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Homogeneous Immunoassay Based on Two-Photon Excitation Fluorescence Resonance Energy Transfer

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A two-photon excitable small organic molecule (abbreviated as TP-NH_2) with large two-photon absorption cross section and competitive fluorescence quantum yield was prepared, which emitted fluorescence in the visible region upon excitation at 800 nm. Using the TP-NH_2 molecule as an energy donor, a two-photon excitation fluorescence resonance energy-transfer (TPE-FRET) based homogeneous immunoassay method was proposed. The donor and the acceptor (DABS-Cl, a dark quencher) were labeled to bovine serum albumin (BSA) separately, and anti-BSA protein was determined by employing an antibody bridging assay scheme. Rabbit anti-BSA serum containing other biomolecules was intentionally used as the sample to introduce interference. A parallel assay was performed using the traditional one-photon excitation FRET model, which failed to carry out quantitative determination due to the serious background luminescence arising from those biomolecules in the sample. The TPE-FRET model showed its strong ability to overcome the problem of autofluorescence and provided satisfying analytical performance. Quite good sensitivity and wide linear range (0.05−2.5 nM) for anti-BSA protein was obtained. The results of this work suggest that TPE-FRET could be a promising technique for homogeneous assays excluding separation steps, especially in complicated biological sample matrixes.

The specificity and sensitivity of the antigen−antibody interaction has resulted in the immunoassay being widely used for the detection and quantification of biomolecules in bioanalysis and clinical diagnosis. The methods can be classified into homogeneous and heterogeneous assays. Heterogeneous assays, like enzyme-linked immunosorbent assay (ELISA), are more sensitive due to the separation of unreacted or unbound biomolecules, but the numerous washing steps also make the assay more complicated.[1] The separation-free nature of the homogeneous assay has the assay time shortened and the assay procedure simplified, making high-throughput screening for multiple samples possible. Unfortunately, without the separation step, some background signals may arise from nontarget molecules (especially biomolecules) resulting in a lower sensitivity or even false results. Fluorescence resonance energy transfer (FRET), which is considered as a sensitive and reliable "ruler" over distances of 10−100 Å,[2] is one of the most commonly used methods in homogeneous assays.[3,4] However, in the application of conventional one-photon excitation (OPE)-FRET, where the energy donor is excited with ultraviolet or visible light, autofluorescence or scattering light always arises from biomolecules upon excitation of the energy donor. Another issue is that the energy acceptor is often coexcited with the energy donor because of the overlap of their excitation spectra.[5,6] Clearly, the traditional OPE-FRET technique has been subject to serious interferences in the homogeneous immunoassay, especially in biological samples. Therefore, new energy donor−acceptor pairs are desired to overcome the problem.

In the past few years, a kind of inorganic fluorescent particle, namely, up-converting phosphors (UCPs), has been tried in FRET applications. UCPs can be excited at longer wavelength (near-infrared region) and emit at relatively shorter wavelength (visible region), which is called anti-Stokes photoluminescence.[7,8] Such properties of UCPs suggest that they could be favorable energy donors for FRET-based bioassays. First, the interference from autofluorescence or the scattered excitation light could be eliminated under excitation at the near-infrared region. Second, the direct excitation of an energy acceptor is avoided. The above two advantages are likely to lead to higher signal/noise ratio and thus ensure higher sensitivity in quantitative analysis. The earliest reports on homogeneous FRET bioassays using UCPs as donors

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appeared in 2005 by Kuningas and Li.[9,10] Thereafter, Kuningas and co-workers have contributed much to this field.[11−13] The significance of using the near-infrared light-excitable UCPs as energy donor in bioassays has been proven in these papers. Nevertheless, we may also notice some negative effects of such inorganic particles, such as the reabsorptive energy transfer of ions in the core of particles, nonspecific binding interactions, and the need for surface modifications to ensure the stability of the phosphor bioconjugates, which might sometimes cause reduced sensitivity and somewhat complicated operations, and hence restrict the application of UCPs.[12,14]

Two-photon excitation (TPE) has attracted increasing attention in biological applications for its particular optical properties in recent years. TPE occurs upon the simultaneous absorption of two lower-energy photons to reach an excited state via an intermediate (virtual) state.[15] In such a nonlinear optical process, TPE materials also can be excited in the near-infrared region to give emission in the visible region. The most obvious difference between the two types of anti-Stokes fluorophore is the excitation method, i.e., sequential absorption (UCPs) versus simultaneous absorption (TPE molecules) of the photons. Therefore, there are different requirements for the efficiency of the excitation sources. Normally, the power of excitation sources for UCPs is higher than that for TPE molecules. One can expect that molecules possessing TPE fluorescence could be another type of promising energy donor for FRET-based bioassays. Taking the small-molecule nature of TPE molecules into consideration, the small organic TPE molecules may have some advantages as compared to the inorganic UCPs in some aspects. For instance, the nonspecific binding/adsorbing of biomolecules on the surface of particles will not occur when using TPE molecules. Besides, small molecules are structurally more flexible so that the functionalization of the donor is much easier and the phosphor bioconjugate is more stable.

In 2006, a method based on labeled DNA for determining the TPE-FRET efficiency was reported by Wahlroos et al.[16] Most recently, we developed a TPE-FRET model using a two-photon excitable small organic molecule *trans*-4-(*N*-2-hydroxyethyl-*N*-ethylamino)-4′-(dimethylamino)stilbene (DMAHAS) as energy donor to demonstrate the merits of TPE in FRET-based bioassays.[17] The results succeeded in showing some advantages of TPE-FRET bioassays over conventional OPE-FRET. However, as a start in investigating the applicability of TPE-FRET in bioassay, the fluorescence property of the donor molecule was not considered intensively, and the analytical performance of the model in real biological sample was not examined. Herein in the present work, we synthesized a new TPE molecule 2-((*E*)-4-(dimethylamino) styryl)-5-((*E*)-4-aminostyrul) terephthalonitrile (written as TP-NH_2; structure and synthesis shown in Scheme S1, Supporting Information, SI) with largely improved fluorescence properties, such as the two-photon absorption (TPA) cross section and fluorescence quantum yield. Using this molecule as energy donor, which was covalently linked to bovine serum albumin (BSA) through a tagged amino group on the molecule, a new TPE-FRET-based method for the homogeneous immunoassay was constructed. In a bridge assay for rabbit anti-BSA antibody using antiserum as the sample, a quite good sensitivity and a wide linear range, i.e., 0.05−2.5nM, was obtained. With the same antigen−antibody system, a set of parallel experiments employing the traditional OPE-FRET model was also conducted. The OPE-FRET method thoroughly failed to work due to interference from the background luminescence of the serum matrix. The comparison between the results from the TPE and OPE models strongly confirmed the ability of TPE-FRET to circumvent the problem of autofluorescence in biological samples, which showed the competence of TPE-FRET in homogeneous immunoassay, especially in complicated sample matrixes. To the best of our knowledge, such TPE-FRET based homogeneous immunoassay for quantitative determination of protein has not been reported so far.

EXPERIMENTAL SECTION

Reagents. The donor TP-NH_2 was synthesized in this laboratory (see SI). BSA was purchased from Biosharp Co.; rabbit anti-BSA serum (IgG, 6 mg/mL antibody protein) and normal rabbit serum were both from Beijing Pusenredar Biotechnological Co. (Beijing, China). DABS-Cl was obtained from Alfa Aesor Chemical Co. (Ward Hill, MA). Bradford Protein Assay Kit (Coomassie brilliant blue G-250) was from Tiangen Biotech Co. (Beijing, China). Glutaraldehyde and Rhodamine B were the products of Shanghai Chemical Reagents (Shanghai, China). L-Cysteine amino acid was from Shanghai Ruji Biology Technology Co. Sephadex G-25 gel was the product of Amersham Pharmacia Biotech Ltd. All aqueous solutions were prepared in ultrapure water (purified by Milli-Q biocel from Millipore China Ltd.).

Apparatus for Fluorescence and Absorption Measurements. Two-photon excited fluorescence measurements were carried out as described earlier.[17] Briefly, the samples were excited at 800 nm by a mode-locked Ti:sapphire pulsed laser (Mira 900, Coherent Inc., Santa Clara, CA) with a pulse width of ~3 ps at a repetition rate of 76 MHz. The anti-Stokes photoluminescence was recorded on a liquid nitrogen-cooled CCD detector (SPEC-10, Princeton Instruments, Trenton, NJ) through a monochromator (Spectrapro 2500i, Acton Research Corp., Acton, MA). One-photon excited fluorescence was measured on a Perkin-Elmer LS 55 fluorometer equipped with 1-cm cell, and the signal was recorded with a PMT detector. UV−vis absorption spectra of BSA, TP-NH_2−BSA, and DABS−BSA conjugates were measured using a TU-1900 UV−vis spectrophotometer (Beijing Purkinje General Instrument).

Preparation of TP-NH_2−BSA Conjugate. A one-step glutaraldehyde linkage procedure was used for the preparation of TP-

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NH_2−BSA conjugate.[18] BSA was dissolved in phosphate buffer (0.01 M, pH 8.0) at a concentration of 2 mg/mL. TP-NH_2 was dissolved in anhydrous DMF at a concentration of 5 mg/mL. With gentle shaking, 1 mL of the BSA solution was mixed with 20 μL of glutaraldehyde (25% in water) and 65 μL of the TP-NH_2 solution at 4 °C, where the molar ratio of BSA/TP-NH_2 was ~1:28. After incubating for 4 h, 75 μL of 0.2 M L-cysteine was added as a mild reducing agent to reduce the resultant Schiff base and any excess aldehydes.[19] The mixture was then centrifuged and the supernatant was purified by gel filtration (Sephadex G-25) against phosphate buffer (0.01 M, pH 7.4).

The concentration of BSA was measured using Bradford Protein Assay Kit according to the instructions. The absorbance maximum of the complex of BSA and Coomassie brilliant blue is located at 595 nm; hence, the protein concentration was quantified by measuring the OD 595 value. The concentration of TP-NH_2 in the TP-NH_2−BSA complex was determined using its molar extinction coefficient in phosphate buffer, which was obtained through a calibration curve. And the dye to protein ratio was calculated accordingly.

Preparation of DABS-Cl Labeled BSA. BSA was dissolved in sodium carbonate−sodium bicarbonate buffer (0.1 M, pH 9.1), at a concentration of 1 mg/mL. DABS-Cl was dissolved in DMF at a concentration of 1 mg/mL, protected from light, and used immediately. With gentle shaking, 100 μL of the DABS-Cl solution was slowly added to 1 mL of the BSA solution. After reacting at room temperature for 2 h in the dark, the unbound DABS-Cl molecules were removed by ultrafiltration using an Amicon Ultra-4 Centrifugal Filter Device with a MW cutoff of 50 kDa (Millipore Corp.) at 4 °C. The ultrafiltration residue with molecular mass larger than 50 kDa was collected and diluted in phosphate buffer (0.01 M, pH 7.4).

The dye to protein ratio was determined in the same manner as described in our previous work,[17] using the molar extinction coefficient of 4.39×10^4 M^{-1} cm^{-1} for BSA at 280 nm[20] and 3.3×10^4 M^{-1} cm^{-1} for DABS-Cl at 474 nm.

Homogeneous Bridge Assay for Rabbit Anti-BSA with TPE-FRET and OPE-FRET. In a typical TPE-FRET based homogeneous bridge assay, varying volumes of rabbit anti-BSA antiserum were added to the mixed solution of TP-NH_2−BSA and DABS−BSA, in which the concentrations of TP-NH_2−BSA and DABS−BSA were fixed at 2.2×10^{-7} and 8.0×10^{-7} M (both representing the concentration of BSA), respectively. To point out that, due to the existence of multiple immunocomplexes (vide infra), i.e., D+Q (donor labeled antigen−antibody−quencher labeled antigen), D+D, and Q+Q, the ratio of donor conjugate/quencher conjugate was decided with a preliminary optimization to obtain the highest quenching of the donor fluorescence under TPE excitation. The ultimate concentrations of the antibody protein tested were 0, 0.05, 0.075, 0.1, 0.25, 0.5, 1, 2.5, 5, 10, 50, and 100×10^{-9} M in sequence, of which the total volume was compensated with phosphate buffer. The mixture was mixed thoroughly and incubated at 37 °C for 1 h with gentle shaking.

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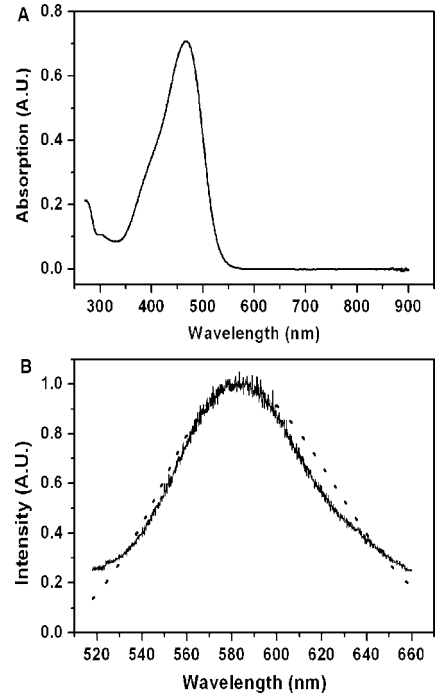

Figure 1. Spectral properties of the donor TP-NH_2. (A) Linear absorption spectrum of TP-NH_2 (2.5×10^{-5} M in DMF). (B) Fluorescence emission spectra of TP-NH_2 under one-photon excitation (dotted line, 7.2×10^{-6} M in DMF, excited at 460 nm) and two-photon excitation (solid line, 7.2×10^{-4} M in DMF, excited at 800 nm); fluorescence intensity was given in normalized form.

Then the TP excited fluorescence spectra were measured for the antigen−antibody complexes. A control experiment using varying amounts of normal rabbit serum (which contains no anti-BSA antibody, and the volumes used were the same as that of antiserum) as samples was conducted similarly.

The above procedure was also followed for the immunoassay under OPE model under the excitation of 460 nm. The excitation and emission slit were set at 2.5 and 15 nm, respectively. And the background luminescence signals of varying amounts of antiserum under both OPE and TPE models were also examined under the above conditions.

RESULTS AND DISCUSSION

Spectral Properties of TP-NH_2. As a potential energy donor for TPE-FRET, the absorption and fluorescence properties of TP-NH_2 were characterized. First, the linear absorption spectrum was examined. As shown in Figure 1A, there is only one absorption band with the maximum locating around 460 nm, and no linear absorption is found from 560 to 900 nm. This confirms that the photoluminescence of TP-NH_2 excited at 800 nm is purely the two-photon induced fluorescence, which comes from the combined energy of two photons at 800 nm that falls into the maximum absorption band of the molecule. It is worth briefly explaining why the 800-nm light was selected as the excitation source. Simply judged from the absorption maximum, it seems that the energy of two 920-nm photons would be matching with the energy level of TP-NH_2 best. In fact, a shorter wavelength is expected according to the prediction that the two-photon allowed states for quadru-

poles are at a higher energy than the Franck−Condon states.[21] That is to say, the maximum absorption wavelength of TPA (λ_{max} [2]) will locate at a somewhat shorter wavelength than the 2-fold peak absorption wavelength of one-photon (λ_{max}). Another reason is that the absorption band of TP-NH_2 is broadened and blue-shifted upon conjugation with BSA (vide infra). Therefore, it would be reasonable to use the 800-nm irradiation to excite the donor in our experiments.

Figure 1B shows the fluorescence emission of TP-NH_2 under OPE (dotted line, excited at 460 nm) and TPE (solid line, excited at 800 nm), respectively. As can be seen, the profiles of the two spectra match exactly to each other, with their maximal emission both locating at 580 nm. The uniformity in the emission spectra indicates that the fluorescence under two excitation models, i.e., TPE and OPE, is emitted from the same excited state of the molecule.

As one can understand, the TPA cross section of the donor could be one of the determinant factors for the detection sensitivity in a TPE-based assay, since it is decisive to the capability of the molecule to upconvert energy, i.e., to emit relatively higher energy photons upon absorbing lower energy photons. The TPA cross section and the fluorescence quantum yield of TP-NH_2 were determined using Rhodamine B as the reference (please refer to SI for detailed procedures). The fluorescence quantum yield was detected to be 0.26, and the TPA cross section of TP-NH_2 was calculated as 417 GM using the method of two-photon induced fluorescence. It is a quite competitive value compared to many fluorophores that are commonly used as fluorescent tags in biological samples. For example, the TPA cross section of fluorescein in water at pH 13 is only 36 GM,[22] and the value for Rhodamine B, the most commonly used standard in determination of the TPA cross section, is just 150 GM under 800-nm excitation.[23] Such favorable TPA cross section and fluorescence quantum yield have made the TP-NH_2 molecule a potential energy donor for TPE-FRET based assays.

Characterization of the Donor and Acceptor Bioconjugates. Glutaraldehyde is one of the most commonly used homobifunctional cross-linking reagents, through which two amino groups covalently combine with each other. In this system, one aldehyde group of glutaraldehyde forms a Schiff base linkage mainly with the ε-amine group of a lysine residue on BSA,[24,25] while the other aldehyde group forms an identical bond with the amine group of TP-NH_2. After a one-step coupling reaction, the TP-NH_2−BSA conjugate was separated from small molecules and protein dimer by centrifugation and gel filtration. Figure 2A shows the UV−vis absorption spectra of the TP-NH_2−BSA conjugate and pure BSA, which confirms that TP-NH_2 successfully combined with BSA through glutaraldehyde coupling since all the small molecules had been exhaustively removed. As has been reported, the interaction of glutaraldehyde with lysine residues often causes a blue-shift of the absorption wavelength of proteins and increases the absorbance.[24] As a result, the protein concentration cannot be simply determined with the absorption at 280 nm. Therefore,

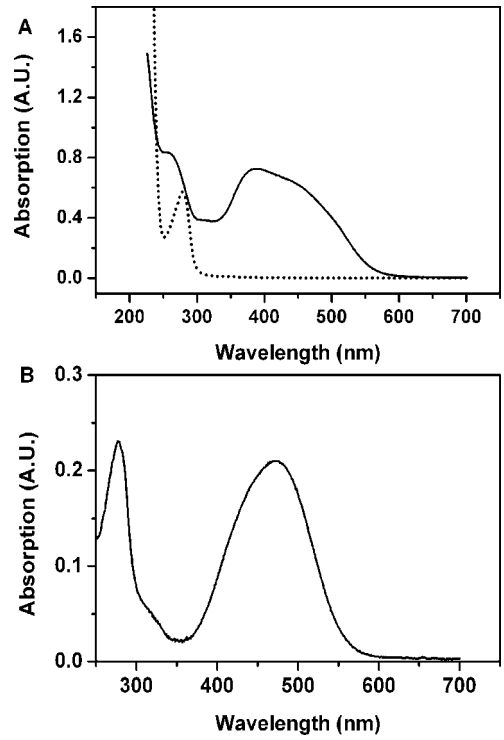

Figure 2. (A) UV−vis absorption spectra of the TP-NH_2−BSA complex (solid line) and pure BSA (dotted line). The concentration of BSA was 1.75×10^{-5} M for both samples. (B) UV−vis absorption spectrum of the DABS−BSA conjugate; the concentration of BSA was 3.28×10^{-6} M. All the spectra were recorded in 0.01 M phosphate buffer, pH 7.4.

the absorption peak at 265 nm in the figure was attributed to the reaction of BSA with glutaraldehyde, and the concentration of BSA in the conjugate was determined according to the Bradford method[26] with free BSA as the standard. Using the molar extinction coefficient of TP-NH_2 at 445 nm in phosphate buffer, i.e., 6.47×10^3 M^{-1} cm^{-1}, which was obtained through a calibration curve (Figure S4, SI), the concentration of TP-NH_2 in the TP-NH_2−BSA complex was determined, and the dye/protein ratio was calculated as 5.6. The figure also shows the broadened absorption band of TP-NH_2 with the maximum located at 385 nm. Such alterations in the absorption spectra are always seen when a hydrophobic dye is linked with hydrophilic biomolecules.

DABS-Cl is a nonfluorescent quencher with high molar extinction coefficient, which makes it a good acceptor in FRET. It is labeled to BSA as the energy acceptor in this work through a straightforward one-step reaction. The UV−vis spectrum in Figure 2B exhibits two distinct absorption peaks at 474 and 280 nm, corresponding to the characteristic absorption of DABS-Cl and BSA, respectively. The dye-to-protein ratio was determined based on the absorbance at 474 and 280 nm. Generally, rational increase of the dye-to-protein ratio will result in increased quenching of the donor (i.e., the fluorescence intensity of donor is decreased more) due to the existence of multiple nonfluorescent acceptors. But on the other hand, coupling more water-insoluble fluorophores to the surface of proteins may result in lower solubility and activity of the complex owing to the hydrophobicity of the conjugate. According to our previous experience in using

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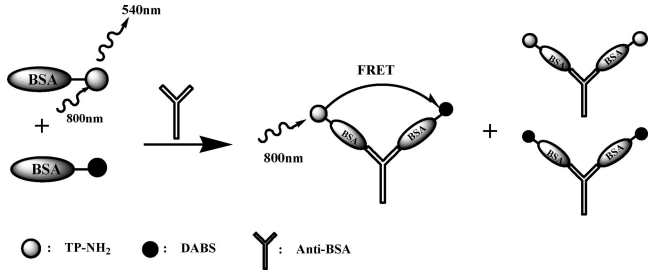

Figure 3. Schematic description of the TPE-FRET based homogeneous bridge assay for rabbit anti-BSA antibody with TP-NH_2-conjugated BSA as donor and DABS-Cl-conjugated BSA as acceptor.

DABS-Cl, the DABS-to-BSA ratio was controlled as 1.9 in subsequent immunoassay.

Investigation of the Antigen−Antibody System. As one of the best known and widely studied proteins, the detailed structural information of BSA has made it a good antigen in immunoassays. Therefore, BSA and the corresponding antibody were chosen to build the antigen−antibody system. A bridge assay model was employed for the determination of the antibody (Figure 3). In the presence of rabbit anti-BSA (Ab), the two labeled antigen conjugates, namely, TP-NH_2−BSA (Ag1) and DABS−BSA (Ag2), will form the bridging immunocomplex with the antibody, i.e., Ag1−Ab−Ag2 (accompanied with complexes Ag1−Ab−Ag1 and Ag2−Ab–Ag2). Hence, the FRET between TP-NH_2 and DABS-Cl occurs since they are in proximity due to the specific immunoreaction. With fixed concentrations of Ag1 and Ag2, the amount of the immunocomplex is decided by the amount of Ab added. As a result, the fluorescence of the donor is quenched by the acceptor in an Ab-dependent manner.

Notably, rabbit anti-BSA serum was used here rather than a purified antibody protein. That is to say, the antibody was analyzed in a real antiserum sample rather than a spiked analyte-free serum. In such a way, the concentrations of interfering components also changed with the concentration of the analyte, which made the interfering level inconstant and made the sample matrix more complicated. It was designed to elucidate the capability of TPE-FRET to overcome the interference from the serum matrix. As we know, the serum sample contains many kinds of biomolecules including proteins. Therefore, the background luminescence of the antiserum was first examined under the OPE model. The solid line in Figure 4 is the emission spectrum of TP-NH_2−BSA conjugate excited at 460 nm (the maximal absorption wavelength), which exhibits a maximum emission at 540 nm. When the antiserum was excited at the same wavelength (460 nm), it presented a quite strong and complicated luminescence signal throughout a rather wide wavelength range (the dotted line in Figure 4). Such background signals might be fluorescence emission or scattering light or a mixture of them from other macromolecules in the serum. Although the background signal was not clearly identified (which we think is not necessary), it is likely to hinder spectroscopic analysis, such as FRET-based assays, performed in such samples.

OPE-FRET Based Homogeneous Immunoassay for Anti-BSA. An OPE-FRET immunoassay following the above bridge assay scheme was then conducted to test whether the traditional FRET-based homogeneous immunoassay still works under such a situation. The fluorescence emission intensity at 540 nm was

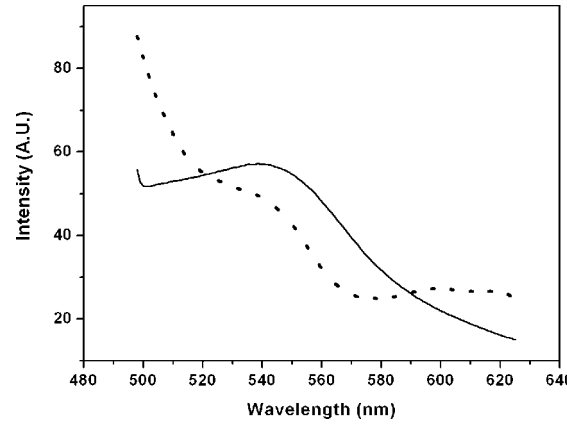

Figure 4. Fluorescence emission spectra of TP-NH_2−BSA conjugate (solid line, BSA concentration 2.2 × 10^{-7} M) and rabbit anti-BSA antiserum (dotted line, concentration of antibody protein 1 × 10^{-9} M) under one-photon excitation. Excitation wavelength, 460 nm; ex/em slit, 2.5/15 nm. Spectra were recorded in 0.01 M phosphate buffer, pH 7.4.

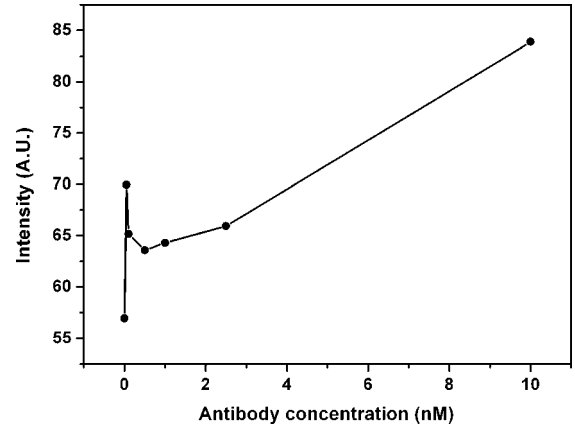

Figure 5. OPE-FRET homogeneous immunoassay for rabbit anti-BSA. The fluorescence was detected at 540 nm under excitation at 460 nm, with the ex/em slit set as 2.5/15 nm. Varying amounts of antiserum were used as samples with the concentrations of antibody being controlled at 0, 0.05, 0.1, 0.5, 1, 2.5, and 10 nM. The experiment was performed in 0.01 M phosphate buffer, pH 7.4.

recorded after the introduction of various amounts of rabbit anti-BSA serum to the mixture of the two labeled antigens. Considering the nature of the immunoreaction, FRET should have occurred upon the formation of the antibody bridging immunocomplex, and the fluorescence of TP-NH_2 should have been quenched by the acceptor. Assuming that no interference occurs, the fluorescence intensity of the donor should be decreasing gradually according to the increase of the amount of antibody. Unfortunately but predictably, under the OPE-FRET model excited at 460 nm, the signal intensity at 540 nm did not change in such an expected way (Figure 5), and it was impossible to perform any quantitative analysis with such irregular data. From Figure 5, the decline of the signal can be seen at relatively lower antibody concentrations, i.e., from 0.05 to 0.5 nM, while the signal intensity keeps increasing with higher antibody concentrations. A quantificational explanation to this experimental fact can be obtained from the antiserum amount dependence of the background signal. Panels A and B in Figure 6 (curve ●) have shown the change of background signal with varying amounts of the antiserum, which was detected under

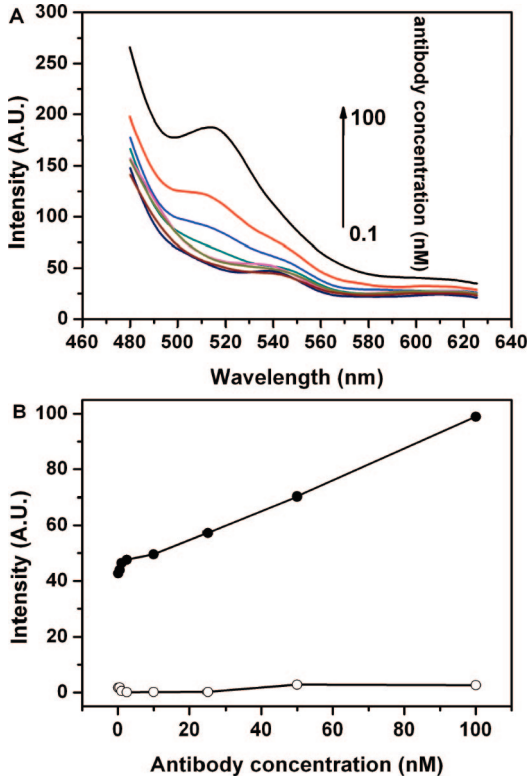

Figure 6. (A) Background luminescence of the antiserum under the OPE model. Concentrations of the antibody protein in the samples were 0.1, 0.5, 1, 2.5, 10, 25, 50, and 100 nM, respectively. (B) The relationship between the background luminescence intensity at 540 nm and the concentrations of the antibody (●) under the OPE model and (○) under the TPE model. All experiments were performed in 0.01 M phosphate buffer, pH 7.4.

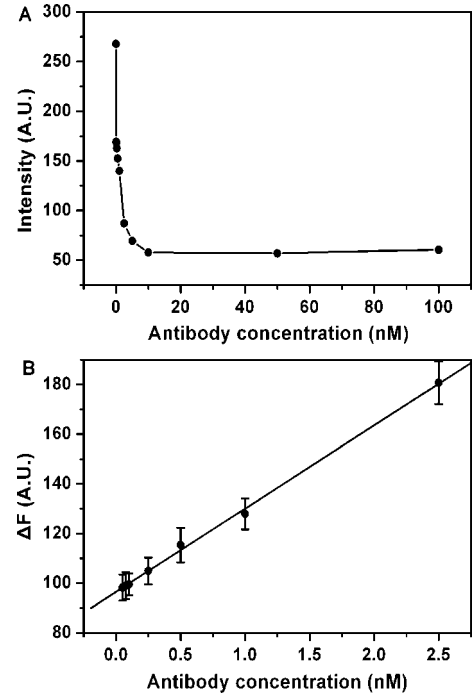

Figure 7. TPE-FRET homogeneous immunoassay for rabbit anti-BSA. (A) The change of fluorescence intensity of the donor according to the antibody concentration. The fluorescence was excited at 800 nm and measured at 540 nm. The concentrations of antibody tested were 0, 0.05, 0.075, 0.1, 0.25, 0.5, 1, 2.5, 5, 10, 50, and 100 nM. (B) The linear relationship between fluorescence intensity decrease of the donor and the concentration of antibody protein within the range of 0.05−2.5 nM. Data were presented as average ± sd from three repeated measurements. All experiments were performed in 0.01 M phosphate buffer, pH 7.4.

the same conditions as the OPE-FRET experiments. In fact, the recorded signal at 540 nm in the OPE-FRET assay was mainly the background luminescence of the antiserum sample, which seriously disturbed (actually destroyed) the traditional OPE-FRET based assay.

TPE-FRET Based Homogeneous Immunoassay for Anti-BSA. The same samples were then analyzed following the identical procedure using the TPE-FRET model excited at 800 nm. First, the background signals of the varying amounts of antiserum were examined under TPE. As is shown in Figure 6B (curve ○), no interference was observed under TP excitation, no matter how many interfering components there were in the sera. With increasing concentration of antibody, the two-photon excited fluorescence intensity of the donor decreased accordingly in a regular way (Figure 7A). A sharply declining section in the curve is observed from 0.05 to 2.5 nM antibody, and the decrease of the fluorescence intensity of the donor slows down with the antibody concentration ranging from 2.5 to 10 nM. The fluorescence intensity value of the donor shows a platform with higher antibody concentrations (10−100 nM), which is possibly due to saturation of the antigen−antibody binding. In the control experiment using varying amounts of normal rabbit serum as samples, no obvious signal change was observed when the sera containing no anti-BSA antibody were incubated with the two labeled antigens (data not shown). It indicated that the fluorescence quenching of the donor was specific to the anti-BSA antibody. Values of ΔF ($\Delta F = FL_0 - FL$, where FL_0 represents the fluorescence intensity in the absence of the antibody and FL represents the fluorescence intensity with different concentrations of the antibody) were calculated, and the dependence of the ΔF value on the concentration of antibody is illustrated in Figure 7B. Within the range of 0.05−2.5nM, a good linearity between ΔF and the anti-BSA concentration was obtained with a correlation coefficient of 0.9991, based on which quantitative determination could be performed. Relative standard deviations of less than 7.0% were obtained between three repeated measurements. We did not try optimizing detection conditions in detail to seek an accurate lower limit of the determination, since the sensitivity of the method is decided with multiple factors, such as the concentration of antigen proteins, the fluorescence property of the donor, the FRET efficiency, and also the performance of the instrument. For example, when higher concentrations of antigen are used, the signal-to-noise ratio (where the noise is mainly from the dark current of the instrument) is enhanced, but it will need more antibodies to form the immunocomplex so as to produce detectable fluorescence quenching. On the other hand, lower antigen concentration may need fewer antibodies in terms of the immunoreaction, but the decreased signal-to-noise ratio will prevent the sensitivity from improving. Therefore, the lowest detectable concentration of target molecule (antibody) at a given concentration of reagent (antigen) could be a more significative index for evaluating the sensitivity of this method. In this TPE-FRET based homogeneous immunoassay, with the donor labeled antigen being fixed at 2.2×10^{-7} M, the antibody can be quantitatively determined to as low as 5×10^{-11} M.

As compared to our last TPE-FRET assay for avidin using DMAHAS as donor,[17] the detection sensitivity of the present work is increased by several orders of magnitude. Such advancement of the sensitivity, in our opinion, can be largely attributed to the improvement of the fluorescence properties of TP-NH_2 due to the amended molecular structure. Briefly, the adoption of a symmetrical D-π-A-π-D quadrupole motif in TP-NH_2, instead of the D-π-D motif of DMAHAS, has increased the conjugation length and the distance over which charge is transferred. The donor-substituted styryl groups can ensure symmetric charge transfer from the ends of the molecule to the middle, and the electron-accepting ability of cyano groups attached to the central ring of the benzene backbone further enhances the extent of such charge transfer. As a result, the intramolecular charge transfer is notably facilitated; therefore, the TPA cross section of the molecule is remarkably enhanced. Meanwhile, the fluorescence quantum yield of TP-NH_2 is also increased due to the enlarged conjugate plane. Such a structural comparison suggests the possibility to further improve analytical performances of TPE-FRET methods through optimizing the molecular structure of donors. And it also demonstrates one of the merits of small molecules as anti-Stokes energy donor, which is the high structural flexibility.

As has been well documented,[27] the luminescence from UCP particles is so intense that it is not a feasible approach to set up a quenching-based assay when aiming for a sensitive assay principle. Therefore, it is hard to directly compare the analytical performance between these two types of anti-Stokes donors in this kind of assay. Anyway, the satisfying sensitivity and linear range obtained with this TPE-FRET model have shown that TPE molecules could be another type of promising anti-Stokes energy donor for FRET-based homogeneous bioassays.

CONCLUSIONS

The TPE-FRET technique has shown its strong ability to circumvent the problem of autofluorescence in biological samples. It may be a promising model to ensure the accuracy and sensitivity of quantitative analysis, especially in complicated biological sample matrixes. We have not tested the ability of TPE-FRET to avoid the coexcitation of the acceptor since a dark quencher is used in this work, but such ability is reasonably predictable due to the near-IR light-excitation nature of the donor. For further development of the method in future studies, better donor−acceptor pairs are to be discovered. New TPE molecules with improved TPA cross section, fluorescence quantum yield, water solubility, and biocompatibility should be synthesized. Especially, as in vivo or intracellular analysis is concerned, an as-long-as-possible emission wavelength should also be pursued to obtain deeper penetration in tissues or cells. Meanwhile, more efficient energy acceptors, either fluorophores or dark quenchers, should be selected or produced to gain maximal FRET efficiency.

(27) Rantanen, T.; Järvenpää, M.-L.; Vuojola, J.; Kuningas, K.; Soukka, T. *Angew. Chem., Int. Ed.* **2008**, *47*, 3811–3813.

SUPPORTING INFORMATION AVAILABLE

Scheme for synthesizing TP-NH_2, synthesis procedures, determination of TPA cross section and fluorescence quantum yield of TP-NH_2, and calibration curve for determining the molar extinction coefficient of TP-NH_2. This material is available free of charge via the Internet at http://pubs.acs.org.

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