

Metal-enhanced fluorescent detection for protein microarrays based on a silver plasmonic substrate†

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This paper presents an ultrasensitive fluorescent detection method through fabricating a silver microarray substrate. Silver nanoparticles (AgNPs) and Ag@Au core-shell nanoparticles with different sizes were first synthesized by a seed-mediated growth method and the metal-enhanced fluorescence of these nanoparticles on different fluorescent dyes was investigated. The results indicated that AgNPs could act as a versatile and effective metal-enhanced fluorescence material for various fluorophores, whereas the enhanced fluorescence from Ag@Au was limited only to certain fluorophores. When the AgNPs were functionalized with aptamers and fluorescent dyes, a good analytical performance for simultaneous detection of human IgE and platelet-derived growth factor-BB (PDGF-BB) could be obtained. AgNPs were not only used as detection tags but also used to fabricate the plasmonic microarray substrate to further enhance the sensitivity of fluorescent detection. As a result, a linear response to PDGF-BB concentration was obtained in the concentration range of 16 pg mL⁻¹ to 50 ng mL⁻¹, and the detection limit was 3.2 pg mL⁻¹. In addition, the AgNP modified plasmonic microarrays showed remarkable recovery and no significant interference from human serum when applied to 2 ng mL⁻¹ PDGF-BB concentration. The plasmonic microarray substrate demonstrated both high specificity and sensitivity for protein microarray detection and this novel approach has great potential for ultrasensitive detection of protein biomarkers in the bio-medical field.

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Introduction

The protein microarray-detection system provides an attractive bioanalytical platform with enormous potential for biomedical analysis, proteome research, biomarker discovery and validation, disease diagnosis and personalized medicine.^{1,2} Microarray detections usually require low sample consumption, with high-throughput for protein analysis and have great potential for miniaturization.³ Detection techniques used for microarray analysis are generally classified into two categories:⁴ the label-free detection techniques such as surface plasmon resonance imaging (SPRi) and atomic force microscopy (AFM); and the labeling detection techniques such as radioisotope methods and fluorescence imaging. The fluorescence detection method is widely used in biotechnology and biomedical diagnosis due to its high level of sensitivity.^{5,6} Fluorescence detection has also been the preferred choice for data quantification in biomedical microarrays.⁷ Therefore, improving the fluorescence sensitivity is a critical component in the development of protein

microarray systems. There are only two approaches to improve the fluorescence sensitivity of protein microarrays: one is enhancing the detection performance of fluorescent probes. Here, fluorescent nanoparticles are used as probes to enhance the sensitivity of protein microarrays.^{8–11} For example, bio-conjugated fluorescent zeolite L nanocrystals have been used as labels in protein microarrays to increase the sensitivity and decrease the detection limit to the femtomolar range.¹⁰ Metal-enhanced fluorescence developed to enhance the quantum yield and photo-stability of the nearby fluorophore, also prevents the auto-fluorescence of the sample and therefore promotes further reduction in the detection limit.^{12,13} The metal-enhanced fluorescence (MEF) is attributed to the localized surface plasmon resonance (LSPR) of metallic nanostructures, which results in an enhanced electromagnetic field when excited resonantly with photons.^{14,15} The LSPR is strongly dependent on the nature of the metal, the size, and the shape of metallic nanostructures.^{16,17} Modulating the size and shape of the noble metals will change the LSPR, which results in different fluorescence enhancement effects on nearby fluorescent dyes. Therefore, designing and synthesizing biomolecule-functionalized nanostructures with optimal fluorescence enhanced effect will improve the sensitivity of fluorescence analysis, which will facilitate the development of biomedicine and biotechnology. Nanostructures of noble metals (mainly gold and silver) have been used for enhancing the fluorescence

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† Electronic supplementary information (ESI) available: Additional information such as the synthesis of AgNPs and Ag@Au (Fig. S1), the amount of reagents (Table S1), optimization for functionalization of AgNPs-d (Fig. S2), SEM images (Fig. S3) and contrast data (Fig. S4). See DOI: 10.1039/c3an01875j

intensity of fluorescent molecules to improve the sensitivity of fluorescent biosensors or bioassays.^{9,18–22} Gold nanoparticles with good chemical stability can be easily synthesized with uniform size and modified with biomolecules whereas silver nanoparticles have a narrow surface plasmon band and high scattering efficiency.²³ Core-shell nanostructures are becoming more attractive over monometallic nanostructures due to their improved optical, electronic and catalytic properties.^{24,25} Paria²⁶ extensively studied the synthesis mechanisms, characterization and applications of core-shell nanoparticles. The seed-mediated method has been used to synthesize core-shell nanocomposites with different sizes, shapes and clearly shows different LSPR.^{27,28} In this seed mediated method, with small nanoparticles as the core, with the reducing and oxidizing agent, Ag⁺ or Au³⁺ is reduced to form a silver shell or gold shell with different thicknesses.²⁹ The nanomaterials synthesized by the seed-mediated method can be used as tags for fluorescence enhancement to enhance the sensitivity of fluorescence.

The other approach to improve fluorescence sensitivity is the fabrication of a special substrate, such as a metal enhanced substrate for fluorescence enhancement. Fluorescent nanoparticle probes with large sizes tend to increase the nonspecific absorption on the solid support and increase the chances of aggregation in solution. However, the nanomaterials used as a substrate for metal-enhanced fluorescence on neighboring fluorescent probes may avoid these limitations due to large particle sizes. The metal plasmonic substrate has been found to enhance the fluorescence sensitivity, for example, a gold plasmonic substrate has been used for multiplexed protein microarrays with femtomolar sensitivity and broad dynamic range.¹⁸ In addition, nanoparticle coupled to nanoparticle can further enhance the electromagnetic field, which results in the enhanced fluorescence effect.^{30–32} Similarly, nanoparticles coupled to films also enhances the electromagnetic field, which was used to enhance the sensitivity of surface-enhanced Raman scattering (SERS).^{33,34} Yi³³ constructed a composite plasmonic substrate, in which silver nano-cubes coupled with silver ground plane to enhance the SERS signals. Kang³⁴ developed a gold nanoparticle-on-wire system to enhance SERS signals for detecting multiplex DNA with high specificity and sensitivity. However, a substrate nanostructure together with probe nanoparticle coupling with enhanced electromagnetic field for fluorescence microarrays has rarely been reported. Therefore, developing a sensitive nanoparticle-on-nanostructure system for protein microarrays is an important field to exploit. We focused on fabricating new fluorescent tags on plasmonic substrates to increase the fluorescence sensitivity.

To fabricate highly effective fluorescent assays for protein microarrays. The AgNPs and Ag@Au nanocomposites were synthesized and the fluorescence enhancement effect on different fluorescent molecules is investigated. The fluorescence properties of AgNPs and Ag@Au are reported. Detection tags based on AgNP-d were synthesized through self-assembly and applied for the simultaneous detection of IgE and PDGF-BB. The optimized AgNPs were then used to design a sensitive and specific plasmonic microarray substrate. The plasmonic microarray substrate was used to detect PDGF-BB.

The linear range response and limit of detection were established using PDGF-BB.

Experimental

Materials and reagents

Aldehyde-modified slides (Shanghai Biotechnology Co. Ltd), silver nitrate (AgNO₃), sodium borohydride (NaBH₄), sodium L-ascorbate, polyvinylpyrrolidone (MW, 40 000), and hydroxylamine hydrochloride (NH₂OH·HCl) were purchased from Sigma Aldrich (St. Louis, MO, USA), and chloroauric acid (HAuCl₄·3H₂O) (Sinopharm Chemical Reagent Co., Ltd, China), PDGF-BB (R&D System, Minneapolis, MN), anti-human PDGF-BB (Abcam, USA), anti human IgE produced in goat (Sigma Aldrich), IgE kappa, myeloma, and Human (United States Biological, USA) were used. Normal human serum (Zhongkechenyu Biotech, Beijing, China), phosphate buffered saline (PBS) (Shanghai Sangon Biotechnology Co.), 1× PBS (137 mmol L⁻¹ NaCl, 2.7 mmol L⁻¹ KCl, 10 mmol L⁻¹, Na₂HPO₄·12H₂O, 2 mmol L⁻¹ KH₂PO₄, pH 7.4), 0.1 M PBS (0.1 M NaCl + 0.1× PBS), 0.1 M PBST (0.1 MPBS + 0.05% Tween-20), and 0.2 M PBST (0.2 M NaCl + 0.1× PBS + 1 mM MgCl₂) were used. All the synthetic oligonucleotides used in this study were purchased from Shanghai Sangon Biotechnology Co. The oligonucleotides were derived with an alkyl thiol group at the 5' terminus and their sequences were as follows:

TAMRA (carboxytetramethylrhodamine), ROX (6-carboxyl-X-rhodamine), Cy3 (cyanine 3), Cy5 (cyanine 5).

5'SH-oligo(d)A₂₄-TAMRA: 5'SH-AAAAAAAAAAAAAAAAAAAAAAA
AAA-TAMRA,

5'SH-oligo(d)A₂₄-ROX: 5'SH-AAAAAAAAAAAAAAAAAAAAAAA
ROX,

5'SH-oligo(d)A₂₄-Cy3: 5'SH-AAAAAAAAAAAAAAAAAAAAAAA
Cy3,

5'SH-oligo(d)A₂₄-Cy5: 5'SH-AAAAAAAAAAAAAAAAAAAAAAA
Cy5,

5'SH-oligo(d)A₁₅: 5'SH-AAAAAAAAAAAAAAAAAAAAA,

5'SH-oligo(d)T₁₅: 5'SH-TTTTTTTTTTTTTTTTTT,

Apt-PDGF: 5'SH-AAAAAAAAAAAAAAAAAATACTCAGGGCACTGC
AAGCAATTGTGGTCCCAATGGGCTGAGTA,

Apt-IgE: 5'SH-AAAAAAAAAAAAAAAAAGGGGCACGTTTATCCGT
CCCTCCTAGTGGCGTGCCCC,

Za-1: 5'SH-AAAAAAAAAAAAAAAAAGGGTGCTCCCCCTAGTGG,

Za-2(A): 5'SH-AAAAAAAAAAAAAAAAACCACTAGGGGGAGCACCC,

Za-2(T): 5'SH-TTTTTTTTTTTTTTTTCCACTAGGGGGAGCACCC.

Apparatus

The absorption for LSPR spectra was measured on a UV-vis spectrophotometer (Shimadzu UV3600, Japan), fluorescence spectra of 5'SH-oligo(d)A₂₄-Cy3 and 5'SH-oligo(d)A₂₄-TAMRA and 5'SH-oligo(d)A₂₄-ROX and 5'SH-oligo(d)A₂₄-Cy5 were collected on a spectrofluorometer (RF-5301PC, Shimadzu, Japan). The immunological arrays were made by using a Smart Arrayer (Beijing CapitalBio Co. Ltd, China) and scanned by using a Luxscan-10K/A Microarray Scanner (532 nm laser source for TAMRA, ROX and Cy3, and 635 nm laser source for Cy5, Beijing CapitalBio

Co. Ltd, China). A scanning electron microscope (SEM) and a transmission electron microscope (TEM) (JEM-200CX, Japan) were used for collecting SEM and TEM images.

Preparation of nanoparticles

Silver nanoparticles (AgNPs-a) were prepared using our published method.^{9,35} AgNPs and Ag@Au nanoparticles with different sizes were synthesized according to the published method with some modification.²⁹

Preparation of fluorescent dye-functionalized AgNPs-a

The Cy3-functionalized AgNPs-a were prepared by mixing 1 mL AgNPs-a with the following oligonucleotides; SH-oligo(d)A₂₄-Cy3 (25 μ L, 10 μ M), SH-oligo(d)A₁₅ (25 μ L, 10 μ M), Apt-IgE (25 μ L, 10 μ M) and Za-1 (25 μ L, 10 μ M), the preparation procedure is as described in our published work.^{9,35} The mixture was left to stand for 18 h, then, 1 $\times$ PBS and NaCl were added to bring the total NaCl concentration to 0.2 M. After standing for 48 h, the solution was centrifuged at 15 000 rpm (3 times), at 15 min/time. The precipitate was re-dispersed in 0.2 M PBSM. The Cy5-functionalized AgNPs-a were prepared by mixing 1 mL AgNPs with SH-oligo(d)A₂₄-Cy5, SH-oligo(d)A₁₅, Apt-IgE and Za-1. ROX-functionalized AgNPs were prepared by mixing 1 mL AgNPs-a with SH-oligo(d)A₂₄-TAMRA, SH-oligo(d)A₁₅, Apt-IgE and Za-1. TAMRA-functionalized AgNPs-a were prepared by mixing 1 mL AgNPs-a, with SH-oligo(d)A₂₄-ROX, SH-oligo(d)A₁₅, Apt-IgE and Za-1. Scanning electron microscopy (SEM) was used for collecting images.

Preparation of oligonucleotide-functionalized nanoparticles

Five kinds of AgNPs were modified by Za-2(A) (25 μ L, 10 μ M) and SH-oligo(d)A₁₅ (75 μ L, 10 μ M), named Tag-A, Tag-B, Tag-C, Tag-D, and Tag-E, in addition, three kinds of Ag@Au were modified by Za-2(T) (25 μ L, 10 μ M) and SH-oligo(d)T₁₅ (75 μ L, 10 μ M), named Tag-F, Tag-G, and Tag-H. The other step was the same as preparation of fluorescent dye-functionalized AgNPs-a.

Optimizing the nanoparticles for detection tags

Human IgE was spotted on the aldehyde-modified slides by using a Smart Arrayer, fluorescent dye-functionalized AgNPs-a (Cy3-functionalized AgNPs-a, or Cy5-functionalized AgNPs-a, or ROX-functionalized AgNPs-a, or TAMRA-functionalized AgNPs-a) were first reacted with the IgE sample for 1 h. After washing, different tags (Tag-A, or Tag-B, or Tag-C, or Tag-D, or Tag-E, or Tag-F, or Tag-G, or Tag-H) were added on the slides, left for 1 h and then subjected to the same washing step as before. Then the arrays were scanned and the data were collected by using a Luxscan-10K/A Microarray Scanner.

Preparation of Tag-A

Tag-A was prepared by mixing 1 mL AgNPs-d with SH-oligo(d)A₂₄-TAMRA (25 μ L, 20 μ M), SH-oligo(d)A₁₅ (25 μ L, 20 μ M), Apt-IgE (12.5 μ L, 20 μ M), Apt-PDGF (12.5 μ L, 20 μ M), and Za-1 (25 μ L, 20 μ M). The rest of the steps were the same as in the preparation of fluorescent dye-functionalized AgNPs-a.

Simultaneous detection of IgE and PDGF-BB with Tag-A

0.1 mg mL⁻¹ of anti-IgE and anti-PDGF-BB antibodies were spotted on the aldehyde modified slide and made to stand for 2 h. The slide was then blocked by 10 mg mL⁻¹ BSA for 1 h and washed with 0.1 M PBST three times. Then 50 ng mL⁻¹ IgE and PDGF-BB were added separately for 1 h, after washing, Tag-A was added and made to stand for 1 h, followed by washing, then the prepared slide was scanned using the Luxscan-10K/A Microarray Scanner.

Preparation of Tag-B and Tag-C

Tag-B was prepared by mixing AgNPs-a with SH-oligo(d)A₂₄-TAMRA (50 μ L, 10 μ M) and Apt-PDGF (50 μ L, 10 μ M), and Tag-C was prepared by mixing AgNPs-d with SH-oligo(d)A₂₄-TAMRA (50 μ L, 10 μ M) and Apt-PDGF (50 μ L, 10 μ M), the preparation method was the same as Tag-A.

Preparation of antibody-AgNPs

200 μ L of AgNPs-a (or AgNPs-d) was mixed with 0.1 mg mL⁻¹, 20 μ L antibody at 37 $^{\circ}$ C for 2 h with gentle shaking, then the antibody-AgNPs-a and antibody-AgNPs-d were centrifuged at 15 000 rpm, 15 min/time. The precipitates were washed with 1 mg mL⁻¹ BSA and re-dispersed in 20 μ L, 1 mg mL⁻¹ BSA.

Optimizing the plasmonic properties of the substrate

0.1 mg mL⁻¹ anti-PDGF-BB antibody, antibody-AgNPs-a and antibody-AgNPs-d were spotted on the aldehyde slide and immobilized for 2 h at 37 $^{\circ}$ C. After blocking for 1 h by 10 mg mL⁻¹ BSA, the slides were washed with 0.1 M PBST (3 times), at 5 min/time. Then PDGF-BB was added for 1 h followed by the washing step as mentioned before. Finally, Tag-B (or Tag-C) was added to bind with the protein for 1 h. A Luxscan-10K/A Microarray Scanner was used to scan the slide and collect data. In addition, the same slide was also used for scanning electron microscope image measurements.

Developed strategy for detection of PDGF-BB on microarrays

Antibody-AgNPs-d were spotted on the aldehyde-modified slide and immobilized for 2 h at 37 $^{\circ}$ C. After blocking for 1 h by 10 mg mL⁻¹ BSA, the slide was washed with 0.1 M PBST (3 times), at 5 min/time. For the determination of linear relationship, the PDGF-BB concentrations from 50 ng mL⁻¹ to 3.2 pg mL⁻¹ were applied to the prepared slide for 30 min. For the recoveries, 2 ng mL⁻¹ PDGF-BB was added to normal human serum (10% serum, 2% serum, 1% serum) for 30 min, then Tag-B was added for 30 min, followed by the rest of steps stated above.

Results and discussion

Optimizing the nanoparticles for metal-enhanced fluorescence

Silver nanoparticles of about 20 nm diameter were used as seeds to synthesize larger AgNPs and Ag@Au nanoparticles with different localized surface plasmon resonance (LSPR) for enhanced fluorescence. The amount of reagents for synthesis is

shown in Table S1.[†] Depending on the amount of reducing agent (sodium L-ascorbate, hydroxylamine hydrochloride) and oxidizing agent (silver nitrate, chloroauric acid), various colors of AgNPs and Ag@Au were synthesized (Fig. S1B[†]). The solutions of a–d and f–h were clear; however, precipitates were easily produced in solution e. Therefore, the concentrations of oxidizing and reducing agents have to be controlled. The seed-mediated growth method was simple, rapid and versatile as for nanoparticles, by which AgNPs of different LSPR properties could be easily obtained. The functionalization of nanoparticles would facilitate the applications of nanoparticles in biotechnology and biomedicine. The synthesis of biomolecule-functionalized nanomaterials can be achieved by four approaches: physical adsorption of sulfhydryl groups, electrostatic binding of proteins and nanomaterials, covalent coupling by carboxyl or amino groups of nanomaterials and specific affinity interaction.³⁶ The AgNPs and Ag@Au can be functionalized with different oligomers by self-assembly of sulfhydryl. The oligo(d)A and oligo(d)T were used as spacers and were separately used to stabilize the AgNPs and Ag@Au. These spacer oligonucleotides also increase the yield of tags as poly-A has a better adsorption ability to AgNPs and also stabilizes the nanoparticles more effectively than other polynucleotides (C,G,T).^{37–39} Thus, Za-2(A) and SH-oligo(d)A₁₅ were used to bind with AgNPs through the sulfhydryl group (SH) at the 5' terminus to prepare Tag-A, Tag-B, Tag-C, Tag-D, and Tag-E, while Za-2(T) and SH-oligo(d)T₁₅ were chosen to bind with Ag@Au to prepare Tag-F, Tag-G, and Tag-H because of the higher surface coverage of ploy-thymine(T) on gold nanoparticles.³⁹ The fabricated tags have different sizes (Fig. S1C[†]), and they were estimated as $D_a = 21.2 \pm 6.3$ ($N = 92$), $D_b = 24.9 \pm 7.5$ ($N = 76$), $D_c = 28.9 \pm 6.5$ ($N = 102$), $D_d = 55.9 \pm 12.5$ ($N = 100$), $D_f = 26.5 \pm 6.8$ ($N = 93$), $D_g = 40.9 \pm 8.1$ ($N = 83$), $D_h = 57.2 \pm 10.5$ ($N = 169$), respectively.

Silver and gold nanoparticles are good candidates for metal-enhanced fluorescence, especially gold nanoparticles, since they have better chemical stability than silver nanoparticles, and can be easily modified and bound by biomolecules. Alternatively, gold nanoparticles are commonly regarded as ultra-efficient quenchers.¹² To explore the most optimal metal nanoparticles for enhanced fluorescence detection of proteins, the enhanced factors of AgNPs and Ag@Au nanoparticles on different fluorescent dyes were estimated based on our reported method,⁹ the distance between fluorescent dyes and nanoparticles was constant (8 nm), shown in Fig. 1A. The results showed that all tags could enhance the fluorescence intensity of Cy3 (cyanine 3)-functionalized AgNPs-a, the highest enhanced effect was attained from Tag-D (7.0-fold). Tag-E also exhibited a relatively high enhanced effect at 4.1-fold. Tag-A, B, and C showed similar enhanced effects as 2.7-fold, 2.1-fold and 2.9-fold, respectively (Fig. 1B). The fluorescence intensity would increase with the size of the non-aggregated nanoparticles; Tag-D was the largest one without any aggregation or precipitation during the experiment. The largest particle Tag-E aggregated easily and could only be used with strong ultrasonic processing. Additionally, the LSPR spectra of Tag-D showed better overlap with the Cy3 spectrum (Fig. 1D). This spectra overlap could explain the enhanced mechanism of tags on Cy3. The effect of

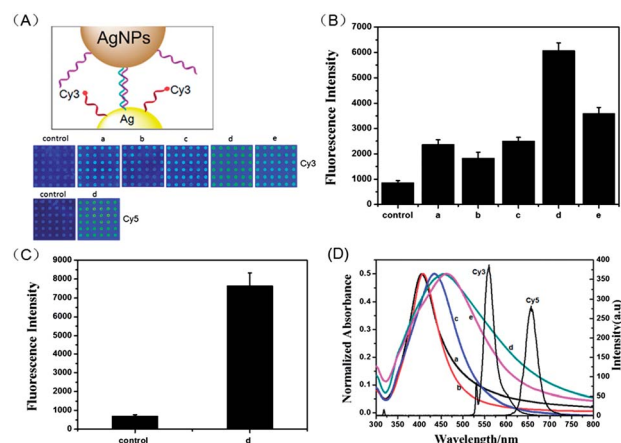


Fig. 1 (A) Schematic illustration of AgNPs with different sizes attached to dye-functionalized AgNPs-a by hybridization of oligomer (up), and fluorescence images of dye-functionalized AgNPs-a hybridized with AgNPs (down). (B) Fluorescence intensity of Cy3-functionalized AgNPs hybridized with AgNPs. (C) Fluorescence intensity of Cy5-functionalized AgNPs-a hybridized with AgNPs. (D) LSPR spectra of AgNPs and emission spectra of Cy3 and Cy5.

Tag-D on fluorescent dye Cy5 (cyanine 5) was also investigated, and results showed an enhancement effect of 10.8-fold for Cy5 which could also be attributed to the spectrum overlap (Fig. 1D). The increased metal enhanced fluorescence opens more possibilities for ultrasensitive detection in bio-analytical fields.

The fluorescence enhanced effect of Ag@Au on Cy3 and Cy5 was also investigated to explore the regularity of fluorescence enhancement. The experiments were performed under the same conditions as before and the results are shown in Fig. 2. The fluorescence enhanced effects of Tag-F, Tag-G, Tag-H for Cy3 were 1.1-fold, 1.7-fold, 2.2-fold (Fig. 2A) and 3.1-fold, 10.7-fold, 14.3-fold for Cy5 (Fig. 2B), respectively. The fluorescence enhanced effect for Cy3 was much weaker than that of Cy5, possibly because the excitation wavelength (532 nm) and emission wavelength of Cy3 (566 nm) were too close to the

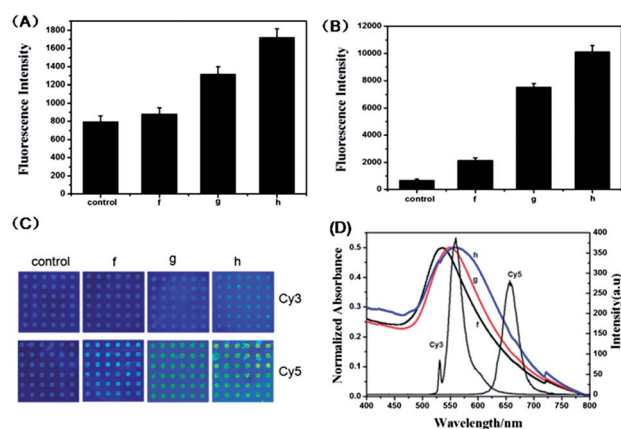


Fig. 2 Fluorescence intensity of dye-functionalized AgNPs hybridized with different sizes of Ag@Au (A) Cy3-functionalized AgNPs-a. (B) Cy5-functionalized AgNPs-a. (C) Fluorescence images of dye-functionalized AgNPs-a hybridized with different sizes of Ag@Au. (D) LSPR spectra of Ag@Au and emission spectra of Cy3 and Cy5.

maximum LSPR wavelength of Tag-F (528.5 nm). The Ag@Au may not be suitable for dye molecules whose maximum excitation wavelength is smaller than the maximum absorbance wavelength of Ag@Au. Therefore, the applications can be limited. It is not versatile fluorescence enhanced nanoparticles and may be only used for fluorescent dyes with longer excitation wavelength.

To find highly effective and more economical fluorescent dyes for fluorescence enhancement, two other fluorescent dyes, TAMRA (carboxytetramethylrhodamine) and ROX (6-carboxyl-X-rhodamine) were chosen as their excitation wavelengths are close to 532 nm. The fluorescence enhanced effects of Tag-D for TAMRA and ROX were 11.2-fold and 6.3-fold (Fig. 3), the LSPR spectra could overlap with the emission spectra of TAMRA and ROX. Though the fluorescence enhanced effect of Tag-D on ROX was 6.3-fold, the fluorescence intensity of ROX was weaker compared to Cy3 and TAMRA. The fluorescence enhanced effect of Tag-D on TAMRA was higher than that of Cy3, and the fluorescence intensity of TAMRA was not weaker than Cy3 and Cy5. Additionally, TAMRA was much cheaper than Cy3 and Cy5. As a consequence, TAMRA will be a good candidate for fluorescence dyes used in the detection of significant proteins related to human disease.

Simultaneous detection of IgE and PDGF-BB by Tag-A

Aptamers are single-stranded DNA or RNA which rival antibodies to specifically recognize target molecules as they are easily synthesized and modified, nontoxic and stable.⁴⁰ Aptamer-conjugated nanomaterials as capture and detection probes have attracted greater attention and have been widely used in biomedicine and biotechnology.^{41–44} A fluorescent detection tag (Tag-A) based on AgNPs-d, aptamers and TAMRA was prepared and the preparation process is shown in Fig. 4A. Aptamers and TAMRA bind with AgNPs-d by self-assembly of sulfhydryl at the 5' end of aptamers and 5'SH-oligo(d)₂₄-TAMRA. Transmission electron microscopy images of Tag-A, showed that Tag-A was mostly spherical and sparsely triangular with the diameter of the spherical nanoparticle to be $D = 60.1 \pm 7.7$ nm (Fig. 4C). Tag-A could simultaneously recognize protein

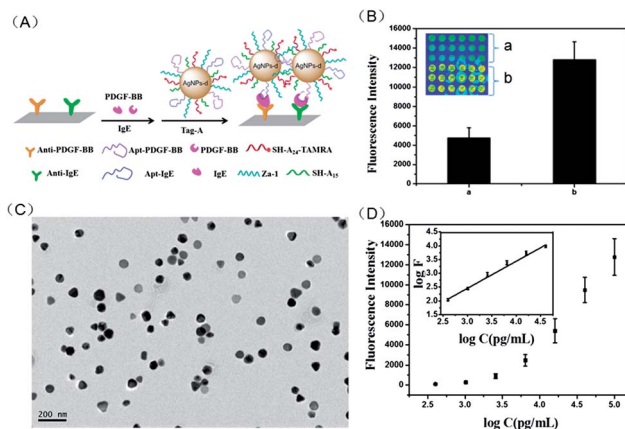


Fig. 4 Strategy for the simultaneous detection of IgE and PDGF-BB. (A) Schematic illustration. (B) Fluorescence intensity and images (inset), a: IgE, b: PDGF-BB. (C) TEM images of Tag-A. (D) Fluorescence intensity vs. PDGF-BB concentration in the range 0.41–50 ng mL⁻¹, and the linear relationship between log *F* and log *C*.

IgE (100 ng mL⁻¹) and PDGF-BB (100 ng mL⁻¹) by Apt-IgE and Apt-PDGF with high fluorescence intensity (Fig. 4B). As the method for detection of human IgE had been well established in our previous research,⁹ more attention was devoted to another equally important protein, PDGF-BB, which is an indicator of tumor angiogenesis.⁴⁵ Tag-A was used to explore PDGF-BB to find out the potential application of Tag-A in bioanalysis and biomedicine. The results showed that the fluorescence intensity increased with the increase of PDGF-BB concentration, and an exponential relationship between log *C* (PDGF-BB concentration) and fluorescence intensity (*F*) was obtained when PDGF-BB concentrations (*C*) range from 0.41 ng mL⁻¹ to 100 ng mL⁻¹. The inset showed a good linear relationship between log *C* and log *F*. From the linear curve, it could be inferred that the detection limit for Tag-A was below 0.41 ng mL⁻¹. Therefore, Tag-A was an excellent fluorescent tag for the detection of PDGF-BB. In addition, with an optimal design of aptamers, the tags based on aptamers and fluorescent dyes could be exploited for other important protein biomarkers.

Optimising the plasmonic properties of the substrate

Formation of biomolecule–nanoparticle conjugates is a significant step in bioanalysis and biomedicine. The biomolecules used in this study were aptamers and other oligonucleotides. In order to find the optimal method to reduce the production cost of functionalization, other approaches for functionalization were carefully examined, including the influence of other oligonucleotides as well as the ratios of aptamers to fluorescent dyes. The results showed that the optimal ratio for Apt-PDGF to TAMRA was 1 : 1 (Fig. S2†). Based on the optimal ratio, the Tag-B based on AgNPs-a, Apt-PDGF and TAMRA was prepared, and Tag-C based on AgNPs-d, Apt-PDGF and TAMRA was also prepared and these were used to investigate further improvement in the sensitivity of PDGF-BB detection.

To further improve the sensitivity of PDGF detection, AgNPs-a-on-AgNPs-d plasmonic microarrays were designed and tested,

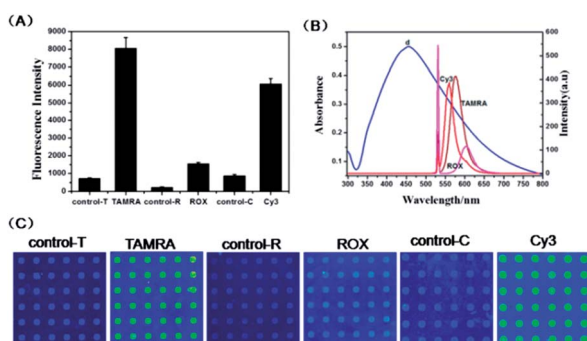


Fig. 3 (A) Fluorescence intensity of dye-functionalized AgNPs-a hybridized with Tag-D. (B) LSPR spectra of Tag-D and emission spectra of Cy3 and Cy5. (C) Fluorescence images of different dye-functionalized AgNPs-a hybridized with Tag-D.

as the coupling between nanoparticles could further enhance the fluorescence intensity of nearby fluorescent dyes. Antibody-conjugated nanomaterials have been widely used as the capture agent or detection agent, specifically for recognizing target molecules.^{46–49} Antibody–AgNPs-a conjugates and antibody–AgNPs-d conjugates were prepared and spotted on the aldehyde-modified slide, by the interaction of the amino group and the aldehyde group. The conjugates were immobilized on the aldehyde-modified slide. The zeta potential of AgNPs-a and AgNPs-d was estimated to be $-26.0 (\pm 2.9)$ mV ($n = 3$) and $-12.8 (\pm 0.3)$ mV ($n = 3$) respectively. The zeta potential of antibody–AgNPs-a and antibody–AgNPs-d was estimated to be $-29.9 (\pm 0.3)$ mV ($n = 3$) and $-24.9 (\pm 1.6)$ mV ($n = 3$) respectively. The decreased zeta potential proved that the antibody molecules were bound onto the surface of AgNPs-a and AgNPs-d. Based on the interaction of antibody–protein–aptamers, the Apt–PDGF and TAMRA functionalized detection tags (Tag-B, Tag-C) could couple with plasmonic microarrays. To further improve the analytical performance of Tag-C, antibody, antibody–AgNPs-a and antibody–AgNPs-d were spotted respectively on the slides for contrast experiments, and the results showed that the fluorescence intensities with the antibody–AgNPs-a and antibody–AgNPs-d substrate were decreased when the concentration of PDGF-BB was 50 ng mL^{-1} . The decreased intensity was

attributed to the strong steric hindrance of antibody–AgNPs-a and antibody–AgNPs-d to Tag-C, at high concentration of (50 ng mL^{-1}) of PDGF-BB (Fig. 5A). When the PDGF-BB concentration was decreased to 400 pg mL^{-1} , steric hindrance was still evident on the antibody–AgNPs-d plasmonic microarrays. The steric hindrance on antibody–AgNPs-a plasmonic microarrays was decreased as the number of Tag-C on antibody–AgNPs-a plasmonic microarrays was decreased, and the enhanced fluorescence was observed. Although, the fluorescence enhancement was evident at lower PDGF-BB concentration, the fluorescence intensity was too low, thus limiting its application. On the other hand, we found that Tag-B on the plasmonic microarrays could exhibit the increased fluorescence intensity with the enhanced effect reaching the highest point on antibody–AgNPs-d plasmonic microarrays. This result clearly shows that AgNPs-d can act as a fluorescent detection tag, and also as a plasmonic substrate to enhance sensitivity of the fluorescence detection method. The SEM images showed that the antibody–AgNPs-a and antibody–AgNPs-d were well immobilized on the slides, which indicated that the antibody was successfully bound with AgNPs-a and AgNPs-d (Fig. S3†). The size of the antibody ($\sim 12 \text{ nm}$)⁵⁰ may affect the MEF of the antibody–AgNPs-d plasmonic substrate on TAMRA, however, the size of the antibody could not be tuned, in addition, our aim was to optimize the three kinds of substrates and obtain the optimal one, therefore, the effect of antibody size was ignored.

Developed strategy for detection of PDGF-BB

A highly sensitive and specific detection strategy was developed based on antibody–AgNPs-d plasmonic microarrays, PDGF-BB

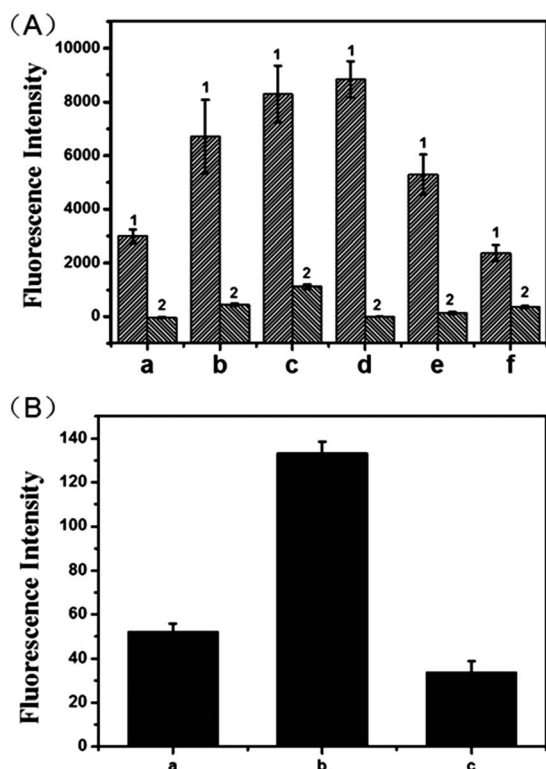


Fig. 5 (A) Fluorescence intensity of Tag-B on different microarray substrates, (a) antibody, (b) antibody–AgNPs-a, (c) antibody–AgNPs-d, fluorescence intensity of Tag-C on different microarray substrates, (d) antibody, (e) antibody–AgNPs-a, and (f) antibody–AgNPs-d, 1: PDGF-BB (50 ng mL^{-1}), 2: BSA (1 mg mL^{-1}). (B) Fluorescence intensity of Tag-C on different microarrays (a) antibody, (b) antibody–AgNPs-a, and (c) antibody–AgNPs-d when the concentration of PDGF-BB was 400 pg mL^{-1} .

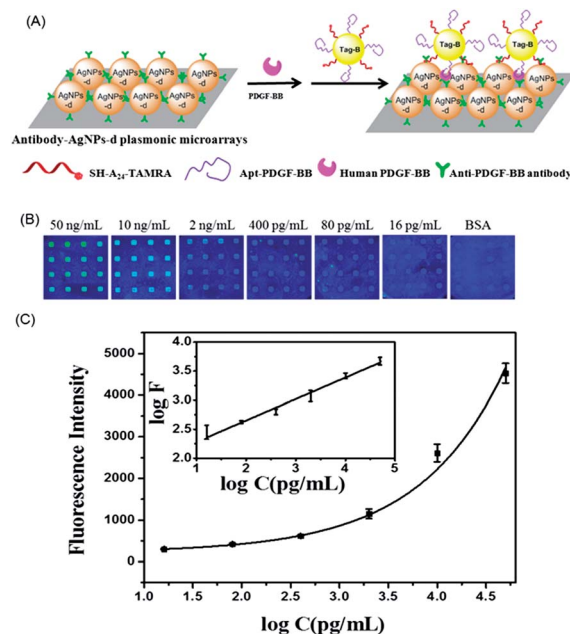


Fig. 6 (A) Schematic illustration of Tag-B-on-antibody–AgNPs-d plasmonic microarrays for ultrasensitive detection of PDGF-BB. (B) Fluorescence images of different PDGF-BB concentrations. (C) The relationship between PDGF-BB concentration and fluorescence intensity, the inset is the linear relationship between log F and log C.

and Tag-B, the schematic illustration is shown in Fig. 6A. The reaction time between the antibody and PDGF-BB, PDGF-BB and Tag-B was decreased to 30 min to reduce the nonspecific adsorption of Tag-B on antibody-AgNPs-d plasmonic microarrays.

The fluorescence images show that the intensity increased with the increase of PDGF-BB concentration (Fig. 6B). Fig. 6C shows an exponential relationship between $\log C$ (C was PDGF-BB concentration) and fluorescence intensity (F means having subtracted the intensity of BSA) when PDGF-BB concentration (C) was from 16 pg mL^{-1} to 50 ng mL^{-1} . The inset showed a good linear relationship between $\log C$ and $\log F$. The data of fluorescence intensity using the linear relationship is usually shown by subtracting the background signal and the measurement noise is subtracted automatically when collecting original data on the computer. The Y-axis fluorescence intensity in Fig. 6 (fluorescence intensity means having subtracted the intensity of BSA) and the detection limit was calculated to be 3.2 pg mL^{-1} ($F_{3.2 \text{ pg mL}^{-1}} \approx F_{1 \text{ mg mL}^{-1} \text{ BSA}} + 2\text{SD}$). The detecting performance of Tag-B and Tag-C on the antibody substrate was estimated to be a control, and the results are shown in Fig. S4,† the detection limits of Tag-B and Tag-C for PDGF-BB were approximately 2 ng mL^{-1} and 0.4 ng mL^{-1} respectively. The method based on the antibody-AgNPs-d substrate and Tag-B rivaled the method based on the antibody substrate using Tag-B or Tag-C. The detection limit was about 625-fold lower than that of Tag-B, and approximately 125-fold lower than that of Tag-C. Therefore, the method based on the antibody-AgNPs-d substrate and Tag-B showed remarkable increase in sensitivity. Theoretically, as the concentration of antibody on the antibody substrate is higher than that of antibody on the antibody-AgNPs-d plasmonic substrate, the antibody surface density on the antibody substrate is higher than that on the antibody-AgNPs-d plasmonic substrate. As shown in Fig. S3,† there are more numbers of Tag-B on the antibody substrate (Fig. S3A†) than on antibody-AgNPs-d (Fig. S3C†), therefore, the surface density of the captured PDGF-BB on the antibody substrate is higher. As the metal-enhanced fluorescence of the antibody-AgNPs-d plasmonic substrate on Tag-B, the final sensitivity was higher with the antibody-AgNPs-d substrate.

To demonstrate the specificity and practicality of the method, the specificity and recovery of PDGF-BB in different concentrations of serum were also investigated to study the interference effect of serum. Human serum samples were diluted by 0.2 M PBSM to make 10%, 2% and 1% serum. The fluorescence intensity of PDGF-BB (50 ng mL^{-1}) and serum are shown in Fig. 7A, the PDGF-BB had much higher intensity than serum indicating good specificity of the method. In addition, when 2 ng mL^{-1} PDGF-BB was added to three serum samples of 10% serum, 2% serum and 1% serum, a good recovery was observed and the increase in respective fluorescence intensities is shown in Fig. 7B. The fluorescence intensity of PDGF-BB in 10% serum was the lowest and the highest intensity was observed in 1% serum, and the recoveries in three diluted samples were estimated to be 80.5%, 94.3%, 103.7% respectively. Therefore, human serum would not have interference with the PDGF-BB detection when PDGF-BB was 2 ng mL^{-1} .

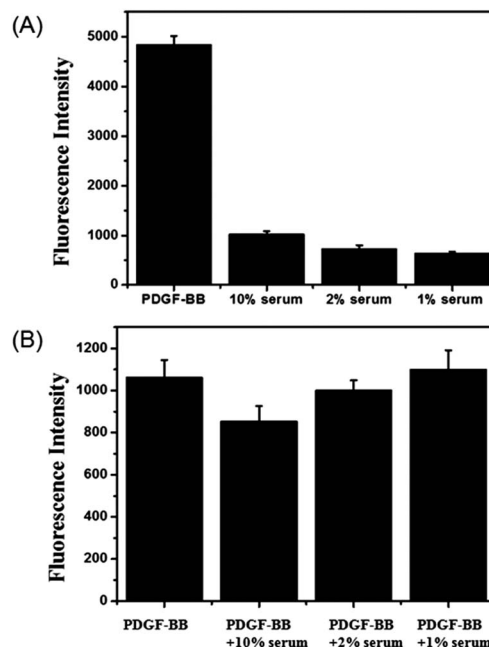


Fig. 7 (A) The specificity of PDGF-BB (50 ng mL^{-1}) in different concentrations of human serum and (B) the recovery of PDGF-BB (2 ng mL^{-1}) in different concentrations of human serum.

Conclusions

We developed a silver-enhanced fluorescence method with high sensitivity and specificity for protein microarrays. AgNPs were screened out as versatile fluorescence enhancement materials. The AgNPs are not only suitable for detection probes, but also as substrates to enhance the fluorescence detection. Aptamer-functionalized AgNPs were used as detection probes for the simultaneous detection of IgE and PDGF-BB. To further enhance the sensitivity, antibody-conjugated AgNPs were used to establish a plasmonic microarray substrate for ultrasensitive detection of PDGF-BB. The plasmonic microarray substrate showed remarkable specificity and sensitivity for protein biomarker, PDGF-BB. Linear response to PDGF-BB concentrations was in the range of 16 pg mL^{-1} to 50 ng mL^{-1} and detection limit was 3.2 pg mL^{-1} . The plasmonic microarray substrate showed excellent recovery when PDGF-BB concentration was 2 ng mL^{-1} in normal human serum, indicating no interference of human serum.

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