analytical chemistry

ARTICLE

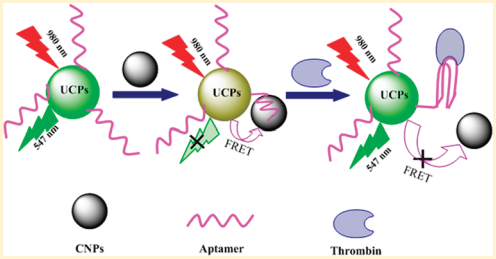
pubs.acs.org/ac

Aptamer Biosensor Based on Fluorescence Resonance Energy Transfer from Upconverting Phosphors to Carbon Nanoparticles for Thrombin Detection in Human Plasma

Yuhui Wang, Lei Bao, Zhihong Liu,* and Dai-Wen Pang*

Key Laboratory of Analytical Chemistry for Biology and Medicine (Ministry of Education), College of Chemistry and Molecular Sciences, Wuhan University, Wuhan 430072, China

ABSTRACT: We presented a new aptamer biosensor for thrombin in this work, which was based on fluorescence resonance energy transfer (FRET) from upconverting phosphors (UCPs) to carbon nanoparticles (CNPs). The poly(acrylic acid) (PAA) functionalized UCPs were covalently tagged with a thrombin aptamer (5′-NH_2- GGTTGGTGTGGTTGG-3′), which bound to the surface of CNPs through $\pi-\pi$ stacking interaction. As a result, the energy donor and acceptor were taken into close proximity, leading to the quenching of fluorescence of UCPs. A maximum fluorescence quenching rate of 89% was acquired under optimized conditions. In the presence of thrombin, which induced the aptamer to form quadruplex structure, the $\pi-\pi$ interaction was weakened, and thus, the acceptor was separated from the donor blocking the FRET process. The fluorescence of UCPs was therefore restored in a thrombin concentration-dependent manner, which built the foundation of thrombin quantification. The sensor provided a linear range from 0.5 to 20 nM for thrombin with a detection limit of 0.18 nM in an aqueous buffer. The same linear range was obtained in spiked human serum samples with a slightly higher detection limit (0.25 nM), demonstrating high robustness of the sensor in a complex biological sample matrix. As a practical application, the sensor was used to monitor thrombin level in human plasma with satisfactory results obtained. This is the first time that UCPs and CNPs were employed as a donor−acceptor pair to construct FRET-based biosensors, which utilized both the photophysical merits of UCPs and the superquenching ability of CNPs and thus afforded favorable analytical performances. This work also opened the opportunity to develop biosensors for other targets using this UCPs-CNPs system.



Determination of disease-related biomolecules in body fluids such as serum or plasma holds significant applications in clinical diagnosis. Taking advantage of high specificity of some intermolecular recognition reactions, such as antibody−antigen interaction, protein−aptamer recognition, and so on, it is possible to directly detect these biomolecules without pre-extracting them from the matrix, simplifying the assay procedures notably. Generally, two catalogs of assay protocols, i.e., heterogeneous and homogeneous assay, are applied in biological and clinical analysis. Heterogeneous assays, such as enzyme-linked immunosorbent assay (ELISA), are widely used and known for their high sensitivity because of the separation of unreacted or unbound biomolecules, but the tedious washing (separating) steps are time-consuming and make the procedure somewhat complicated.[1] In contrast, homogeneous methods are separation free and have the advantages of shorter assay time and simpler assay procedure, which makes high-throughput and high-speed screening possible. However, some background signals may arise due to the existence of numerous nontarget biomolecules, which lead to a lower signal-to-noise ratio and/or cause interference with the detection. Therefore, it would be of great significance to improve the analytical performances of homogeneous bioassays via facile and straightforward strategies.

Fluorescence resonance energy transfer (FRET) is a typical homogeneous assay technique, which is built on the basis of nonradiative energy transfer from energy donors to energy acceptors within close proximity (normally 1−10 nm) via long-range dipole−dipole interactions.[2] In a fine designed FRET model, the energy donors and acceptors are brought to a proper distance exclusively through the recognition of target substances; hence, FRET-based assays always show pronounced specificity. In combination with the high sensitivity of fluorescence, FRET methods have found broad applications in bioassays.[3−5] Nonetheless, as a kind of spectroscopic technique, FRET is much likely to suffer from some (mainly two) optical shortcomings, as described in quite a lot of literature. One major concern of FRET is related to the UV/vis-excitation nature of the energy donors, which normally are fluorescent dyes or proteins or semiconductor quantum dots. In this excitation window, unfortunately, rather strong autofluorescence and scattering light always arise from biomolecules when the assay is conducted in biological sample matrixes. Another issue of FRET assays is the unexpected coexcitation of the energy donor and the acceptor because of the overlap of their excitation spectra,[6,7] which at present is quite hard to eliminate due to the relatively small Stokes-shift of most down-converting fluorophores. Obviously, these limitations would

Received: June 26, 2011
Accepted: September 16, 2011
Published: September 16, 2011

lower the sensitivity or even reduce the reliability of FRET assays, especially in biological matrixes. In addition, the energy transfer efficiency between a selected donor−acceptor pair is also an important concern, which is a determinant factor for the detection sensitivity. Therefore, it is continuously desired to look for new energy donor−acceptor pairs, so as to circumvent the above shortcomings and to acquire improved FRET efficiency and analytical performances.

In the past few years, anti-Stokes fluorophores including two-photon excitable organic molecules and inorganic upconverting nanocrystals, which show visible emission with near-infrared (NIR) excitation, have attracted increasing interest in bioassays and been adopted as FRET donors.[8−13] Although the physical mechanisms underlying the excitation of two-photon molecules and upconverting phosphors (UCPs) are different, i.e., two-photon molecules must simultaneously absorb two NIR photons while UCPs absorb them sequentially because UCPs possess a stable intermediate state,[11] these two kinds of anti-Stokes fluorescent materials have the common advantage in bioassays conducted in complex biological matrixes. In those reports, anti-Stokes fluorophores have shown their capability of reducing autofluorescence and scattering light, thus enhancing the signal-to-noise ratio and sensitivity. In addition, the relatively low energy of NIR photons also leads to less photodamage of biological substances and less photobleaching of fluorophores, which is also valuable for FRET-based biological or clinical assays. Particularly, a remarkable merit of UCPs as FRET donors is the ability to avoid the coexcitation of the energy donor and the acceptor. With an NIR continuous wave laser (normally 980 nm for UCPs) as the light source, the direct excitation of energy acceptors (including organic molecules and inorganic particles) is physically impossible since they do not possess such an intermediate electronic energy state. Another notable advantage of upconverting materials is the very large Stokes shift (actually, it might be named as anti-Stokes shift), which produces neat emission spectra without any interference from the exciting light. For example, it is >400 nm for $NaYF_4$, Yb, and Er, the most commonly used UCPs. Considering the above features of UCPs as well as their high photoluminescence efficiency and chemical stability, upconverting nanocrystals have shown their promising prospects as candidates of energy donors for FRET assays.[14−16] By now, the upconverting-FRET technique has found successful applications in protein detection,[17,18] DNA sensing,[19,20] and metal ion detection.[21]

In another aspect, it is also of great importance to find energy acceptors with as large as possible power to quench the emission of donors. Generally, a larger quenching efficiency will lead to higher detection sensitivity in quantitative assays. As reported in the literature,[18] those normally used organic quenchers always cannot adequately quench the fluorescence of UCPs. This could be attributed to the structure of upconverting materials that only the emitters (rare earth ions) at or near the surface of the nanocrystals can be quenched. In previous studies, gold nanoparticle was found to be a good energy acceptor of UCPs, which offered a fluorescence quenching efficiency of up to 80%.[9,17] These years, nanosized carbon materials like carbon nanotubes, graphene, and graphene oxide have drawn increasing attention in bioassay, because of their unique structure and electronic properties, low biotoxicity, and easy acquisition.[22] In particular, the graphite-based structures with sp^2 electronic hybrid and a large conjugate plane have exhibited so-called superquenching ability to fluorescence, which may be explained with their strong electron-capturing tendency.[23,24] Using this superquenching power, several FRET sensors have been developed, employing graphene or graphene oxide as energy acceptors.[25−30] Most recently, we have constructed a FRET-based biosensing platform utilizing UCPs as an energy donor and graphene oxide as an acceptor.[31] Li et al. also employed the UCPs-graphene oxide system as an energy donor−acceptor pair with a high energy transfer efficiency.[32]

In comparison to the two-dimensional graphene (or graphene oxide) with a relatively large plane, the size of zero-dimensional carbon nanoparticles (CNPs) is more comparable with biomolecules. Lately, CNPs have inspired intensive research efforts in various fields, such as bioimaging and light energy conversion.[33,34] Since CNPs hold the similar sp^2 electronic structure with graphene,[33] it is reasonable to expect for their fluorescence quenching ability. However, the use of CNPs as FRET acceptor has rarely been reported.[35] In the present work, we have developed a new FRET sensor using UCPs and CNPs as the energy donor−acceptor pair for the first time. The sensor is designed for direct determination of thrombin, a specific serine protease playing an important role in physiological and pathological coagulation,[36] in human serum and plasma. Owing to the aforementioned merits of UCPs as an energy donor and the strong quenching ability of CNPs, satisfactory analytical performances have been acquired in both aqueous buffers and the body fluid samples.

EXPERIMENTAL SECTION

Materials and Instrumentations. All chemicals were of analytical grade or better and were used as received without further purification. Polyacrylic acid (with an average molecular weight of 1800), 1-ethyl-3-(3-dimethyllaminopropyl) carbodiimide hydrochloride (EDC.HCl), and sodium dodecyl benzene sulfonate (SDBS) were purchased from Sigma-Aldrich. Amine modified thrombin aptamer (5′-NH_2-GGTTGGTGTGGTTGG-3′) was supplied by Sangon Biotechnology Co., Ltd. (Shanghai, China). Thrombin, human IgG antibody, and bovine serum albumin (BSA) were from Zhongshan Golden Bridge Biotechnology Co., Ltd. (Beijing, China). 2-(*N*-morpholino) ethanesulfonic acid (MES) were obtained from Biosharp (USA). 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was the product of Alfa Aesar (Ward Hill, MA). Human factor Xa was purchased from New England Biolabs Ltd. The rest of the chemical reagents were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). All aqueous solutions were prepared using ultrapure water (Mill-Q, Millipore, 18.2 MΩ resistivity).

The crystal phase of UCPs were identified by a Bruker D8 Discover X-ray diffractometer (XRD) with 2θ range from 10° to 70° at a scanning rate of 4° per minute, with Cu Ka irradiation (k = 1.5406 Å). The sizes and morphologies of poly(acrylic acid) (PAA) modified $NaYF_4$:Yb, Er upconverting phosphors and carbon nanoparticles were characterized by a JEM-2010 transmission electron microscope (TEM) operated at 200 kV. FT-IR spectra of PAA-UCPs were measured on Magan-IR Spectrometer 500 (Nicolet, Madision, WI, USA) with the KBr pellet technique. The UV−vis absorption spectrum of carbon nanoparticles was acquired using a UV-2550 UV−vis spectrometer (Shimadzu, Japan). A 980 nm diode CW laser (Beijing Hi-Tech Optoelectronic Co., Ltd.) was used as the excitation source with the power being set at 500 mW. The upconverting fluorescence spectra were recorded on DCS200PC Photon Counting with single-photon sensitivity through an Omni-λ300 monochromator (Beijing Zolix Instruments Co., Ltd.).

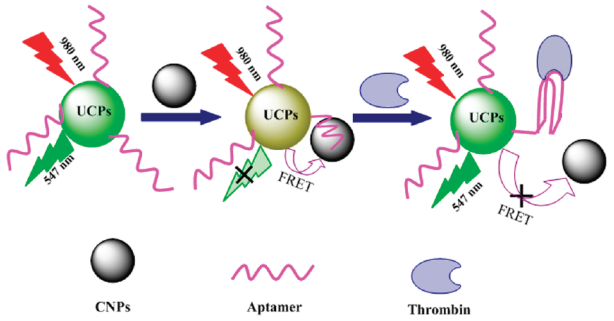
Preparation of PAA Modified UCPs. Water-soluble Yb, Er codoped $NaYF_4$ with dominant hexagonal phase was prepared using a one-pot hydrothermal method as described in our previous work.[31,37] Briefly, 0.25 mmol of lanthanide oxides Ln_2O_3 (Y/Yb/Er = 0.78:0.2:0.02, mole-to-mole ratio) was dissolved in hot nitric acid (65 °C) to acquire $Ln(NO_3)_3$, and the solvent was evaporated after a 6 h reaction. The as-obtained nitrate salts were added to the solution containing 900 mg poly(acrylic acid). Then, another aqueous solution containing 0.21 g of NaF was added dropwise to the above solution under vigorous stirring. Note that both the PAA solution and NaF solution were prepared using a mixed solvent ($V_{ethanol}/V_{water}$ = 1.0). Subsequently, the mixture (with a total volume of 36 mL) was transferred into a 50 mL Teflon autoclave and heated to 240 °C for a 10 h hydrothermal treatment. After the autoclave was cooled down to room temperature, a precipitate was obtained by centrifuging and washed several times with ultrapure water and absolute ethanol. The product was dried under vacuum before use.

Preparation of SDBS Stabilized Carbon Nanoparticles. Carbon nanoparticles were prepared with candle soot as starting material according to reported methods.[35,38] Candle soot (8 mg) was suspended in 20 mL of mixed solvent ($V_{water}/V_{ethanol}$ = 1:1), and the solution was sonicated for several hours. Then, the black mixture was centrifuged with 3000 rpm for 2 min to remove large-size particles. The supernatant was collected and centrifuged again for 6 min with 6000 rpm. A black precipitate, with a dry weight of ca. 2 mg, was obtained and dissolved in 20 mL of water containing 0.02 wt % SDBS under the sonication condition for 2 h. Finally, a black aqueous solution was acquired, and the concentration of CNPs was calculated as ca. 0.1 mg/mL.

Attachment of Thrombin Aptamer to Upconverting Nanoparticles. The amine modified thrombin aptamer was covalently conjugated to PAA-UCPs following the standard EDC protocol.[39] Briefly, 5 mg of PAA-UCPs was dissolved in 2 mL of MES buffer (10 mM, pH 5.5), and 0.8 mg of EDC.HCl (2 mM) and 2.2 mg of Sulfo-NHS (5 mM) were introduced to the solution to activate the carboxyl groups of PAA. The mixture was incubated at 30 °C with gentle shaking for 2 h. After centrifugation, the activated PAA-UCPs were collected and washed with ultrapure water for three times. The precipitate was then dispersed in 2 mL of HEPES buffer (10 mM, pH 7.2) containing 2.5 μM amine modified thrombin aptamer. After a 4 h reaction at 30 °C with slow shaking, 50 mg of Tris was added to block the excess NHS. Subsequently, the PAA-UCPs particles tagged with thrombin aptamer were harvested by centrifugation. The product was washed with ultrapure water for three times and finally diluted with 2.5 mL of Tris-HCl buffer (10 mM, 150 mM NaCl, pH 7.4) and stored at 4 °C in icebox for further use. The concentration of UCPs-aptamer was calculated as 2 mg/mL.

Thrombin Detection in Aqueous Solution and Serum. In a typical FRET assay in aqueous buffer, 0.036 mg/mL CNPs was added to 0.03 mg/mL UCPs-aptamer in Tris-HCl buffer (10 mM, pH 7.4, 150 mM NaCl). The mixture was incubated at 30 °C for 1.5 h. Thereafter, various concentrations of thrombin were added to the UCPs-aptamar-CNPs complexes, and the mixture was incubated for another 1.5 h at 30 °C. The reaction mixture was then subject to fluorescence measurement with the excitation of 980 nm. To examine the specificity of the FRET sensor, some other biomolecules and ions were added into the UCPs-aptamar-CNPs system in place of thrombin with the same experimental conditions. For the detection in serum matrix, newly obtained serum from a healthy man (provided by School of Medicine, Wuhan University) was 40-fold diluted with Tris-HCl buffer, which was then used as the assay medium. An identical detection procedure as in aqueous solution was followed for thrombin. The upconverting fluorescence was recorded with the excitation of 980 nm, and the emission intensity at 547 nm was taken for quantitative analysis.

Scheme 1. Schematic Illustration of the Thrombin Sensor Based on Fluorescence Resonance Energy Transfer from Aptamer-Modified Upconverting Phosphors to Carbon Nanoparticles[a]



[a] Note that the sizes of substances do not represent their real proportion.

Procedure for Monitoring Thrombin Level in Human Plasma. A normal human plasma sample collected in a citrate anticoagulated tube was provided by School of Medicine, Wuhan University. Fibrinogen was removed through precipitation according to the method reported in the literature.[40] In short, 0.25 mL of plasma was quickly mixed with 1.25 mL of ammonium sulfate (2 M) and 1 mL of NaCl (0.1 M). Four minutes later, the mixture was centrifuged and the upper supernatant solution was taken and used for further experiments. The protein amount was evaluated by absorbance at 280 nm, and a loss of protein content (40%) was calculated after precipitation. $CaCl_2$ (0.03 M) with 8 nM human factor Xa was then added to the plasma to promote the transformation from prothrombin to thrombin. The as-obtained plasma containing thrombin was then immediately introduced to the UCPs-aptamar-CNPs for detection. The subsequent incubation and fluorescence measurement procedures were the same as above.

RESULTS AND DISCUSSION

Principle of the UCPs-CNPs FRET Sensor for Thrombin. The thrombin sensor was constructed on the basis of aptamer-bridged fluorescence resonance energy transfer from UCPs to CNPs, as shown in Scheme 1. As a catalog of single-stranded (ssDNA) oligonucleotides which specifically react with certain proteins, ions, or viruses, aptamers have been broadly used in biosensing.[41−43] The thrombin-specific aptamer (5′-NH_2-GG-TTGGTGTGGTTGG-3′)[41] was tagged at 5′ end with an amino group, which was utilized to covalently link the aptamer to UCPs through the PAA carboxyl on the surface. Previous studies using graphene as an energy acceptor have illustrated the $\pi-\pi$ stacking interaction between single-stranded DNA and the π electron-rich carbon material.[25] In our design, the same $\pi-\pi$ interaction is expected to occur between the aptamer and CNPs, because of the sp^2 electronic hybrid structure of CNPs. On the basis of such interactions, the donor and the acceptor are taken into close proximity, which results in the occurrence of FRET and the

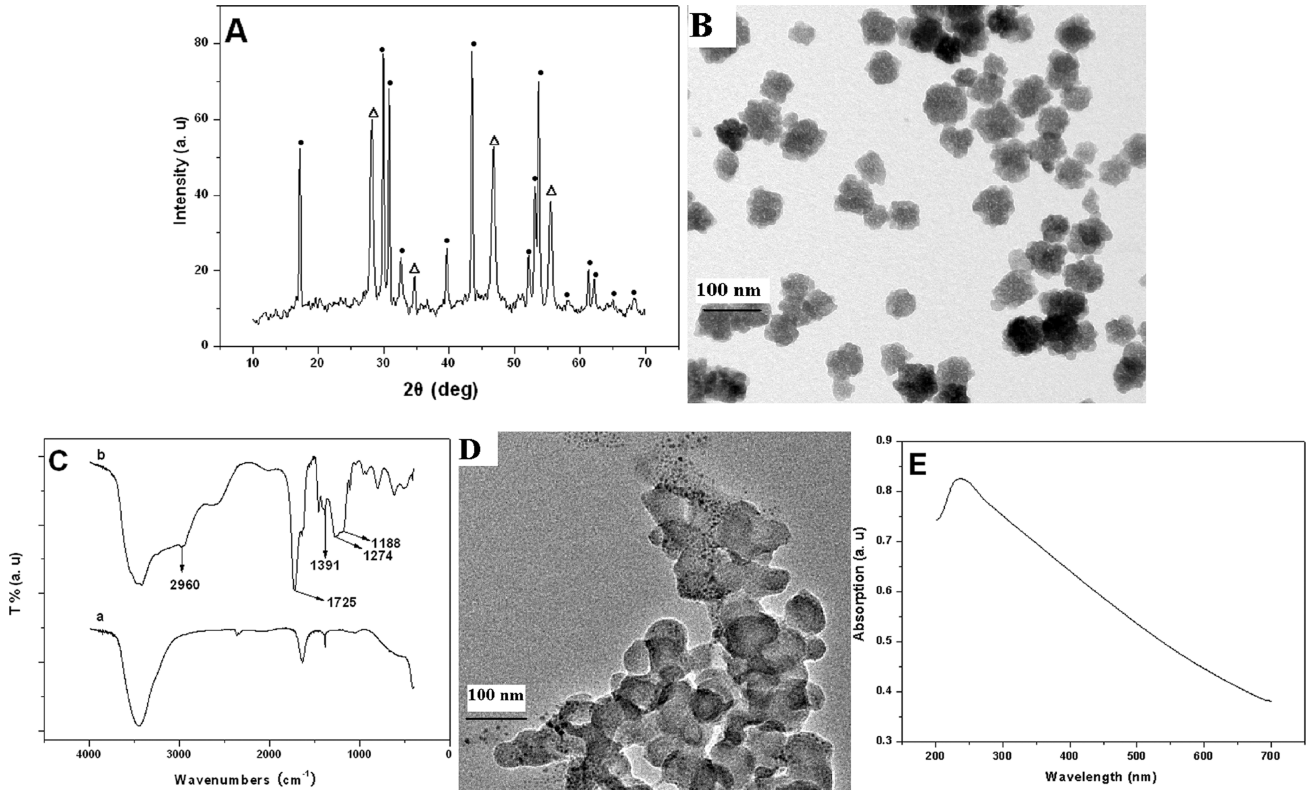


Figure 1. (A) XRD pattern of $NaYF_4$:Yb, Er nanocrystals. Δ, cubic phase (JCPDS file no. 77-2042); ●, hexagonal phase (JCPDS file no. 28-1192). (B) TEM image of the polycrylic acid coated $NaYF_4$:Yb, Er nanoparticles. (C) FT-IR spectra of UCPs without (curve a) and with (curve b) polycrylic acid functionalization. (D) TEM image of carbon nanoparticles. (E) UV−vis absorption spectrum of the as-prepared carbon nanoparticles with a concentration of 0.02 mg/mL.

fluorescence quenching of UCPs. It can be seen that the FRET system is quite simple in construction since only one labeling step is involved. In addition, because the size of the label (aptamer) is small, the distance between the donor and the acceptor can be adequately close, which ensures high energy transfer efficiency. With the introduction of thrombin to the as-formed UCPs-aptamer-CNPs system, the formation of quadruplex-thrombin complex will lead to the disappearance of the $\pi-\pi$ stacking interaction, thus CNPs separating from UCPs. Therefore, the FRET process is inhibited, and the fluorescence of UCPs is recovered, which is the foundation of the quantification of thrombin.

Characterization of the Energy Donor and Acceptor. To realize the above design, the energy donor and acceptor were first synthesized and characterized. $NaYF_4$:Yb, Er was selected as the energy donor since it is so far the most efficient upconverting material due to the low phonon energy of $NaYF_4$ as host matrix. Employing the one-pot synthesis recently developed in our lab,[31] $NaYF_4$:Yb, Er nanocrystals were obtained consisting of a dominant hexagonal phase with a small amount of cubic phase, as demonstrated by the XRD pattern in Figure 1A. It is known that the hexagonal phase $NaYF_4$:Yb, Er exhibits an order of magnitude higher upconverting luminescence efficiency than cubic phase.[44] Polyacrylic acid was used to functionalize UCPs via the chelation between PAA carboxyl and surface Ln^{3+} of UCPs. The carboxyl groups on PAA could endow the nanoparticles with both water-solubility and reactive sites for coupling of biomolecules, which is essential for bioanalytical applications. The TEM image in Figure 1B shows that the PAA coated $NaYF_4$:Yb, Er particles, which possess fairly uniform size and an average diameter of ca. 50 nm, are highly dispersible in water. To further verify the existence of carboxyl on the surface of UCPs, FT-IR spectra of PAA modified and bare UCPs were measured and compared in Figure 1C. The spectrum of PAA functionalized UCPs (curve b) shows typical absorption peaks of methylene asymmetric C−H stretching (2960 cm^{-1}), C═O stretching vibration (1725 cm^{-1}), C−O stretching (1188−1274 cm^{-1}), and asymmetric and symmetric COO^- stretching (1391 cm^{-1}), which approves successful coating of PAA molecules on surface of UCPs. Figure 1D shows the TEM image of the acceptor, i.e., carbon nanoparticles, which exhibit diameter distribution from 40 to 60 nm. As compared to the previously reported graphene or graphene oxide which generally have micrometer length and width, the nano-sized carbon particles would be more comparable to biological substances. Besides such biocomparability, the size of carbon particles also exhibits higher uniformity as compared to graphene. As we can see in the literature, graphene materials always show quite broad dimensions, e.g., from nanometers to micrometers in sheet width.[29] It is recognized that more uniform size of assay agents would be more favorable for analytical performances, such as the reproducibility of detections. We know that pure carbon materials are insoluble in aqueous solutions and that the use of amphiphilic surfactants could be a simple yet effective solution to this problem. Considering the acidity of poly(acrylic acid) (pK_a = 4.75), which makes the UCPs particles negatively charged at neutral pH (the appropriate pH for bioassay), anionic surfactants should be chosen to exclude electrostatic attraction between UCPs and CNPs. Therefore, sodium dodecyl benzene sulfonate, a widely used solubilizer, was used to acquire dispersibility of

dx.doi.org/10.1021/ac201631b |*Anal. Chem.* 2011, 83, 8130–8137

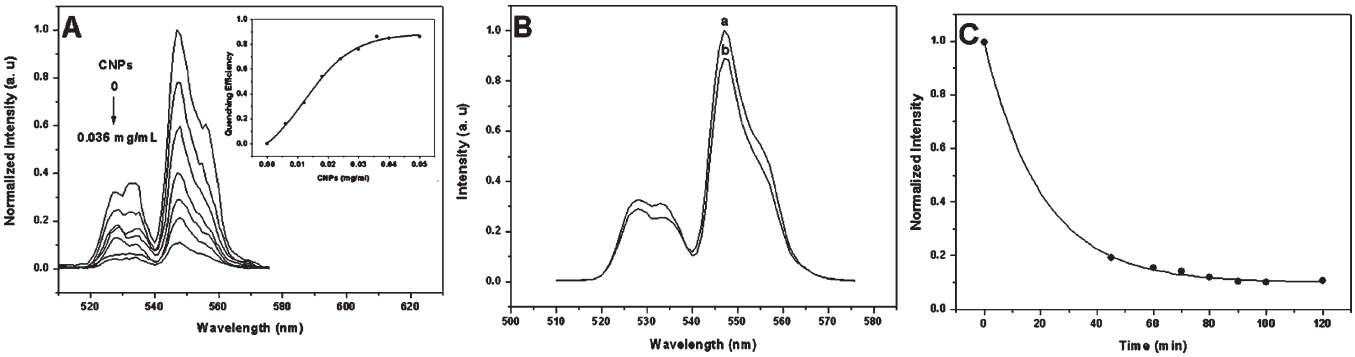


Figure 2. (A) Fluorescence quenching of UCPs-aptamer (0.03 mg/mL) with varying amounts of CNPs. Inset: upconverting fluorescence intensity versus CNPs concentration (0, 0.006, 0.012, 0.02, 0.03, 0.036, 0.04, and 0.05 mg/mL). (B) Fluorescence of 0.03 mg/mL PAA coated UCPs in the absence (curve a) and presence (curve b) of 0.036 mg/mL CNPs. (C) Time dependence of the fluorescence quenching with 0.03 mg/mL UCPs-aptamer and 0.036 mg/mL CNPs. All experiments were performed in Tris-HCl buffer (0.01 M, 0.15 M NaCl, pH 7.4) under excitation at 980 nm. Fluorescence was given in normalized form.

CNPs in water in subsequent experiments. Figure 1E shows the UV−vis absorption spectrum of the as-prepared carbon nanoparticles. The optical band spans a wide range of wavelength (approximately 200−700 nm), which is similar with that of graphene materials.[26,32] Such a broad absorption band makes CNPs possible energy acceptors for a variety of donors.

FRET between Aptamer Labeled UCPs and Carbon Nanoparticles. ssDNA has been reported to noncovalently bind to and assembly on the surface of aromatic nanocarbon including graphene and carbon nanotubes[28,45,46] In our experiments, as expected, the fluorescence intensity of UCPs exhibited gradual decrease with adding increasing amounts of CNPs to the UCPs-aptamer solution (Figure 2A). With the UCPs concentration being fixed at 0.03 mg/mL, the fluorescence quenching percentage reached a maximum of ca. 89% with 0.036 mg/mL of CNPs, and a plateau was observed when further increasing the concentration of CNPs (inset in Figure 2A). Such a quenching efficiency of UCPs fluorescence is better than all reported cases with UCPs as energy donors and organic dyes as energy acceptors (quenchers), revealing the superquenching ability of CNPs. In another hand concerning the donor, not surprisingly, such a quenching rate is not so high as that of an organic donor-CNPs system, which could reach to nearly 100% at certain concentrations.[35] This can be explained with the structural feature of UCPs materials as mentioned above, that is, a small amount of interior emitters (rare ions) cannot be quenched because of the protection of outer layers as well as the relatively long distance from quenchers. Although, as an energy donor, the fluorescence quenching rate of UCPs is inherently lower than organic fluorophores, the unmatched photoluminescence efficiency and the aforementioned optical merits of UCPs still make them competitive FRET donors. According to the theoretical predictions by Swathi et al.[23,24] and some experimental results,[30] the energy transfer from fluorophores to carbon acceptors can be defined as a long-range interaction named surface energy transfer (SET). The fluorescence quenching can be observed with a distance of up to 300 Å, and the rate of this long-range SET is suggested to have a $(\text{distance})^{-4}$ dependence, which is different with the Forster resonance energy transfer where the rate has a $(\text{distance})^{-6}$ dependence. Such SET process was also revealed in FRET systems with other nanomaterials (gold nanoparticles) as the energy acceptor.[47] Therefore, the fluorescence quenching of UCPs by CNPs could probably be attributed to a long-range surface energy transfer, and a detailed mechanism investigation is right now in process in our lab.

A control experiment was performed to investigate nonspecific quenching of UCPs fluorescence, in which 0.03 mg/mL PAA-UCPs (no aptamer tagged) were mixed with 0.036 mg/mL CNPs. After an identical incubation procedure, the fluorescence was lowered by ca. 11% (Figure 2B). The results show that slight nonspecific UCPs-CNPs interaction occurred, but the quenching of UCPs fluorescence can be mainly attributed to the aptamer-bridged FRET from the donor to acceptor, as a result of the $\pi-\pi$ stacking between aptamer and CNPs. Since the subsequent thrombin determination is based on the enhancement of fluorescence of the UCPs-aptamer-CNPs sensor (which is a fixed value), the existence of such nonspecific interaction would not affect the accuracy of thrombin quantification. Figure 2C shows the time dependence of fluorescence quenching, which reveals that the maximal quenching rate was achieved with ca. 90 min incubation, and a plateau was observed with further longer reaction time. To ensure complete quenching and obtain stable signal, 90 min of incubation was adopted in this step.

Thrombin Sensing in Aqueous Buffer. Determination of thrombin with the above aptamer sensor was first conducted in Tris-HCl buffer. With the addition of free thrombin to the UCPs-aptamer-CNPs system, the aptamer was induced to form quadruplex structure,[48] which dramatically weakened the $\pi-\pi$ interaction. Therefore, the acceptor was separated away from the donor, resulting in the restoration of the donor fluorescence. The time course of fluorescence restoration was measured and shown in Figure 3A, which demonstrates that the donor fluorescence could recover to the maximal extent with ca. 75 min reaction and thereafter kept quite stable. It is worthy to point out here the very stable luminescence of UCPs as exhibited in this experiment, which also has been repeatedly verified in the reported literature and is considered as an outstanding advantage of upconverting phosphors. With a fixed amount of the aptamer sensor (0.03 mg/mL UCPs-aptamer plus 0.036 mg/mL CNPs), the donor fluorescence was enhanced, dependent on thrombin concentration (Figure 3B). The donor fluorescence restoration was linearly related to thrombin within the concentration range from 0.5 nM to 20 nM (Figure 3C). The detection limit of thrombin was calculated as 0.18 nM according to the $3s_b/m$ criterion, where m is the slope for the range of the linearity used and s_b is the standard deviation of the blank (n = 11).

dx.doi.org/10.1021/ac201631b |*Anal. Chem.* 2011, 83, 8130–8137

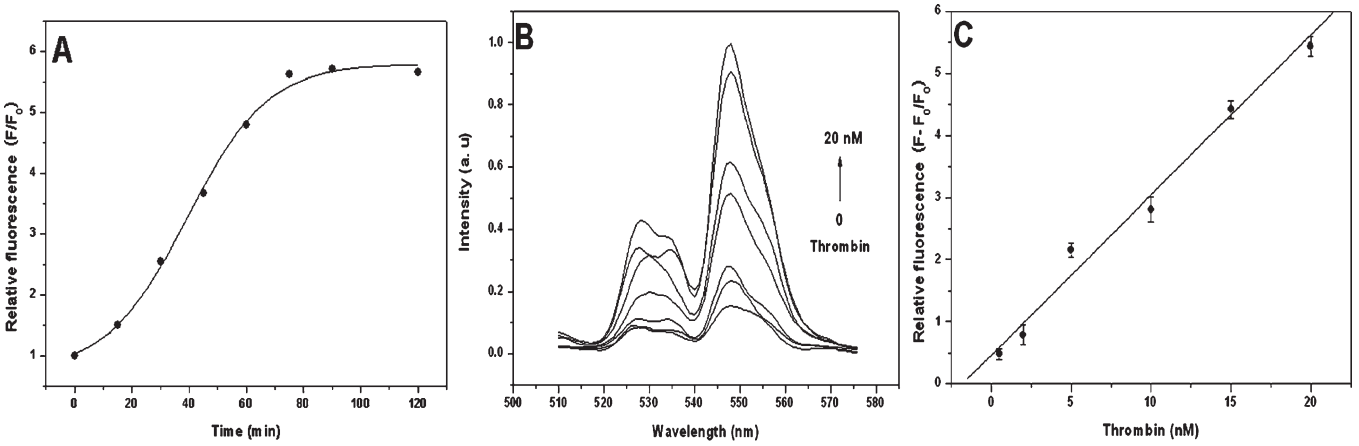


Figure 3. (A) Time course of the restoration of UCPs fluorescence. F and F_0 represent the upconverting fluorescence intensity in the presence and absence of 20 nM thrombin, respectively. (B) The fluorescence recovery of aptamer sensor with various concentrations of thrombin (0, 0.5, 2, 5, 10, 15, and 20 nM). (C) The linear relationship between the fluorescence recovery and the concentration of thrombin within the range of 0.5−20 nM, data were presented as average ± sd from three independent measurements. All experiments were performed in Tris-HCl buffer (0.01 M, 0.15 M NaCl, pH 7.4) under excitation at 980 nm, in the presence of 0.03 mg/mL UCPs-aptamer and 0.036 mg/mL CNPs. Fluorescence was given in normalized form.

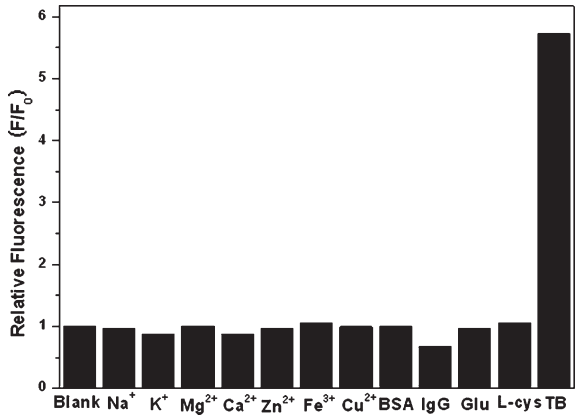


Figure 4. Relative fluorescence intensity (F/F_0) of the aptamer biosensor in the presence of different substances. The concentration of all interfering species was 1 μM, and thrombin was 20 nM. Experiments were performed in Tris-HCl buffer (0.01 M, 0.15 M NaCl, pH 7.4) under excitation at 980 nm, in the presence of 0.03 mg/mL UCPs-aptamer and 0.036 mg/mL CNPs.

Specificity of the Aptamer Sensor for Thrombin. To assess the specificity of the FRET-based aptamer sensor for thrombin, the influences of some biological species including metal ions, amino acids, and proteins were examined in aqueous buffer. To the above constructed aptamer sensor, 1 μM inspected species were added individually and incubated, the fluorescence of which were compared to that of the biosensor (denoted as blank in Figure 4). It is seen from Figure 4 that none of these substances caused obvious fluorescence alteration even with a concentration as high as 1 μM, while only 20 nM of thrombin resulted in significant fluorescence enhancement. The results have thus clearly illustrated the specificity of the aptamer sensor for thrombin.

Thrombin Sensing in Human Serum. A set of more meaningful thrombin determination with the aptamer sensor was conducted in spiked human serum, which was aimed at investigating the ability of the UCPs-CNPs FRET system to overcome interference from background fluorescence and scattered light. Serum is a recognized complex biological matrix which contains a large number of biomolecules but no coagulation proteins such as thrombin or other factors.[49] With 40-fold diluted (with Tris-HCl buffer so as to keep the pH at 7.4) human serum as the assay medium, the same thrombin-dependent fluorescence restoration as that in aqueous buffer was observed (Figure 5A), except for a slightly higher background signal (compare the curve with zero thrombin to that of Figure 3B). This little amount of background could not autofluorescence from any biomolecules since the excitation of the molecules with 980 nm light is impossible. It might probably be the scattered exciting light which is substantially scaled down as compared to the case with shorter-wavelength UV/vis light excitation. Significantly, a linear range for thrombin was also obtained in the diluted serum, which was identical to that in aqueous buffer, that is, from 0.5 to 20 nM (Figure 5B). Besides, good reproducibility was also obtained in the serum matrix, similar with that in Tris-HCl buffer. The only difference in analytical performance between the two assay media was the slightly higher limit of detection in the serum matrix, which was 0.25 nM ($3s_b/m$ criterion). These results have shown high robustness of the UCPs-CNPs FRET-based biosensor in the complex matrix, suggesting that the sensor may be competent for monitoring thrombin in situ in blood samples.

Monitoring Thrombin Level in Plasma with the Aptamer Sensor. The FRET-based aptamer sensor was subsequently applied to monitor the thrombin level in human plasma. In order to avoid clotting which will deplete thrombin, fibrinogen in plasma samples was preremoved with ammonium sulfate precipitation. Thrombin was then generated from its precursor, prothrombin, with Ca^{2+} activation.[50,51] The thus obtained plasma samples containing thrombin, also 40-fold diluted with Tris-HCl buffer, were introduced to the aptamer sensor. Three independent plasma samples were tested with the results shown in Table 1. Taking the 40-fold dilution into calculation, the concentrations of thrombin of the three samples were determined as 128, 72, and 112 nM, respectively, using the calibration curve obtained in the human serum matrix, which are consistent with the reported levels.[40,52] Furthermore, standard addition experiments were performed with these plasma samples to validate the determination. The recoveries were from 96% to 116% with RSD around 5% (Table 1), which

dx.doi.org/10.1021/ac201631b |*Anal. Chem.* 2011, 83, 8130–8137

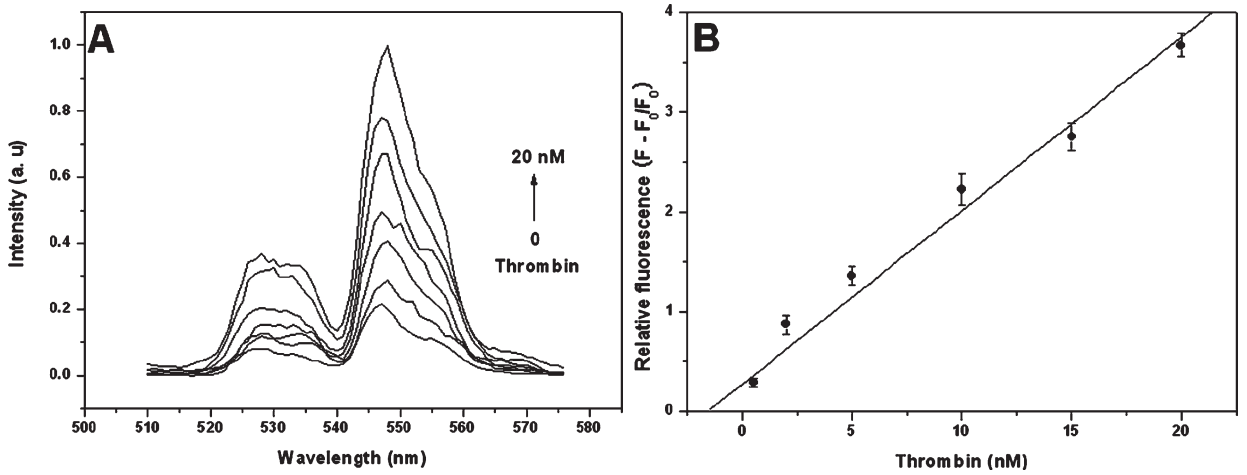


Figure 5. (A) Fluorescence recovery of aptamer sensor with various concentrations of thrombin (0, 0.5, 2, 5, 10, 15, and 20 nM). (B). The linear relationship between the fluorescence recovery and the concentration of thrombin within the range of 0.5−20 nM, data were presented as average ± sd from three independent measurements. Experiments were performed in human serum 40-fold diluted with Tris-HCl buffer (0.01 M, 0.15 M NaCl, pH 7.4) with the excitation at 980 nm.

Table 1. Analytical Results of the Direct Determination of Thrombin in Three Human Plasma Samples Using the Aptamer Biosensor

sample	measured (nM)	added (nM)	found (nM)	recovery	RSD ($n = 3$)
1	3.2	10	12.8	96%	4.8%
2	1.8	10	13.4	116%	5.2%
3	2.8	10	13.3	105%	4.2%

are acceptable for quantitative assays performed in biological samples.

Previously, some interesting aptamer-based thrombin biosensors built on various platforms have been constructed. Tennico et al. developed an on-chip aptamer-based fluorescence assay for thrombin detection and quantification using sandwich ELISA principles.[53] This strategy integrated microfluidics with the merits of magnetic beads and quantum dots, resulting in the accelerated assay procedures and the possibility of high throughput screening for multiple samples. A thrombin biosensor using time-resolved fluorescence technique was contributed by Huang et al., which could be utilized in both buffer solution and serum taking advantages of long-life fluorophores.[54] Compared to these methods which either involve rather tedious operations or require expensive instrument, the proposed UCPs-CNPs FRET method is a simple and low-cost alternative for homogeneous thrombin determination, with the sensitivity higher than these reports. Another homogeneous assay for thrombin based on graphene FRET platform was recently developed by Chang et al. with remarkably high sensitivity.[28] Using a fluorescein dye as the energy donor and graphene as the acceptor, a detection limit of as low as 31.3 pM was acquired in an aqueous solution. However, in that work, the applicability of the sensor in complex sample matrixes was not explored and no quantitative determination was conducted in real samples. In sharp contrast, in our work using the UCPs-CNPs FRET, we have successfully performed thrombin quantification in both an aqueous solution and human serum. Also, quantitative thrombin assay was achieved in plasma samples. In one word, as a brand new analytical method, UCPs-CNPs FRET has already shown its potential in bioanalytical chemistry. Moreover, there is no doubt that the analytical performances including the detection sensitivity can be further improved in future studies, through the optimization of the properties of UCPs and CNPs as well as detection conditions.

CONCLUSIONS

We have constructed a new aptamer biosensor based on fluorescence resonance energy transfer from upconverting phosphors to carbon nanoparticles. The sensor can be used for thrombin sensing both in an aqueous buffer and in a serum matrix with comparable performances, proving that the UCPs-CNPs FRET system is capable of overcoming background interference in complex biological samples. The sensor has been applied to monitor thrombin level in human plasma with satisfactory results. The anti-Stokes emission and NIR-excitation nature of UCPs makes them a promising energy donor for FRET assay in complex biological samples. In combination with the strong fluorescence quenching ability and good biocomparability of carbon nanoparticles, the UCPs-CNPs system could be a competitive energy donor−acceptor pair, which will contribute to FRET technique as well as FRET-based analytical applications. Owing to the facile fabrication, the sensor could be readily developed to build up sensing platforms for various targets by linking different aptamers or other ligands to UCPs. Further studies looking into the energy transfer mechanism between UCPs and the carbon material would be desired to gain more comprehensive understanding and better applications.

AUTHOR INFORMATION

Corresponding Author

*Z.L.: e-mail, zhhliu@whu.edu.cn; tel, 86-27-8721-7886; fax, 86-27-6875-4067. D.-W.P.: e-mail, dwpang@whu.edu.cn; tel, 86-27-68756759.

ACKNOWLEDGMENT

The authors thank the National Natural Science Foundation of China (Nos. 21075094 and 20833006), the Science Fund for

Creative Research Groups (20621502 and 20921062), and the National Basic Research Program of China (973 Program, No. 2011CB933600) for financial support.

REFERENCES

(1) Swartzman, E. E.; Miraglia, S. J.; Mellentin-Michelotti, J.; Evangelista, L.; Yuan, P. M. *Anal. Biochem.* **1999**, *271*, 143–151.
(2) Sapsford, K. E.; Berti, L.; Medintz, I. L. *Angew. Chem., Int. Ed.* **2006**, *45*, 4562–4588.
(3) Pickup, J. C.; Hussain, F.; Evans, N. D.; Rolinski, O. J.; Birch, D. J. S. *Biosens. Bioelectron.* **2005**, *20*, 2555–2565.
(4) Wang, S.; Mamedova, N.; Kotov, N. A.; Chen, W.; Studer, J. *Nano Lett.* **2002**, *2*, 817–822.
(5) Oswald, B.; Lehmann, F.; Simon, L.; Terpetschnig, E.; Wolfbeis, O. S. *Anal. Biochem.* **2000**, *280*, 272–277.
(6) Schmid, J. A.; Scholze, P.; Kudlacek, O.; Freissmuth, M.; Singer, E. A.; Sitte, H. H. *J. Biol. Chem.* **2001**, *276*, 3805–3810.
(7) Willard, D. M.; Carillo, L. L.; Jung, J.; Orden, A. V. *Nano Lett.* **2001**, *1*, 469–474.
(8) Kuningas, K.; Rantanen, T.; Ukonaho, T.; Lövgren, T.; Soukka, T. *Anal. Chem.* **2005**, *77*, 7348–7355.
(9) Wang, L.; Yan, R.; Huo, Z.; Wang, L.; Zeng, J.; Bao, J.; Wang, X.; Peng, Q.; Li, Y. *Angew. Chem., Int. Ed.* **2005**, *44*, 6054–6057.
(10) Clapp, A. R.; Pons, T.; Medintz, I. L.; Delehanty, J. B.; Melinger, J. S.; Tiefenbrunn, T.; Dawson, P. E.; Fisher, B. R.; O'Rourke, B.; Mattoussi, H. *Adv. Mater.* **2007**, *19*, 1921–1926.
(11) Liu, L.; Shao, M.; Dong, X.; Yu, X.; Liu, Z.; He, Z.; Wang, Q. *Anal. Chem.* **2008**, *80*, 7735–7741.
(12) Liu, L.; Wei, G.; Liu, Z.; He, Z.; Xiao, S.; Wang, Q. *Bioconjugate Chem.* **2008**, *19*, 574–579.
(13) Liu, L.; Dong, X.; Lian, W.; Peng, X.; Liu, Z.; He, Z.; Wang, Q. *Anal. Chem.* **2010**, *82*, 1381–1388.
(14) Kuningas, K.; Ukonaho, T.; Päkkilä, H.; Rantanen, T.; Rosenberg, J.; Lövgren, T.; Soukka, T. *Anal. Chem.* **2006**, *78*, 4690–4696.
(15) Rantanen, T.; Päkkilä, H.; Jämsen, L.; Kuningas, K.; Ukonaho, T.; Lövgren, T.; Soukka, T. *Anal. Chem.* **2007**, *79*, 6312–6318.
(16) Kuningas, K.; Päkkilä, H.; Ukonaho, T.; Rantanen, T.; Lövgren, T.; Soukka, T. *Clin. Chem.* **2007**, *53*, 145–146.
(17) Wang, M.; Hou, W.; Mi, C. C.; Wang, W. X.; Xu, Z. R.; Teng, H. H.; Mao, C. B.; Xu, S. K. *Anal. Chem.* **2009**, *81*, 8783–8789.
(18) Rantanen, T.; Järvenpää, M. L.; Vuojola, J.; Kuningas, K.; Soukka, T. *Angrew. Chem., Int. Ed.* **2008**, *47*, 3811–3813.
(19) Zhang, P.; Rogelj, S.; Nguyen, K.; Wheeler, D. *J. Am. Chem. Soc.* **2006**, *128*, 12410–12411.
(20) Chen, Z.; Chen, H.; Hu, H.; Yu, M.; Li, F.; Zhang, Q.; Zhou, Z.; Yi, T.; Huang, C. *J. Am. Chem. Soc.* **2008**, *130*, 3023–3029.
(21) Kumar, M.; Zhang, P. *Biosens. Bioelectron.* **2010**, *25*, 2431–2435.
(22) Yang, W.; Ratinac, K. R.; Ringer., S. P.; Thordarson, P.; Gooding., J. J.; Braet., F. *Angew. Chem., Int. Ed.* **2010**, *49*, 2114–2138.
(23) Swathi, R. S.; Sebastian, K. L. *J. Chem. Phys.* **2008**, *129*, 054703.
(24) Swathi, R. S.; Sebastian, K. L. *J. Chem. Phys.* **2009**, *130*, 086101.
(25) Lu, C. H.; Yang, H. H.; Zhu, C. L.; Chen, X.; Chen, G. N. *Angew. Chem., Int. Ed.* **2009**, *48*, 4785–4787.
(26) Dong, H.; Gao, W.; Yan, F.; Ji, H.; Ju, H. *Anal. Chem.* **2010**, *82*, 5511–5517.
(27) Wu, W.; Hu, H.; Li, F.; Wang, L.; Gao, J.; Lu, J.; Fan, C. *Chem. Commun.* **2011**, *47*, 1201–1203.
(28) Chang, H.; Tang, L.; Wang, Y.; Jiang, J.; Li, J. *Anal. Chem.* **2010**, *82*, 2341–2346.
(29) Jang, H.; Kim, Y. K.; Kwon, H. M.; Yeo, W. S.; Kim, D. E.; Min, D. H. *Angew. Chem., Int. Ed.* **2010**, *49*, 5703–5707.
(30) Liu, M.; Zhao, H.; Quan, X.; Chen, S.; Fan, X. *Chem. Commun.* **2010**, *46*, 7909–7911.
(31) Zhang, C.; Yuan, Y.; Zhang, S.; Wang, Y.; Liu, Z. *Angew. Chem., Int. Ed.* **2011**, *50*, 6851–6854.
(32) Liu, C.; Wang, Z.; Jia, H.; Li, Z. *Chem. Commun.* **2011**, *47*, 4661–4663.
(33) Baker, S. N.; Baker, G. A. *Angew. Chem., Int. Ed.* **2010**, *49*, 6726–6744.
(34) Wang, X.; Cao, L.; Lu, F.; Meziani, M. J.; Li, H.; Qi, G.; Zhou, B.; Harruff, B. A.; Kermarrec, F.; Sun, Y. *Chem. Commun.* **2009**, 3774–3776.
(35) Li, H.; Zhang, Y.; Wang, L.; Tian, J.; Sun, X. *Chem. Commun.* **2011**, *47*, 961–963.
(36) Hu, J.; Zheng, P.; Jiang, J.; Shen, G.; Yu, R.; Liu, G. *Anal. Chem.* **2009**, *81*, 87–93.
(37) Wang, Y.; Cai, R.; Liu, Z. *CrystEngComm* **2011**, *13*, 1772–1774.
(38) Liu, H.; Ye, T.; Mao, C. *Angew. Chem., Int. Ed.* **2007**, *46*, 6473–6475.
(39) Hermanson, G. T. *Bioconjugate Techniques*, 2nd ed.; Academic: San Diego, 2008, 595.
(40) Chen, X.; Liu, H.; Zhou, X.; Hu, J. *Nanoscale* **2010**, *2*, 2841–2846.
(41) Rahman, M. A.; Son, J. I.; Won, M. S.; Shim, Y. B. *Anal. Chem.* **2009**, *81*, 6604–6611.
(42) Zhang, Y.; Huang, Y.; Jiang, J.; Shen, G.; Yu, R. *J. Am. Chem. Soc.* **2007**, *129*, 15448–15449.
(43) Huang, Y.; Chang, H. T.; Tan, W. *Anal. Chem.* **2008**, *80*, 567–572.
(44) Krämer, K, W.; Biner, D.; Frei, G.; Güdel, H. U.; Hehlen, M. P.; Lüthi, S. R. *Chem. Mater.* **2004**, *16*, 1244–1251.
(45) Zheng, M.; Jagota, A.; Semke, E. D.; Diner, B. A.; Mclean, R. S.; Lustig, S. R.; Richardson, R. E.; Tassi, N. G. *Nat. Mater.* **2003**, *2*, 338–342.
(46) Zheng, M.; Jagota, A.; Strano, M. S.; Santos, A. P.; Barone, P.; Chou, S. G.; Diner, B. A.; Dresselhaus, M. S.; Mclean, R. S.; Onoa, G. B.; Samsonidze, G. G.; Semke, E. D.; Usrey, M.; Walls, D. J. *Science* **2003**, *302*, 1545–1548.
(47) Yun, C. S.; Javier, A; Jennings, T.; Fisher, M.; Hira, S.; Peterson, S.; Hopkins, B.; Reich, N. O.; Strouse, G. F. *J. Am. Chem. Soc.* **2005**, *127*, 3115–3119.
(48) Willner, I.; Zayats, M. *Angew. Chem., Int. Ed.* **2007**, *46*, 6408–6418.
(49) Bini, A.; Minunni, M.; Tombelli, S.; Centi, S.; Mascini, M. *Anal. Chem.* **2007**, *79*, 3016–3019.
(50) Shuman, M. A.; Majerus, P. W. *J. Clin. Invest.* **1976**, *58*, 1249–1258.
(51) Zolton, R. P.; Seegers, W. H. *Thromb. Res.* **1973**, *3*, 23–33.
(52) Centi, S.; Tombelli, S.; Minunni, M.; Mascini, M. *Anal. Chem.* **2007**, *79*, 1466–1473.
(53) Tennico, Y. H.; Hutanu, D.; Koesdjojo, M. T.; Bartel, C. M.; Remcho, V. T. *Anal. Chem.* **2010**, *82*, 5591–5597.
(54) Huang, D. W.; Niu, C. G.; Qin, P. Z.; Ruan, M.; Zeng, G. M. *Talanta* **2010**, *83*, 185–189.