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Upconversion Fluorescence Resonance Energy Transfer Based Biosensor for Ultrasensitive Detection of Matrix Metalloproteinase-2 in Blood

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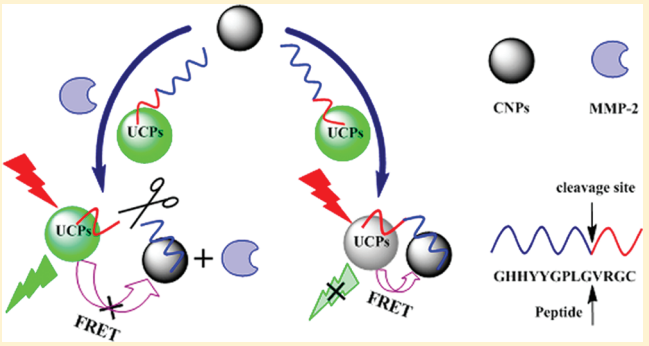
ABSTRACT: Matrix metalloproteinase-2 (MMP-2) is a very important biomarker in blood. Presently, sensitive and selective determination of MMP-2 directly in blood samples is still a challenging job because of the high complexity of the sample matrix. In this work, we reported a new homogeneous biosensor for MMP-2 based on fluorescence resonance energy transfer (FRET) from upconversion phosphors (UCPs) to carbon nanoparticles (CNPs). A polypeptide chain (NH_2-GHHYYGPLGVRGC-COOH) comprising both the specific MMP-2 substrate domain (PLGVR) and a π-rich motif (HHYY) was designed and linked to the surface of UCPs at the C terminus. The FRET process was initiated by the $\pi-\pi$ interaction between the peptide and CNPs, which thus quenched the fluorescence of the donor. Upon the cleavage of the substrate by the protease at the amide bond between Gly and Val, the donor was separated from the acceptor while the π-rich motif stayed on the acceptor. As a result, the fluorescence of the donor was restored. The fluorescence recovery was found to be proportional to the concentration of MMP-2 within the range from 10−500 pg/mL in an aqueous solution. The quantification limit of this sensor was at least 1 order of magnitude lower than that of other reported assays for MMP-2. The sensor was used to determine the MMP-2 level directly in human plasma and whole blood samples with satisfactory results obtained. Owing to the hypersensitivity of the method, clinical samples of only less than 1 μL were needed for accurate quantification, which can be meaningful in MMP-2-related clinical and bioanalytical applications.



Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptides that are able to degrade extracellular matrix and basement membrane components.[1] Particularly, MMP-2 (also known as gelatinase A) is able to degrade type VI collagen and thus not only plays a key role in physiological and pathological states including morphogenesis, reproduction, and tissue remodeling, but also is one of the crucial MMPs in tumor growth, invasion, and metastasis.[2−4] Studies have shown that almost every type of human cancer,[5] diabetes,[6,7] and hypertension[8,9] has a close relationship with MMP-2, with its concentration in blood increasing to varying degrees. Hence, the quantification of MMP-2 is significant for disease diagnosis and therapy. Due to the rather low level of MMP-2 in human blood as well as the high complexity of the clinical samples such as serum, plasma, or even the whole blood, it is imperative and still challenging to develop analytical methods with high sensitivity and promising ability to circumvent the interferences arising from the complex biological sample matrixes.

Like many other blood proteins, the conventional and most widely used method for MMP-2 detection is enzyme-linked immunosorbent assay (ELISA), which has been well recognized in clinical assay and can afford satisfying sensitivity and good selectivity.[10] Nonetheless, an obvious limitation of such a heterogeneous assay is the requirement of tedious separation (washing) operations, which inevitably complicate the assay procedure; another concern of ELISA could be the involvement of costly antibody proteins. The antigen−antibody immunoreaction was also adopted in some other analytical models for MMP-2 detection. For example, a particle-enhanced sandwich assay for the quantification of MMP-2 using surface plasmon resonance (SPR) was reported with relatively easier operations as compared to ELISA,[11] but it was still restricted by the requirement of those monoclonal antibodies and inhibitors, which are not easy to acquire and tend to lose their activities quickly. Gel electrophoresis-based gelatin zymography is a useful tool for measuring MMP-2 activity in biological samples such as cells and body fluids,[12,13] whereas it is not competitive

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in quantitative determination. Notably, a series of methods based on hydrolytic cleavage of substrate peptides by the active protease have been reported for the detection of MMP-2 activity and quantity, and these methods normally can provide good selectivity because of the high specificity of the enzymatic cleavage. A charge-changing fluorescent peptide substrate was designed by Lefkowitz et al. for the purpose of detecting the activity of MMP-2 directly in whole blood.[14] In that approach, a positively charged fragment was produced upon the cleavage of the substrate by the target enzyme. The fragment was then separated rapidly from blood components by gel electrophoresis, and the fluorescence of the gel was subsequently detected. The enzymatic cleavage strategy was also combined with superparamagnetic nanoparticles[15] and magnetic resonance imaging[16] for MMP-2 determination. Despite the strong ability of these magnetism-based assays in cellular and in vivo analysis, the requirement of special instruments somewhat restrains their widespread application.

Since the cleavage of a substrate peptide by the protease is necessarily accompanied by the production of two fragments as well as a change of the distance between the two ends of the peptide, we may naturally conceive of resonance energy transfer (RET), a well-known distance-dependent physical process, for the assay of the protease. Indeed, several RET-based models have already been constructed for MMP-2 detection, including both fluorescence resonance energy transfer (FRET) and bioluminescence resonance energy transfer (BRET) employing various fluorophores as energy donors and acceptors.[17−21] As is well established, in a FRET process energy is transferred nonradiatively from the excited donor to the acceptor by means of intermolecular long-range (typically 1−10 nm) dipole−dipole coupling, and the intervening solvent or macromolecule has little effect on the efficiency of the energy transfer.[22] Therefore, FRET-based assays can be performed homogeneously with no need to separate coexisting substances, which remarkably simplifies the assay procedure. Moreover, the FRET technique has also been proven to have high sensitivity, which is another reason why this technique has found broad applications in chemical/biological analysis.[23]

Nonetheless, as a kind of optical measurement, the FRET signal is very likely to be interfered with by background signals in complex clinical samples, which mainly include autofluorescence of biomolecules and scattered excitation light. To overcome the problem of background interference in complex samples, it is a promising choice to use anti-Stokes fluorophores as the energy donor, which has been recognized in previous studies.[24−26] Upconversion phosphors (UCPs) are lanthanide-doped inorganic phosphors, which can be excited with near-infrared (NIR) light and emit anti-Stokes fluorescence. The advantages of using UCPs in bioconjugation and bioimaging have already been well reviewed by Wolfbeis et al.[27] In our previous work, we constructed several upconversion (UC)-FRET biosensors for the determination of various biomolecules in clinical samples.[26,28,29] In the most recent work,[29] CNPs were used as the energy acceptor to quench the fluorescence of UCPs. CNPs are a kind of newly emerging nanomaterials that are attracting increasing attention in bioassay because of their easy acquisition as well as the high biocomparability of the carbon element. The π-rich electronic structure of CNPs, which results from sp^2 orbital hybridization, endows the carbon material with strong fluorescence quenching power as a result of electron transfer from excited donors to the π orbital of carbon atoms. Meanwhile, such a π-rich structure also enables easy assembly of biomolecules containing an aromatic motif on the surface of CNPs via $\pi-\pi$ stacking interaction, which helps to bring the labeled donor and the acceptor (CNPs) into close distance. On the basis of these properties, a few FRET systems using CNPs as the energy acceptor have lately been built up, which tagged fluorescent donors to single-stranded oligonucleotides (ssDNA) and utilized the $\pi-\pi$ interaction between ssDNA and CNPs.[29−31]

In this study, we have developed a new FRET sensor for MMP-2 determination employing UCPs and CNPs as the energy donor−acceptor pair. A polypeptide chain (GHHYYGPLGVRGC) containing both the MMP-2 specific substrate domain (PLGVR) and a π-rich motif (HHYY, which consisted of four aromatic residues) was designed and linked to the surface of UCPs. Unlike the previous CNP-involved FRET systems that all relied on ssDNA, a peptide chain is revealed to self-assemble on CNPs for the first time in this work. The FRET process was initiated by the $\pi-\pi$ interaction between the peptide and CNPs. The cleavage of the substrate peptide separated UCPs from CNPs and therefore inhibited the FRET process, which provided a quite simple and straightforward approach to quantify MMP-2. Owing to the NIR excitation nature of UCPs, this FRET sensor was successfully used to directly detect MMP-2 in human plasma and whole blood samples. In combination with the high photoluminescence efficiency of UCPs and the strong quenching ability of CNPs, the developed method was able to determine MMP-2 with a concentration as low as 10 pg/mL, which is so far the lowest quantification limit among all reported MMP-2 determinations. Because of such hypersensitivity of the method, the MMP-2 level can be accurately quantified in less than 1 μL of blood sample, which would be considerably significant for clinical diagnosis and related biological research.

EXPERIMENTAL SECTION

Apparatus and Reagents. Poly(ethylenimine) (PEI; with an average molecular weight of 25 000) and sulfosuccinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (Sulfo-SMCC) were purchased from Sigma-Aldrich. The latent format of matrix metalloproteinase-2 (proMMP-2) and MMP-1 were obtained from Sino Biological Inc. (Beijing, China). The substrate peptide was supplied by Rui-Dong Bioscience Co., Ltd. (Shanghai, China). (4-Aminophenyl)mercuric acetate (APMA) was offered by GenMed Medical Science and Technology Co., Ltd. (Shanghai, China). Human IgG antibody and bovine serum albumin (BSA) were from Zhongshan Golden Bridge Biotechnology Co., Ltd. (Beijing, China). The rest of the chemical reagents were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Human plasma and whole blood samples were collected from five healthy male volunteers (provided by ZhongNan Hospital, Wuhan University). All aqueous solutions were prepared using ultrapure water (Mill-Q, Millipore, 18.2 MΩ resistivity).

The crystal phases of UCPs were identified by a Bruker D8 Discover X-ray diffractometer with a 2θ range from 10° to 70° at a scanning rate of 4 deg/min, with Cu Kα irradiation (k = 1.5406 Å). The sizes and morphologies of PEI-modified $NaYF_4$:Yb,Er upconversion phosphors and carbon nanoparticles were characterized by a JEM-2010 transmission electron microscope with an acceleration voltage of 200 kV. FT-IR spectra of PEI−UCPs were measured on a Magan-IR spectrometer 500 (Nicolet, Madision, WI) with the KBr pellet technique. A 980 nm diode continuous-wave (CW) laser

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(Beijing Hi-Tech Optoelectronic Co., Ltd.) was used as the excitation source, with the power being set at 800 mW. The upconversion fluorescence spectra were recorded on a DCS200PC photon counter with single-photon sensitivity through an Omni-λ300 monochromator (Beijing Zolix Instruments Co., Ltd.).

Synthesis of PEI-Modified UCPs. The water-soluble PEI-modified $NaYF_4$:Yb,Er was synthesized using a one-pot hydrothermal method according to the literature.[32,33] In brief, 0.25 mmol of lanthanide oxides Ln_2O_3 (Y:Yb:Er = 0.78:0.2:0.02, mol/mol ratio) was dissolved in hot nitric acid (65 °C) to acquire $Ln(NO_3)_3$, and the solvent was evaporated after 6 h of reaction. The obtained nitric salt was added to an aqueous solution containing 340 mg of PEI. Then another aqueous solution containing 0.1260 g of NaF (with F^-/Ln^{3+} = 6, mol/mol) was added to the above mixture under vigorous stirring. The whole reaction (36 mL, $V_{ethanol}$:V_{water} = 1:1) was transferred to a 50 mL Teflon autoclave and heated to 200 °C for 10 h. Thereafter, the Teflon autoclave was cooled to room temperature naturally, and a precipitate was obtained by centrifuging and washed several times with ethanol and water, respectively. The product was dried under vacuum before use.

Preparation of CNPs Stabilized with Triton X-100. Carbon nanoparticles were synthesized with candle soot as the starting material following literature reports.[30,34] In a typical procedure, 6 mg of candle soot was added to 20 mL of an ethanol−water mixture ($V_{ethanol}$:V_{water} = 1:1), and then the solution was sonicated for 4.5 h. The as-obtained black solution was centrifuged at 3000 rpm for 1 min to remove large-size carbon particles, and the black supernatant was sonicated for another 2 h. Thereafter, the obtained black solution was centrifuged at 10 000 rpm for 5 min to collect the precipitate, which was finally dissolved in water containing 0.05 wt % Triton X-100.

Preparation of UCP−Peptide Bioconjugates. The polypeptide probe was linked with UCPs using Sulfo-SMCC as the cross-linking agent.[35] A 2 mg portion of PEI-modified UCPs was added to 5 mL of PBS buffer solution (pH 7.4, 10 mM) containing 0.2 mg of Sulfo-SMCC, and the mixture was gently shaken for 1 h at room temperature. Then the solution was centrifuged to remove excess Sulfo-SMCC, and thus, a precipitate was harvested and washed with PBS buffer solution three times. Thereafter, 3 mg of sulfydryl-containing polypeptide was incubated with the maleimide-activated UCP solution (5 mL) with gentle shaking overnight at room temperature. The excess peptide was removed by centrifuging and washing, and the obtained UCP−peptide bioconjugate was finally diluted with 5 mL of TCNB buffer (pH 7.5, 50 mM Tris, 10 mM $CaCl_2$, 150 mM NaCl, 0.05% Brij).

MMP-2 Determination in Aqueous Solution and Blood Samples. Latent proMMP-2 was first activated with APMA according to the protocols provided by the manufacturer. Latent proMMP-2 (72 kDa, 10 nM) in TCNB buffer was added with 1 mM APMA (final concentration), and the mixture was incubated in a 37 °C water bath for 1 h with gentle shaking. For the fluorescence quenching experiment, the energy donor, UCP−peptide, was fixed at 0.028 mg/mL, and various concentrations of CNPs were individually introduced into the Eppendorf (EP) tubes. After adjustment of the total volume to 600 μL with TCNB buffer, the mixtures were incubated for 2 h at room temperature followed by upconversion fluorescence measurements.

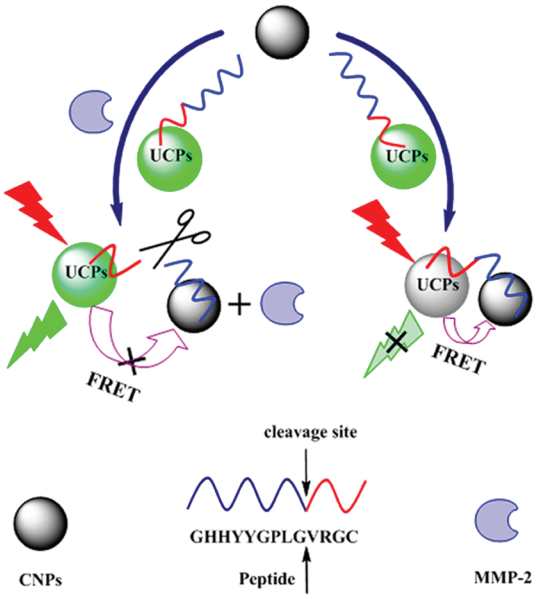
MMP-2 assay was first performed in an aqueous TCNB buffer. To a solution containing 0.028 mg/mL UCP−peptide, a certain amount of activated MMP-2 was added and incubated at 37 °C with gentle shaking for 2 h. Subsequently, 0.05 mg/mL CNPs were added and incubated for another 2 h at room temperature. The reaction mixture was then directly subjected to upconversion fluorescence measurement. To examine the specificity of the UCP−peptide−CNP biosensor toward MMP-2, another matrix metalloproteinase, MMP-1, and some other biomolecules and inorganic ions were added in place of MMP-2 with the same experimental conditions and procedures. For the detection of the MMP-2 level in human plasma and whole blood, 0.2 μL of freshly collected sample was added to the reaction, and a procedure identical to that in aqueous solution was followed. In all upconversion fluorescence measurements, the samples were excited with a 980 nm CW laser and the emission intensity at 547 nm was taken for quantification.

RESULTS AND DISCUSSION

Design of the Polypeptide Probe and the FRET Sensor for MMP-2. For detecting the concentration and/or activity of a protease, it is a highly efficient and commonly employed strategy to make a probe that can be cleaved at a specific site by the protease. The measurement of the cleavage-caused signal change always features easy operation and high specificity.[35,36] To achieve the homogeneous FRET-based MMP-2 determination, we designed the polypeptide GHHYYGPLGVRGC-SH (from N terminus to C terminus) as the probe. The pentapeptide −PLGVR− is a recognized specific substrate for MMP-2 with the amide bond between G and V as the cleavage site.[16,17] The four consecutive residues −HHYY− were designed for the association with CNPs, since each residue contains an aromatic ring. The adsorption of amino acid residues possessing conjugate π electrons was revealed to occur on graphene and graphene oxide,[37,38] in which the carbon atoms have the same sp^2 hybridization as CNPs. This polypeptide probe was linked to UCPs through the sulfydryl on the side chain of the cysteine residue at the C terminus. Since there was only one sulfydryl group existing on the peptide chain, the use of an amine-to-sulfhydryl cross-linker (Sulfo-SMCC) could ensure specific linkage between the PEI-coated UCPs and the peptide chain, yielding a pure UCP−peptide conjugate. The three glycine residues at positions 1, 6, and 12 (starting from the N terminus) were employed to separate different functional regions and to increase the hydrophilicity of the whole peptide.

The principle of this UCP−peptide−CNP FRET sensor for MMP-2 detection is illustrated in Scheme 1. The UCP-labeled peptide noncovalently assembles to the surface of CNPs through π−π stacking interaction. Thus, the energy donor and acceptor are taken into close proximity, resulting in the occurrence of FRET, and the fluorescence emission of UCPs is quenched (the right route in Scheme 1). Upon the introduction of the protease MMP-2, the probe peptide is cleaved into two fragments at the G−V bond leading to the separation of the donor and acceptor (the left route in Scheme 1). Therefore, the FRET process is blocked, and the fluorescence of UCPs is restored in an MMP-2 concentration-dependent manner. It is worth pointing out that the substrate pentapeptide is preferred as −PLGVR− rather than the reverse sequence, i.e., −RVGLP−, in the probe. The positively charged Arg residue (R) was reported to adsorb on graphene oxide through electrostatic attraction,[38] but such adsorption is unlikely to

Scheme 1. Schematic Illustration (Not to Scale) of the MMP-2 Biosensor Based on FRET from Peptide-Linked UCPs to CNPs



occur in the CNP case. This is because CNPs do not carry negatively charged carboxyl groups as graphene oxide does, and the particles are dispersed with nonionic surfactant in water solutions (vide infra). Therefore, keeping the Arg residue on the UCP surface after cleavage will not hinder the separation of the donor from the acceptor. With further consideration of the possible effect of the sequence on the enzymatic recognition, the former sequence was adopted.

Properties of the Energy Donor and Acceptor. Yb,Er-codoped $NaYF_4$ was selected as the upconversion donor since it is so far the most efficient upconversion luminescence material due to the low phonon energy of $NaYF_4$ as the host matrix.[39] In our previous UC-FRET systems where a single-stranded DNA was linked to the surface of UCPs, the luminescent nanoparticles were synthesized using poly(acrylic acid) (PAA) as the ligand such that the surface was functionalized with carboxyl,[28,29] but in the present case, when the UCPs are tagged with a peptide chain, carboxyl is no longer a suitable coupling group since the peptide contains multiple amine groups on its side chain, which could result in many side products when preparing the UCP–peptide conjugate. Considering the higher complexity of the peptide chain than ssDNAs, we synthesized $-NH_2$-coated UCPs using PEI as the ligand. In combination with an appropriate design of a peptide chain which contains a single sulfydryl (cysteine residue), as mentioned above, it is possible to obtain a pure conjugate product using an amine-to-sulfhydryl cross-linker. The existence of PEI molecules on the surface of the particles is confirmed with FT-IR spectra (Figure 1A). As compared to the spectrum of bare UCPs (curve b, without PEI addition in synthesis), the absorption of PEI–UCPs (curve a) shows

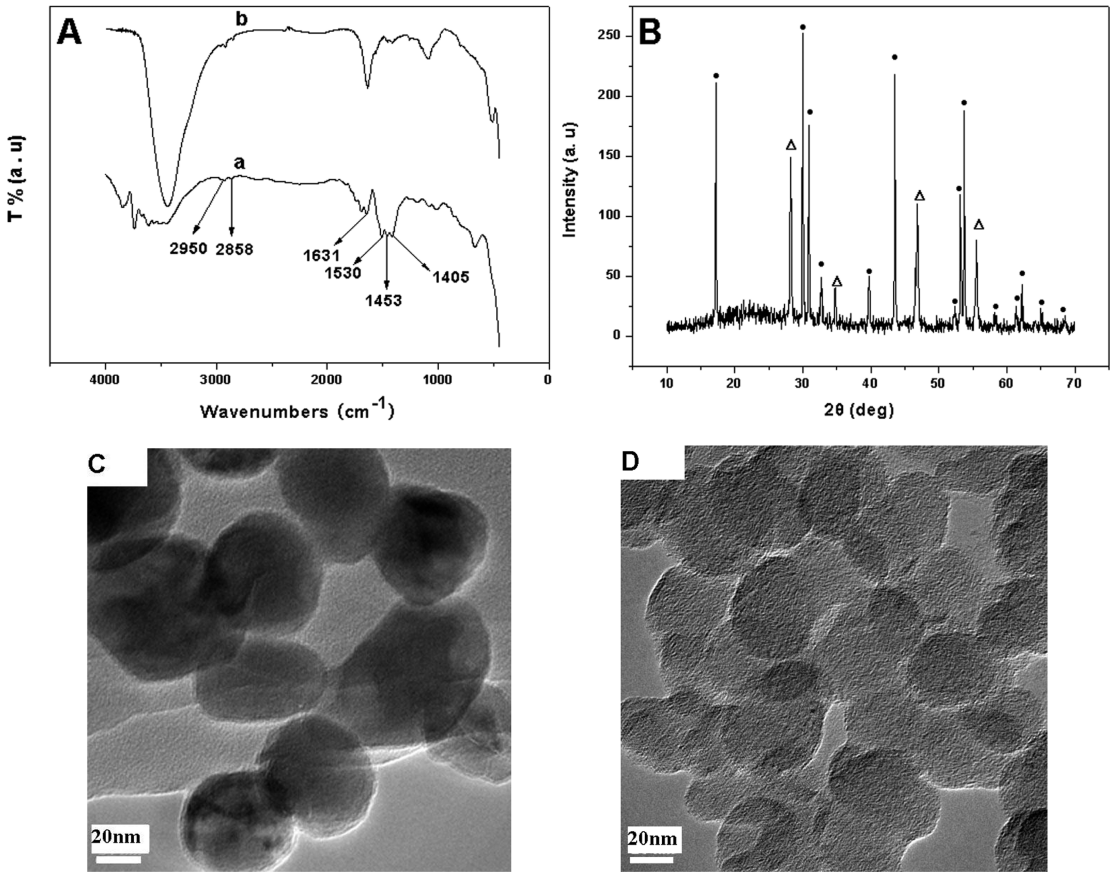


Figure 1. (A) FT-IR spectra of PEI–UCPs (curve a) and bare UCPs (curve b). (B) X-ray diffraction pattern of the as-prepared $NaYF_4$:Yb,Er nanoparticles: Δ, cubic phase (JCPDS file no. 77-2042); ●, hexagonal phase (JCPDS file no. 28-1192). (C) TEM image of the PEI-coated UCPs. (D) TEM image of CNPs.

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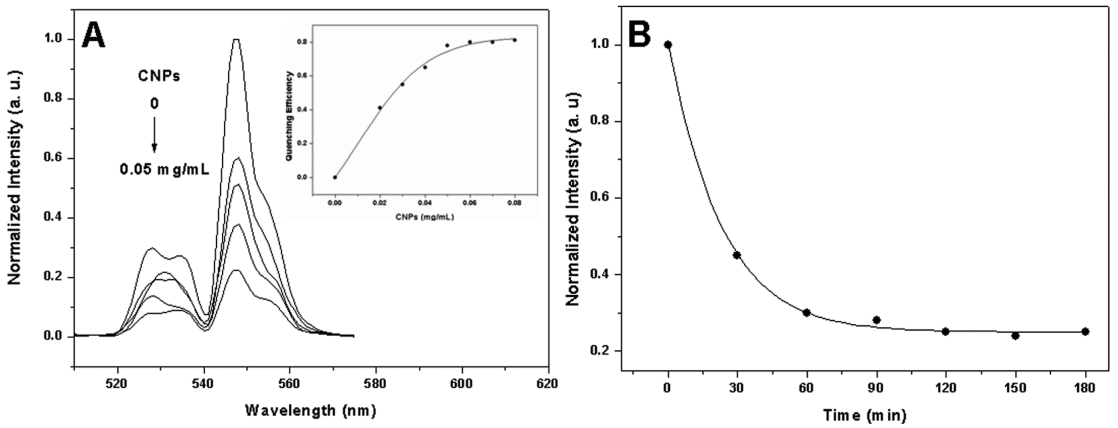

Figure 2. (A) Upconversion fluorescence spectra of UCP−peptide (0.028 mg/mL) in the presence of different concentrations of CNPs. Inset: fluorescence quenching efficiency versus CNP concentration (0, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, and 0.08 mg/mL, respectively). (B) Fluorescence quenching of UCP−peptide (0.028 mg/mL) by 0.05 mg/mL CNPs as a function of time. All experiments were performed in TCNB buffer solution (pH 7.5, 50 mM Tris, 10 mM $CaCl_2$, 150 mM NaCl, 0.05% Brij).

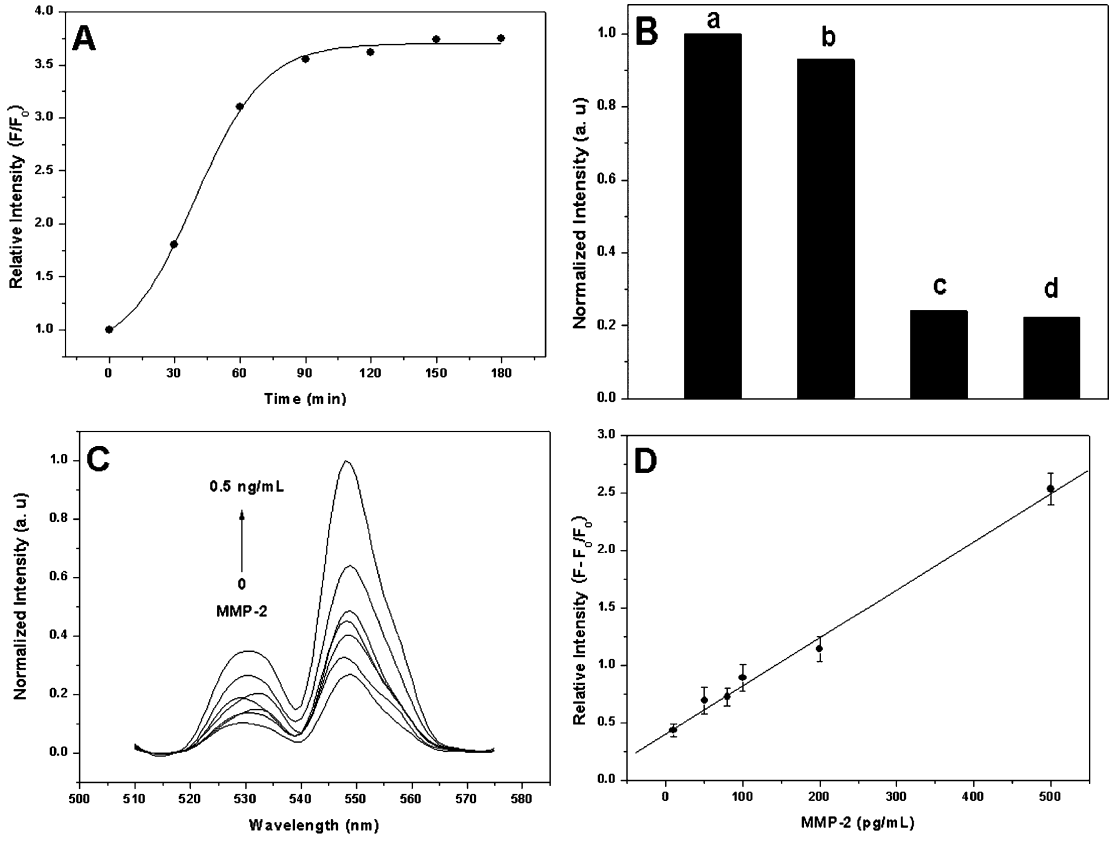

Figure 3. (A) Time course of the fluorescence recovery of the UCP−peptide−CNP complex upon the cleavage by activated MMP-2. (B) Normalized fluorescence intensity (at 547 nm) of UCP−peptide (a), UCP−peptide plus MMP-2 (b), the UCP−peptide−CNP complex (c), and the UCP−peptide−CNP complex plus APMA (d). Concentrations: UCP−peptide, 0.028 mg/mL; CNPs, 0.05 mg/mL; MMP-2, 0.5 ng/mL; APMA, 1 mM. (C) Upconversion fluorescence spectra of the biosensor with various concentrations of MMP-2 (0, 10, 50, 80, 100, 200, and 500 pg/mL). (D) Linear relationship between the fluorescence recovery and the concentration of MMP-2 within the range of 10−500 pg/mL. Data are presented as the average ± SD from three independent measurements. All experiments were performed in TCNB buffer solution (pH 7.5, 50 mM Tris, 10 mM $CaCl_2$, 150 mM NaCl, 0.05% Brij).

unique peaks of methylene asymmetric and symmetric C−H stretching (2950−2858 cm^{-1}), amine N−H bending (1405−1631 cm^{-1}), methylene scissoring (1453 cm^{-1}), and free $-NH_2$ absorption (1530 cm^{-1}), which demonstrates that PEI was successfully loaded to the surface of UCPs. In addition, the large amount of free amine groups also offers good water solubility of the particles, which is beneficial to their further use.

It is well-known that, among the two different crystal phases of $NaYF_4$:Yb,Er, the photoluminescence efficiency of the hexagonal phase is about 1 order of magnitude higher than that of the cubic phase.[39] According to our experiences in making phase-controllable $NaYF_4$:Yb,Er nanocrystals,[32] the PEI-coated $NaYF_4$:Yb,Er nanomaterials were prepared with a dominant hexagonal phase combined with a small amount of

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cubic phase (Figure 1B). The morphology of the as-synthesized PEI-coated UCPs was observed under transmission electron microscopy (TEM), which is shown in Figure 1C. As can be seen, good monodispersibility of the particles in aqueous solution is achieved, which further verifies the solubilizing ability of the surface amine groups. In addition, fairly uniform spheres were obtained with a diameter distribution from 30 to 50 nm, which is better than that of our previously prepared PAA-modified UCPs. This might be due to the chelation of Ln^{3+} with PEI molecules, which contributed to the controlled release of Ln^{3+} into the solution and thus restrained the particle growth and prevented the particles from aggregation. The carbon materials we prepared were spherical nanoparticles with an average diameter of 30 nm (Figure 1D). To facilitate the subsequent bioanalytical usage, their solubility in aqueous solution was enhanced by using surfactants. To avoid introducing any electrostatic effect into the sensing system, the nonionic surfactant Triton X-100 was selected.

Construction of the UCP−Peptide−CNP FRET System. Figure 2A demonstrates the upconversion fluorescence of the donor in the presence of varying amounts of the acceptor. With a fixed amount of UCP−peptide (0.028 mg/mL), the fluorescence is seen to gradually decrease with increasing concentration of CNPs. In several recent reports, the self-assembly of ssDNA on the surface of spherical carbon nanoparticles has been well described,[29−31] which can be attributed to the π-rich electronic structure of oligonucleotides and the $\pi-\pi$ stacking interaction. In this work, the designed probe peptide (GHHYYGPLGVRGC) contained four aromatic amino acid residues, which have a π-rich electronic structure similar to that of oligonucleotides. Apparently, the fluorescence quenching was a result of the FRET process induced by the noncovalent assembly of the peptide chain on CNPs. The fluorescence quenching efficiency reached a maximum of ca. 80% with 0.05 mg/mL CNPs and remained almost unchanged with a further increase of the concentration of CNPs (inset in Figure 2A). For the lanthanide-doped luminescent nanocrystal with the doping ions as the emitter, an 80% fluorescence quenching is a quite high level considering the structural character of the material. That is to say, only the emitting ions located near the surface of the particles can be quenched.[40] Compared to those normally used organic dyes, carbon nanomaterials are more effective quenchers. Actually the so-called superquenching capacity of graphene, another kind of carbon material, has been illustrated with both theoretical predictions and experiments.[41,42] The time dependence of the fluorescence quenching was investigated and is presented in Figure 2B. The fluorescence of the donor was quenched to the maximal degree after 2 h of reaction, and it remained quite stable thereafter, ensuring a constant background for the subsequent MMP-2 sensing.

MMP-2 Sensing in an Aqueous Buffer Solution. In a TCNB buffer, the UCP−peptide (0.028 mg/mL) conjugate was added with activated MMP-2 and incubated followed by the addition of 0.05 mg/mL CNPs. As expected, the upconversion fluorescence of UCPs was enhanced as compared to that of the FRET system (UCP−peptide−CNP). The time course of the fluorescence recovery is shown in Figure 3A. The fluorescence intensity increased gradually along with the cleavage time until it reached a plateau at 2 h. As discussed in the preceding section, only the four residues VRGC were left on the UCP surface after the enzymatic cleavage of the peptide, which consequently broke the UCP−peptide−CNP complex and blocked the energy transfer from UCPs to CNPs, and this must be the origination of the observed fluorescence recovery. To confirm this hypothesis, some control experiments were conducted to preclude possible nonspecific interactions that may cause the fluorescence change. It is known that the fluorescence of some fluorophores may be affected by nearby macromolecules, such as proteins, due to the alteration of the environment. In Figure 3B, we compare the fluorescence intensity of UCP−peptide (column a) with that of the mixture comprising UCP−peptide and MMP-2 (column b). It is found that the MMP-2 protein itself did not cause any fluorescence enhancement of UCP−peptide. Similarly, the effect of APMA (the activator of proMMP-2) was tested. A comparison between column c (the fluorescence intensity of the UCP−peptide−CNP complex) and column d (the complex plus APMA) also indicated that APMA alone had no influence on the fluorescence of the FRET system. The results of these control experiments confirm that the fluorescence recovery was exclusively the result of the hydrolytic cleavage.

As shown in Figure 3C, the fluorescence recovery of the donor was dependent on the amount of MMP-2 introduced. A linear relationship was found between the relative fluorescence intensity ($F - F_0/F_0$, where F and F_0 represent the fluorescence intensity of the system in the presence and in the absence of MMP-2, respectively) and the concentration of MMP-2 within the range from 10 to 500 pg/mL, with a correlation coefficient of 0.9956 (Figure 3D). The relative standard deviations of three independent detections were less than 5% as illustrated with the error bars. To our knowledge, the quantification limit of this UC-FRET-based biosensor for MMP-2 is at least 1 order of magnitude lower than those of all other existing reports.

Selectivity of the UC-FRET Biosensor toward MMP-2. Since the assay was based on the cleavage of a specific substrate peptide by the target protease, a high specificity was to be reasonably expected. Nevertheless, we still investigated the selectivity of the biosensor toward MMP-2 by testing the influence of some species that may exist in biological samples, including another member of the matrix metalloproteinase family, MMP-1, some metal ions, individual amino acids, and proteins. As shown in Figure 4, no obvious fluorescence enhancement was observed even when the concentration of MMP-1 was 10 times higher than that of MMP-2, indicating good specificity of the substrate peptide for MMP-2. The other inspected substances did not cause significant fluorescence alteration of the sensing system either at a concentration (0.1 μM) many times higher than that of MMP-2 (0.5 ng/mL or 6.4 pM). Therefore, the influence of all these species can safely be neglected when their concentrations are comparable with that of the analyte, which suggests quite sound selectivity of the proposed method for MMP-2.

Determination of the MMP-2 Level in Human Plasma and Whole Blood Samples. The UCP−peptide−CNP sensor was applied to detect the MMP-2 level in human plasma and whole blood samples. Five individual real samples including three human plasma samples anticoagulated by heparin and two whole blood samples were analyzed, and the results are presented in Table 1. Taking into consideration the normal MMP-2 level in healthy human blood as well as the linear range of our method, samples of only 0.2 μL were added to the sensing reaction and diluted to 600 μL with buffer, which was a 3000-fold dilution. As shown in Table 1, the MMP-2 levels of the clinical samples were within the range of 500−700 ng/mL taking the dilution into account in the calculation,

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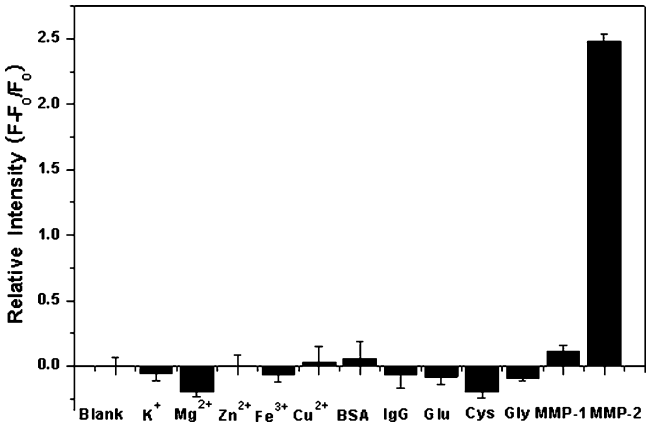

Figure 4. Relative fluorescence intensity $(F - F_0/F_0)$ of the biosensor toward various inspected species, where F_0 represents the fluorescence intensity of the UCP−peptide−CNP complex (denoted as blank), and F is the fluorescence intensity of the complex plus inspected species. The concentration of MMP-2 was 0.5 ng/mL, that of MMP-1 was 5.0 ng/mL, and that of other interfering species was 0.1 μM. Experiments were performed in TCNB buffer solution (pH 7.5, 50 mM Tris, 10 mM $CaCl_2$, 150 mM NaCl, 0.05% Brij) under excitation at 980 nm.

Table 1. Determination of the MMP-2 Level in Human Plasma and Whole Blood Samples Using the UC-FRET Biosensor

sample[a]	measured (pg/mL)	added (pg/mL)	found (pg/mL)	recovery (%)	RSD (%)
1	236.0	100	335.8	99.9	6.1
2	203.2	100	323.6	106.7	1.4
3	178.1	100	279.8	100.6	2.7
4	209.3	100	306.6	99.1	4.7
5	175.2	100	255.5	92.8	2.3

[a]Samples 1−3 were human plasma, and samples 4 and 5 were whole blood.

which are consistent with the reported level obtained with the ELISA method.[43] Standard addition experiments were performed to further validate the developed method. The recoveries ranged from 92.8% to 106.7% for the five real samples with a relative standard deviation (RSD) of around 5%. The above results have exhibited the applicability of this upconversion FRET-based sensor in real samples for MMP-2 quantification. Notably, the ultrahigh sensitivity is of great significance in practical application, since it remarkably reduces the consumption of clinical samples. With further consideration of the quite facile configuration of the sensor and the easy-to-handle assay procedure, the proposed UCP−CNP FRET model is likely to find extensive applications in analytical chemistry.

CONCLUSIONS

In summary, a novel biosensor was constructed for MMP-2 determination in blood samples which was based on fluorescence resonance energy transfer from upconversion phosphors to carbon nanoparticles. A polypeptide probe was designed containing a specific MMP-2 substrate domain and a π-electron-rich region, which was tagged to the energy donor. The FRET process was induced by the $\pi-\pi$ stacking interaction between the probe peptide and carbon nanoparticles, which was realized for the first time in this work. The cleavage of the peptide by the target protease separated the energy donor and the acceptor, which was the basis of MMP-2 quantification. The NIR excitation window eliminates the background interference in complex matrixes and makes the sensor applicable directly in biological samples. The method was facile, highly selective, and ultrasensitive for MMP-2, which could be significant to clinical diagnosis and other MMP-2-related research. Moreover, the flexible construction of the sensor also provides the possibility to develop other sensing systems using this upconversion FRET model through the design and employment of other π-electron-containing substrates or ligands for the targets.

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