE-SEM) with primary electron energy of 3 kV. Atomic force microscopy (AFM) images are 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 are obtained on a Shimadzu UV-3600 spectrophotometer. Fluorescent images are taken by an Olympus Fluoview FV1000 confocal fluorescence microscope with a 60× oil objective. For TRITC, laser wavelength is 515 nm. Laser voltage and laser transmittance are 600 V and 40% respectively.
Results and discussions

Characterizations

Lithography technology was applied to fabricate a long range ordered Ag nanotriangles array. After the Ag nanotriangles array was constructed, immunoreaction was carried out on the surface of each Ag nanotriangle. Due to the interaction of fluorophores from the antibody with the plasmon resonance from the Ag nanotriangle, enhanced fluorescence was obtained, which make it possible for high sensitivity detection of labeled proteins. Each steps of the experiment is shown in Fig. 1. Glass slides were initially cleaned to remove the impurities before 2 μm PS microspheres self-assembled on them. Then, a layer of Ag was deposited on the template by vacuum evaporation. Not only the surface of PS spheres, but also the interspace between each PS spheres was covered with Ag film. After the PS templates were washed away by sonication, Ag nanotriangles on glass slide remained. Then, on this as prepared long range ordered Ag nanotriangles array, antigens and BSA are absorbed. When immune response took place, the surface was covered with labeled antibodies. Confocal fluorescence microscope is chosen to characterize the structure of protein arrays and quantitative analysis of them.

UV–vis absorption spectra of each step are shown in Fig. 2. After this PS microspheres self-assembled on the surface of glass slides, there arise two absorptions at 450 nm and 650 nm (Fig. 2a), corresponding to the diffraction and Bragg diffraction of incident light. After deposition of Ag film, more absorption peaks appeared in UV–vis spectra (Fig. 2b). Band at 380 nm refers to the interband transition of Ag. Other bands are caused both by the surface plasmon resonance (SPR) of Ag film and the multistage diffractions of incident light. The inset image shows the magnified spectra after removing template procedure. Only one band at 440 nm, referring to the surface plasmon resonance of ordered Ag nanotriangles, remains.

Fig. 1. Procedures of silver nanotriangles array fabrication and immune complex formation on it.

Fig. 2. UV–vis absorption spectra of monolayer PS microparticles (a), vacuum deposition Ag film on the PS template (b), the remained Ag nanotriangles array after removing PS microparticles (c), Ag nanotriangles array modified with rabbit IgG and BSA blocking (d), Ag nanotriangles array supported protein pattern immune response with TRITC-antirabbit IgG with concentration of 1 μg/mL (e), inset image is the magnification of spectra (c and d).

Herein, confocal fluorescence microscope with 60× oil objective is used for TRITC-antirabbit IgGs detection. Due to the resolution restriction of the optical microscope, only arrays at microscale can emerge patterned images. Thus, micron scale PS spheres must be chosen as template. That’s the reason why PS spheres with the size of 2 μm were selected in this experiment. SEM images of every step are shown in Fig. 3. Long range ordered PS microspheres were firstly assembled on the surface of glass substrate. Then vacuum deposited Ag layer covered both the surface of PS microspheres and the interspatial room between them. After removing the PS template, Ag nanotriangles in the interspace between PS
microspheres remained, constructing a long range patterned array. The nanotriangles in the pattern are with a height in 100 nm and a length of three equilateral edges in 1 μm. Even after immune response, this ordered pattern maintained.

Generally, proteins absorb on Ag surface through three ways, which are covalent bond, electrostatic interaction, and hydrogen bond. In this experiment, the nitrogen-atoms from amino groups in biomolecules can form covalent bond with the patterned Ag array surface atoms, providing the possibility to make proteins selectively absorbed only on the surface of Ag nanoarrays, rather than on the bare glass surface. As can be seen from the AFM images in Fig. 4, few biomolecules can be found in blank areas of the Ag array. Through section analysis, the heights of patterned Ag nanotriangles and labeled protein nanoarray are determined to be 102 nm and 114 nm respectively, indicating that the total height of the immune complex is 12 nm. The height of immunoglobulin G has already been demonstrated to be 4 nm [35]. That means the distance between the fluorophore in TRITC-antirabbit IgG and Ag surface ranges from 4 nm to 12 nm, and the average distance is 8 nm, which is in accordance with the optimal condition of maximum enhancement [32,33]. In this way, without any other complicated technique, a well enhanced fluorescence could be obtained.

**Laser selection**

Herein, TRITC was used as the signal molecule, whose maximum excitation and emission wavelengths are 555 nm and 580 nm respectively. Usually, the excitation laser wavelength for TRITC is 543 nm, at which there is not any fluorescence exit in patterned Ag nanotriangles. However, patterned Ag nanotriangles could act as a mirror to reflect the incident laser back to CCD and influence the results. In order to reduce the interference of this reflection and enhance the signal to noise ratio of the measurements, laser at 515 nm is chosen. Although excitation laser is not

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**Fig. 3.** SEM images of PS template (a), PS template after Ag film deposition (b), remained Ag nanotriangles array after removing PS microspheres (c), rabbit IgG absorbed on Ag nanotriangles array (d), 1 μg/mL TRITC-antirabbit IgG immunity on the Ag nanotriangles array (e).

**Fig. 4.** AFM section analyses of patterned Ag nanotriangles array (a), 1 μg/mL TRITC-antirabbit IgG immune on the Ag nanotriangles array (b).
located at the maximum excitation line of TRITC, the laser is still in the range of excitation peak, and could achieve the excitation of TRITC. Different lasers are used to irradiate the same area of patterned Ag nanotriangles (Fig. S1). It clearly shows that, under laser at 543 nm, the fluorescence image of patterned Ag nanotriangle exhibits brighter color comparing to image of it under laser at
515 nm. The phenomenon is because that the laser at 543 nm is quite near the signal acquisition area of the equipment, which is 560 nm–660 nm, and the patterned Ag nanotriangles could reflect these light back to the CCD. However, laser 515 nm is far from signal collection region of the equipment, and Ag nanotriangles array reflect little background laser. For further illustration, 3D intensity analyses in one cycle under different laser are shown in Fig. 5. After changing the laser from 543 nm to 515 nm, the detector collects quite weaker signals. For such reason, in the later experiments, excitation laser wavelength for the fluorescent images of labeled proteins is chosen at 515 nm.

Qualitative analysis of labeled proteins

Before immunoreaction took place on the surface of Ag nanotriangles, the still uncovered surface of Ag nanotriangles was blocked by BSA after the antigens was absorbed on the surface of Ag. After blocking, antibodies can only specifically absorb on the surface of these orderly arrays through immunoreaction. By adjusting the concentration of labeled antibodies, different fluorescent images are got, which is shown in Fig. 6e–l. For comparison, nanoscale antibody arrays are fabricated on glass slides without Ag array supported. Samples are prepared in the same way as our previous work [36,37]. Initially, antigen nanoarrays on the surface of aldehyde glass slide were prepared, and blocked by BSA. Then immune responses were taken under antibody solution with different concentrations. Fluorescent images of the substrates are taken under the same condition of antibodies on patterned Ag nanotriangles array (Fig. 6a–d). It can be clearly seen from Fig. 6 that when the concentration of antibodies is lower than 1 μg/mL, labeled antibody nanoarrays constructed on glass slides could not show any patterns. However, protein arrays supported by Ag nanotriangles have the ability to exhibit clearly patterns even concentration reach 1 pg/mL, and the intensity of the fluorescence would become weak when the concentration of antibody decreased. The phenomenon is attributed to the beneath patterned Ag layer, the plasmon resonance from which interacted with the fluorophores and thus enhanced the fluorescent emission from the antibodies. For further

Fig. 7. Selected regions of fluorescence images of TRITC-antirabbit IgG on patterned Ag nanotriangles array with concentration of 10 μg/mL (a), 1 μg/mL (c), 100 ng/mL (e), 10 ng/mL (g), 1 ng/mL (i), 100 pg/mL (k), 10 pg/mL (m), 1 pg/mL (o). And 3D fluorescent intensity analyses of the selected regions with concentration of 10 μg/mL (b), 1 μg/mL (d), 100 ng/mL (f), 10 ng/mL (h), 1 ng/mL (j), 100 pg/mL (l), 10 pg/mL (n), 1 pg/mL (p).
investigation, 3D analyses of the fluorescent intensity of one cycle and the intensities of different protein logarithmic concentrations are shown in Fig. 7 and Fig. S2. In order to increase the representativeness and the accuracy of this data analysis, each data point in Fig. S2 represent the average value from one cycle, and the errors are computed by 10 cycles. When the concentration is lower than 100 pg/mL, the fluorescent intensities became constant, which indicates that the intensity came from the patterned Ag array reflection rather than the fluorophores in antibodies. According to the fitting curve of fluorescent intensities and logarithmic concentrations from 100 pg/mL to 10 µg/mL, which is shown in Fig. 8, it can be concluded that the limit of detection of this method is 100 pg/mL. In Fig. 8, the error bars were calculated by the average intensities of ten cycles and the correlation coefficient can reach 0.9976.

**Enhancement calculation**

The advantage of the patterned Ag nanotriangles array for immunoassay is that it can enhance the fluorescence of the immune complex on it. Such enhancement result derives from not only the coupling of fluorophores with the plasmon resonance from Ag nanotriangles, but also the enrichment effect of it. When the same amount of antibodies was used in the experiment, the density distribution of antibodies for ordinary Ag film and patterned Ag nanotriangles array is different. Due to the high average amount of antibodies on Ag nanotriangles, the fluorescence intensity from array substrate was much stronger than that from Ag film. The fluorescent images and 3D analyses of ordinary Ag film and after immune response of TRITC-antirabbit IgG are shown in Fig. S3. Histogram of fluorescent intensity analysis is displayed in Fig. 9.

The ratio of TRITC fluorescent intensity on ordinary Ag film to glass slide is calculated by the following formula.

\[
I_1 = \frac{I_{\text{labeled molecules on ordinary Ag film}}}{I_{\text{labeled molecules on glass slide}}} = 1.38
\]

Calculation results shows ordinary Ag film gives 1.38 times enhancement of TRITC-antirabbit IgGs. The ratio of biomolecules fluorescent intensity on patterned Ag nanotriangles array to ordinary Ag film can be calculated by the following formula.

\[
I_2 = \frac{I_{\text{labeled molecules on patterned Ag film}}}{I_{\text{labeled molecules on ordinary Ag film}}} = 2.25
\]

The result indicates that, patterned Ag film can achieve 2.25 times enhancement of labeled antibodies comparing to ordinary Ag film.

\[t_1 = \frac{I_{\text{labeled molecules on ordinary Ag film}}}{I_{\text{labeled molecules on glass slide}}} = 1.38\]

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The result indicates that, patterned Ag film can achieve 2.25 times enhancement of labeled antibodies comparing to ordinary Ag film.
binding efficiency of antibody and antigen could not be 100%. Based on the enhancement from plasmon coupling effect and the enrichment effect of the Ag nanotriangle array substrate, the total enhancement factor of the TRITC labeled antibodies is 3.11, a product of \( t_1 \) multiplied by \( t_2 \).

**Conclusions**

In this paper, long range ordered silver nanotriangles array was fabricated for surface-enhanced fluorescent immunoassay. Due to the long range ordered periodicity of this array, when it works as a surface-enhanced fluorescent substrate, the reproducibility of the fluorescent spectrum observed on this platform is improved. After immunoassay was carried on the surface of this Ag nanotriangles array, 3.11 times enhancement of the fluorescent intensity of the target antibodies was obtained, which is because of both the interaction of fluorophores from antibodies with the plasmon resonance from Ag nanotriangles and the enrichment effect of this patterned array. According to the fitting curve of fluorescent intensities and logarithmic concentrations of labeled antibodies from 100 pg/mL to 10 \( \mu \)g/mL, it concludes that the limit of detection by this Ag nanotriangles array for immune complex is 100 pg/mL. Due to the high sensitivity, good reproducibility, and convenient fabrication advantages, the 2D silver nanotriangles array could be a potential platform for not only immunoassay, but also other molecular-fluorescence-based technologies.

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**Appendix A. Supplementary material**

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.saa.2014.01.060.

**References**


