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PAPER

A sensitive and selective quantum dots-based FRET biosensor for the detection of cancer marker type IV collagenase

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A sensitive and selective quantum dots (QDs)-based fluorescence resonance energy transfer (FRET) biosensor was successfully fabricated for the detection of the cancer marker type IV collagenase. The cancer marker type IV collagenase could be determined by linking peptide between the donor-luminescent QDs and the acceptor-gold nanoparticles with small size (SAuNPs) based on FRET. Firstly, peptide terminated with cysteine could be bound to the surface of SAuNPs *via* the formation of Au–S bonds. Then QDs combined with the SAuNPs through the peptide. At this moment, the photoluminescence (PL) of the QDs was quenched. After type IV collagenase was added into the system, the SAuNPs could detach from QDs because the enzyme cleaves the peptide to lead to the disappearance of FRET, which allowed the fluorescence of the QDs to return. The enzymatic activity of type IV collagenase was related to the PL change of QDs-based FRET probes. The concentration of type IV collagenase was determined in the linear range of 0.05–10 μg mL^{-1} with a detection limit of 18 ng mL^{-1}. This sensor opens a new route for monitoring the low activities of type IV collagenase in normal and cancerous cell cultures.

1. Introduction

Type IV collagenase as an extra cellular neutral metalloprotease has drawn much attention because of its application in tumor invasion and metastasis.[1–3] It can both degrade type IV collagen, which is the major component of cell membranes, and degrade type 3, 5 collagen and gelatin. Thus, it is of great significance to develop some methods for the determination of type IV collagenase that is rapid, sensitive and selective in the diagnosis of type IV collagenase-relevant diseases. Up to now, the approaches that have been used to monitor the activity of type IV collagenase, mainly involve gelatin zymography and enzyme linked immunosorbent assay (ELISA).[4] However, these methods have limitations as a multiplexed, high-throughput process, and the application of images for the type IV collagenase in single cell with real time. To resolve this problem, QDs based on FRET were selected as a useful technique to investigate the activity of type IV collagenase in living cells.

FRET has been proven to be a powerful technique for studying bio-molecular interactions and conformational change, because it provides a fast, sensitive, and convenient way to probe the changes in distance between molecules.[5] Recently, quantum dots (QDs) have been used as effective energy donors in FRET for bioassay due to their intrinsic optical properties, including high quantum yield, size-dependent tunable photoluminescence with broad excitation spectra and sharp emission spectra, high photostability, and excellent chemical stability.[6,7] These attractive properties led to the growing number of QDs-based FRET used in targeting immunoassay,[8–11] DNA analysis,[12–15] biological enzymes,[16–19] pH change,[20,21] ion sensing,[21,22] and drug delivery.[23] Usually, organic dyes are used as acceptors in most QDs-based FRET.[9,12–17,19,23] Because of photo-bleaching problems, they are not effective acceptors. Recently, fluorescence proteins have also been used as acceptors.[18] However, the prepared processes of fluorescence proteins may be complex. Compared with organic dyes or fluorescent proteins, AuNPs can act as effective energy acceptors in QDs-based FRET system because of their strong absorbance in the visible range and photobleaching resistance.[23–28] Some evidence suggests that the AuNPs with small size can be more effective for quenching effect.[29,30] In addition, AuNPs are exceptionally attractive in bioassay due to controllable size and good biocapability.[5] Therefore, it is important to develop novel biosensors based on QDs and SAuNPs to improve the detection sensitivity and reduce the influence of background and photobleaching in FRET.[31] On the other hand, FRET efficiency depends on the overlap between the emission spectrum of donor and the absorption spectrum of acceptor.[32] The strong spectral overlap between photoluminescence of QDs and absorption of SAuNPs favors the non-radiative energy transfer within the donor–acceptor assay.

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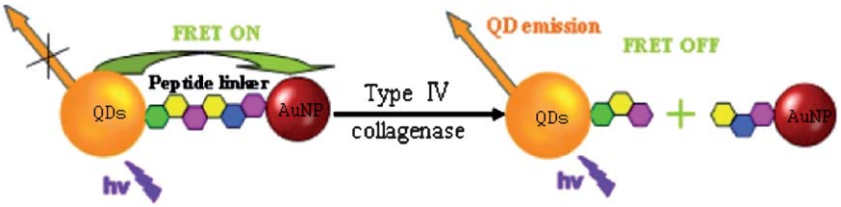

Scheme 1 The procedure for detection of type IV collagenase by QDs-AuNPs FRET system.

Recently, some FRET approaches based on QDs and AuNPs have been explored. For example, Kim reported an inhibition assay based on the modulation in FRET efficiency between QDs and AuNPs in the presence of molecules.[29] Some biosensors based on FRET between QDs and AuNPs for the detection of protein were also reported.[28,30] However, it is necessary to develop a novel technique for cancer clinical diagnosis by the determination of cancer markers. Herein, a sensitive and selective FRET biosensor was fabricated for monitoring the activity of type IV collagenase (Scheme 1) based on the large extinction coefficient of SAuNPs and bright fluorescence of QDs. The spectra overlap between the fluorescence spectra of QDs and absorption spectra of SAuNPs is more than other FRET pairs, which leads to the increase of quenching efficiency. Firstly, peptide was used as the bridge to link QDs donor and SAuNPs acceptor. When the linking process was finished, FRET occurred and the fluorescence of QDs was quenched. However, after the type IV collagenase was added, the fluorescence of QDs recovered because the type IV collagenase could cleave the peptide to hinder the occurrence of FRET. The proposed biosensor showed high sensitivity and selectivity, and could be used for the detection of type IV collagenase in normal and cancerous cell.

2. Experimental section

2.1 Reagents

Type IV collagenase, C-reactive protein (CRP), carcinoembryonic antigen (CEA), α-fetoprotein (AFP), lysozyme and trichloroethyl phosphate (TCEP) were purchased from Kaiji Co., Ltd. The peptide (PLGLCG and GDGDEVDGC) was obtained from Beijing Scilight Biotechnology Co., Ltd. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and 3-mercaptopropionic acid (MPA > 99%) were purchased from Sigma-Aldrich. Potassium borohydride (KBH_4, 96%), tellurium powder (Te, 99.999%) and chloroauric acid ($HAuCl_4 \cdot 5H_2O$) were obtained from Shanghai Reagent Co., Ltd. (Shanghai, China). Disodium hydrogen phosphate, sodium dihydrogen phosphate, and sodium hydroxide were obtained from Nanjing Chemical Reagents Factory (Nanjing, China). The phosphate buffer solution (PBS, 50 mM) with various pH was prepared by mixing stock solution of NaH_2PO_4 and Na_2HPO_4, and then adjusted with NaOH and H_3PO_4 (50 mM). All the other chemicals were of analytical grade and were used as received. SAuNPs were prepared by the reduction of $HAuCl_4$ in the presence of KBH_4.[33] The size of the obtained SAuNPs is 6 ± 2 nm.

2.2 Synthesis of water-soluble CdTe quantum dots

CdTe QDs were synthesized according to references.[34–36] Briefly, cadmium chloride (2 mL, 0.125 M) and MPA (0.0849 g, 0.8 mmol) were added to a 250 mL three-necked flask containing 195 mL doubly distilled water. Then pH was adjusted to 8.5 by adding NaOH solution (5%). Under a condition of pure nitrogen, the above solution was heated to 96 °C. 2.0 mL fresh KHTe solution was added (KHTe solution was prepared by reaction of KBH_4 (0.0480 g, 0.89 mmol) and Te (0.0480 g, 0.375 mmol) in 2.0 mL doubly distilled water. The resulting mixture was refluxed under pure nitrogen at 95–99 °C for 1 h to obtain CdTe QDs with 560 nm maximum emission. The obtained QDs were further purified by ultrafiltration in the 5000 MW size filter to remove unreacted MPA and other impurities.

2.3 Synthesis of SAuNPs-peptide

The SAuNPs-peptide were prepared by the addition of cysteine-terminated peptide solution to the freshly prepared SAuNPs solution which was activated by TCEP. Then the resulting solution was incubated at 4 °C for 18 h. The solution was further purified by ultrafiltration in the 10 000 MW size filter and centrifuged at 3000 *g* for 15 min at 4 °C to remove the unbound peptides from the SAuNPs-peptide, followed by washing with distilled water and resuspending in distilled water.

2.4 Synthesis of QDs-peptide-conjugated SAuNPs

100 μL of 4.2 mg mL^{-1} freshly prepared EDC solution and the SAuNPs-peptide were added to the solution with 1 mL CdTe QDs. The mixture was incubated at room temperature (20 ± 5 °C) for 1 h. The QDs-peptide-conjugated SAuNPs were washed to remove free nonconjugated QDs.

2.5 Enzyme activity assay

Typically, 50 μL of QDs-peptide-conjugated SAuNPs and the type IV collagenase was added to Tris-HCl (50 mM, 7.4) buffer solution to a total volume of 500 μL. Subsequently, the obtained solution was incubated for 30 min at 37 °C and then the fluorescence spectra were measured to monitor the enzymatic reaction.

2.6 Characterization

The UV-Vis absorption spectra were carried on a Shimadzu 3600 UV-Vis spectrometer (Shimadzu, Japan). The XRD analysis was performed with a Philips X-pert X-ray diffractometer at a scanning rate of 4°/min in the 2θ range from 10° to 80°, with graphite monochromatized Cu-Kα radiation ($\lambda = 0.15418$ nm). For XRD characterization, the CdTe QDs were precipitated with the same volume of isopropyl alcohol and centrifuged to collect the precipitate, and then the purified CdTe QDs were dried in a vacuum.

2.7 Fluorescence experiments

Under the excitation wavelength of 300 nm, the fluorescence spectra were recorded at room temperature by using an Edinburgh FLS920P fluorescence spectrometer (Edinburgh Instruments Ltd., UK) and NF920 fluorescence spectrometer. Slit widths were set from 1 to 5 nm for excitation and emission spectra according to the intensity of spectra. Each experiment was carried out at least three times and each data was reproducible, so the data shown in this paper were reproducible spectra rather than averaging spectra data.

3. Results and discussion

3.1 Characterization of CdTe QDs

Fig. 1A shows the X-ray diffraction (XRD) pattern of the CdTe QDs. A peak at 26° (111) and a broad band at *ca.* 45° are the overlap of (220) and (311) diffractions. This result confirmed that the CdTe QDs had a zinc blende crystal structure. The fluorescence and absorption spectra (inset) of the CdTe QDs are shown in Fig. 1B. The emission peak at 560 nm (ex = 300 nm) and the UV-Vis absorption spectrum maximum at 540 nm indicated the quantum confinement and relatively monodispersed particles.[37–39] According to the empirical equation:[40]

$D = (9.8127 \times 10^{-7})\lambda^3 - (1.7147 \times 10^{-3})\lambda^2 + 1.0064\lambda - 194.84$, where D (nm) and λ (nm) represent the diameter of QDs and the wavelength of the UV-Vis absorption spectrum respectively, the average size of CdTe QDs is 3.12 nm.

3.2 Characterization of SAuNPs-peptide

According to the reference,[30] SAuNPs and SAuNPs-peptide were prepared, respectively. UV-Vis spectrum was used to characterize the process, as shown in Fig. 2A. The maximum absorption peak of SAuNPs red shifts from 512.5 to 520 nm after SAuNPs combined with the peptide. The reason may be that the size of the SAuNP-peptide is larger than that of SAuNP. The peptide with the cysteine can bind to SAuNP, which causes the size of SAuNP to be increased, as a result, the maximum absorption peaks red shift. On the other hand, the intensity of UV-Vis absorption spectrum of SAuNPs-peptide is stronger than that of SAuNPs. This can be ascribed to the linking of SAuNPs and peptide to induce the size change of SAuNPs, and the larger concentration of SAuNPs-peptide compared to free SAuNPs.

3.3 FRET between QDs and SAuNPs

FRET occurs when there is appreciable overlap between the emission spectrum of donor and the absorption spectrum of acceptor. Careful selection for an appropriate donor–acceptor pair can ensure high transfer efficiency. Selection for several measurable parameters such as quenched donor photoemission, enhanced acceptor fluorescence, shortened donor exciton lifetime and prolonged acceptor fluorescence lifetime is very important for good signal of FRET. As to non-luminous energy acceptors-SAuNPs which exhibit a high extinction coefficient and broad absorption spectrum, the measurable parameters are reduced to two including quenched donor photoemission and shortened donor excitation. For luminescent QDs, they can be used as energy donors in FRET, because the tunable photoemission makes it possible to adjust FRET efficiency by altering the spectral overlap between QDs and SAuNPs. In order to detect the activity of the type IV collagenase, QDs are first quenched by SAuNPs directly. As shown in Fig. 2B, the fluorescence spectra of QDs and absorption spectra of SAuNPs overlapped favorably. Therefore when the SAuNPs combined with QDs, SAuNPs could absorb the energy emitted by QDs. Therefore, the SAuNPs is a suitable acceptor for FRET-based biosensor while it combined with QDs as donor.

Fluorescence quenching can be described by the Stern–Volmer equation: $F_0/F = 1 + K_{SV}C$, where F_0 is the initial fluorescence intensity of QDs in the absence of quencher, F is the fluorescence intensity in the presence of quencher, K_{SV} is the Stern–Volmer quenching constant, which is related to the quenching efficiency, and C is the concentration of quencher. Fig. 3 shows the SAuNPs concentration-dependent quenching of the fluorescence QDs. In Fig. 3A, linear regression equation can be obtained to be $F_0/F =$ 0.99 + 2.20[AuNPs] and the Stern–Volmer quenching constant is 2.20×10^6 M^{-1}. This value shows that the prepared QDs and SAuNPs could be selected as acceptor and donor in FRET system.

Compared with SAuNPs, SAuNPs-peptide quenching the fluorescence of QDs is more effective. Fig. 3B shows the fluorescence intensity of QDs. The fluorescence intensity of QDs decreases rapidly with the increase of SAuNPs-peptide and is

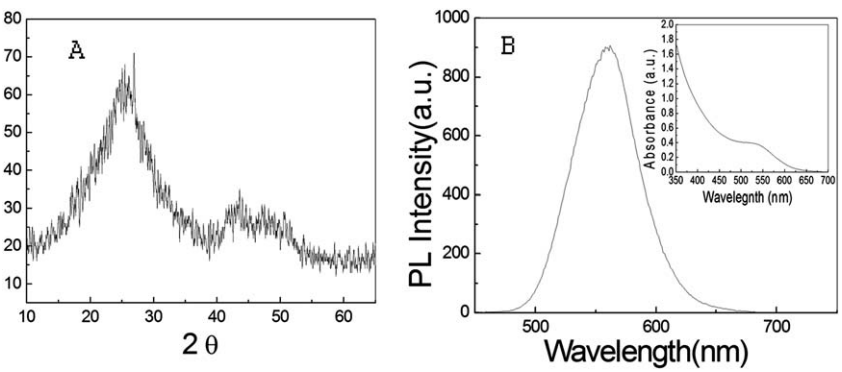

Fig. 1 (A) XRD (Cu-Kα) of CdTe QDs. (B) Photoluminescence (PL) and UV-Visible absorption spectra (inset) of the CdTe QDs aqueous solution (excitation wavelength: 300 nm).

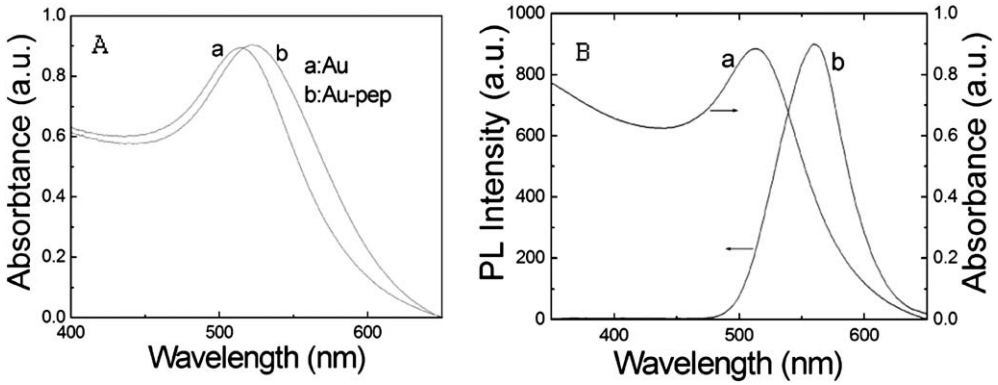

Fig. 2 (A) UV-Visible absorption spectrum of SAuNPs (a) and SAuNPs-peptide (b) in the PBS. (B) UV-Visible absorption spectrum of SAuNPs (a), and fluorescence spectrum of QDs (b).

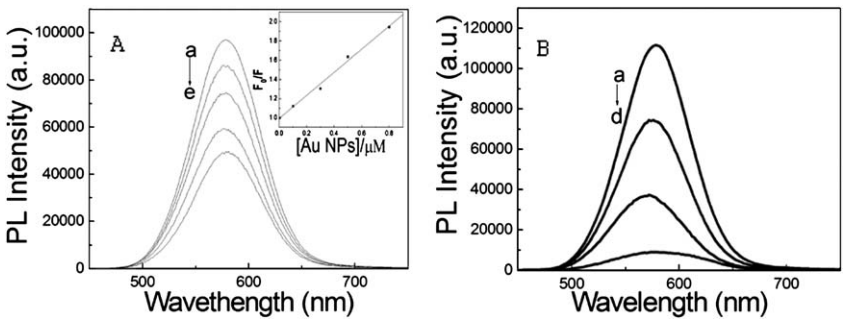

Fig. 3 Changes in the PL intensity of donor QDs by different acceptor as a function molar ratio between donors and acceptors. (A) SAuNPs; (B) SAuNPs-peptide with the concentration of: (a) 0, (b) 0.1, (c) 0.3, (d) 0.5, (e) 0.8 μM. Inset: The Stern–Volmer plot of F_0/F *versus* [AuNPs].

quenched more effectively. This shows that the sensitivity of QDs-SAuNPs FRET sensor is improved to a higher level by linking the QDs and SAuNPs through peptide. For the principle, it is highly desirable for a sensitive enzyme assay if SAuNPs-QDs conjugates have low background fluorescence, and maximized quenching of QDs by SAuNPs-peptide is observed. Obviously, the method can be used to determine type IV collagenase.

3.4 Interference

In order to apply the proposed QDs-based FRET biosensor in a cellular system, the effect of common metal ions, such as K^+, Na^+, Ca^{2+}, Zn^{2+} or Mg^{2+} was investigated. As shown in Fig. 4, all of those metal ions did not interfere the determination, which implied that it could be applied in a cellular system. Besides, the specific details of enzyme and peptide are also investigated. On the one hand, peptide (GDGDEVDGC) was used to replace the peptide (PLGLCG) to determine the specificity, and it was found that the type IV collagenase could not cleave the peptide with DEVD fragment. On the other hand, Some cancer-related proteins such as C-reactive protein (CRP), carcinoembryonic antigen (CEA) and alpha-fetoprotein (AFP), and other proteins such as lysozyme were used as interference to evaluate the specificity through comparing the fluorescence response of pure type IV collagenase and the solution with interference at the concentration of 0.5 μg mL^{-1}. The response changes were 2.6%, 1.9%, −2.5% and 3.1% respectively which indicated that the specificity was acceptable. It also indicated that all of these

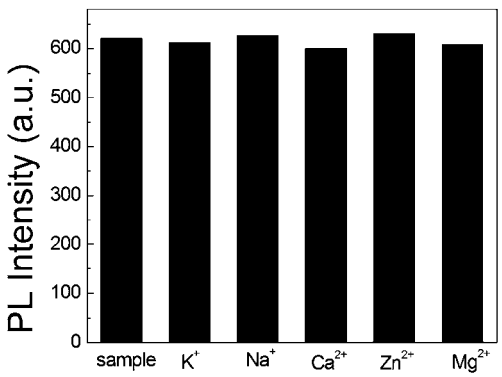

Fig. 4 Effect of common metal ions on the fluorescence intensity of the biosensor (1 mM K^+, Na^+, Ca^{2+}, Mg^{2+} and Zn^{2+}).

proteins could not cleave the peptide which shows that type IV collagenase and the peptide (PLGLCG) had specific functions.

3.5 Enzyme activity measurements

On the basis of the above results, the QDs-based FRET biosensor was fabricated to determine the activity of type IV collagenase. The fluorescence background was very low due to the effective quenching effect of SAuNPs. As expected, the concentration of the type IV collagenase could be determined by monitoring the fluorescence intensity of QDs. When SAuNPs-peptide was added, the QDs fluorescence was quenched

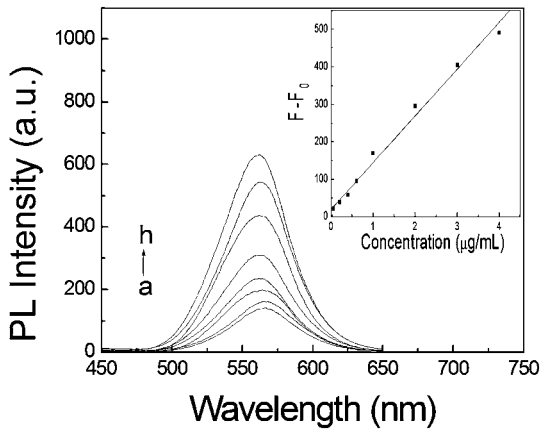

Fig. 5 The fluorescence intensity of the QDs-based FRET probes at increasing type IV collagenase concentrations for a fixed reaction time: (a) 0, (b) 0.05, (c) 0.2, (d) 0.4, (e) 1, (f) 2, (g) 3, (h) 4 μg mL^{-1} (λ_{ex} = 300 nm). Inset: linear plot of relative fluorescence intensity as a function of type IV collagenase concentration.

effectively. However, after the addition of type IV collagenase, the fluorescence was recovered. When the concentration was higher than 10 μg mL^{-1}, the fluorescence intensity reached saturation, which indicated the probes could react with the type IV collagenase to cleave the peptide completely. As shown in Fig. 5, the F-F_0 values were linear with the concentration of type IV collagenase in the range of 0.05 μg mL^{-1} to 10 μg mL^{-1} (F_0 and F represent the fluorescence intensity of QDs-based FRET probes in the absence and presence of type IV collagenase), and the detection limit is 18 ng mL^{-1}.

4. Conclusions

In summary, we have successfully fabricated a QDs-based FRET biosensor by using AuNPs with small size which have a large extinction coefficient and bright fluorescence of QDs to detect the activity of type IV collagenase with high sensitivity and high selectivity. The fluorescence intensity recovery shows linear increase with the concentration of the type IV collagenase from 0.05 μg mL^{-1} to 10 μg mL^{-1} with the 18 ng mL^{-1} detection limit. The novel method can be used to determine the concentration of type IV collagenase in normal and cancerous cells and other applications, such as overexpression of proteolytic activity.

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