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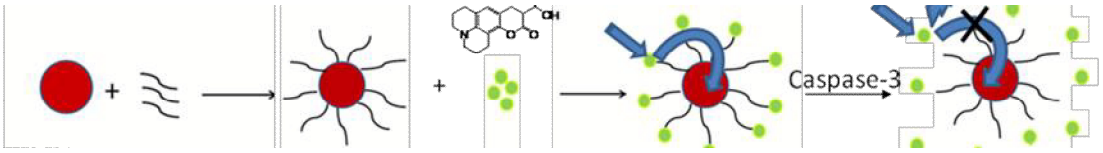
A kind of coumarin-functionalized gold nanosensor by polypeptide chains was developed for sensitive detection of caspase-3 based on FRET process.



Coumarin-modified gold nano probes for sensitive detection of caspase-3

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ABSTRACT

Caspase-3 has been identified as a key mediator and a well-established cellular marker of apoptosis. To increase the sensitivity, a kind of coumarin-functionalized gold nanoparticles (AuNPs) by the connection of polypeptide chains containing specific sequences (DEVD) was designed and synthesized for sensing of caspase-3, because there was a large overlap between the emission of coumarin-343 and the absorption of AuNPs. The fluorescence of coumarin 343 was quenched due to the energy transfer process by the gold nanoparticles. The fluorescence would be restored after the particular polypeptide sequence (DEVD) was cut off by caspase-3. Based on this mechanism, the caspase enzyme activity in vitro could be detected by the fluorescence assay with a high sensitivity. The effect of the different lengths of polypeptide chain on the luminescence quenching efficiency and sensing ability was

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also studied, which is of great importance in designing FRET-based sensing platforms. This kind of sensitive luminescent functional coumarin 343-modified gold nano probes made it suitable for caspase-3 sensing in biological applications.

Keywords**:** gold-nanoparticles; coumarin; luminescent sensor; caspase; energy transfer

Introduction

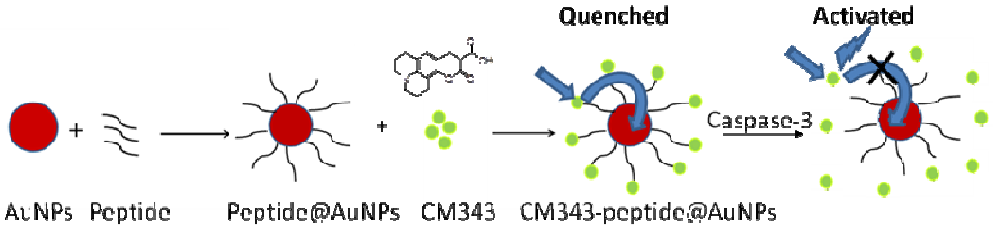
Recently, gold nanoparticle (AuNP) is one of the most widely studied materials due to the advantages of its small size, ease of manufacture, the resonant nature having ion conductivity and biocompatibility, localized surface plasma resonance, special optical activity and surface modification characteristics.[1,2] The applications of Au nanoparticles in bioanalysis have drawn great attention due to their high extinction and strong size- and distance-dependent optical properties.[3-10] One appealing feature of the high extinction coefficients in the visible region enables them to function as efficient quenchers for most fluorophores in the design of biological sensors such as protease, DNA and so on.[11-16]

Apoptosis is an important physiological mechanism to maintain homeostasis of multicellular organisms by elimination of infected or damaged cells and regulation of cell number.[17,18] Caspase was found in recent years, which was a group of cysteine protease existing in cytoplasmic sol, and the protease can catalytically crack (ADP ribose) polymerase and lead to cell apoptosis.[19] There are 13 known species, including caspase-3 and caspase-7 which are similar substrate and inhibitor specificity.[20,21] They can specifically cut off the aspartic acid residue of peptide bond (DEVD) in a specific way and degrade the PARP (DNA repair enzyme) and DFF-45 (DNA fragmentation factor), which results in inhibition of DNA repairing and starting the degradation of DNA. Caspase-3 is activated in the apoptotic cell both by extrinsic and intrinsic pathways, and has been identified as a key mediator and a well-established cellular marker of apoptosis.[22] Accordingly, the development of a highly sensitive and specific detection system for caspase activities will provide new

insights into the roles of proteases in the biological events. Based on the special activity of the caspase-3, various caspase assays were developed by utilizing fluorescence resonance energy transfer (FRET) between a pair of an donor and acceptor, which involved in the form of conventional organic dyes,[23-25] fluorescent proteins,[26-30] or quantum dots (QDs)[31] that are paired with quencher moieties. A simple strategy for the detection of proteolytic activities was designed by the use of peptide substrates containing a FRET pair-fluorescein and tetramethylrhodamine.[25] Genetically encoded fluorescent indicators that include green-fluorescent protein (GFP) derivatives also became valuable tools for studying temporal caspase activities in single living cells.[13,14] A near-infrared fluorescence dye was attached to AuNP surface through the bridge of peptide substrate (DEVD) to fabricate an apoptosis imaging probe.[32] A specific FRET-based protease sensor for caspase-3 was also developed based on quantum dots (QDs) as energy donors and the fluorescent protein mCherry as energy acceptors.[31] Recently, some hybrid nanomaterials-based sensors and probes were successfully developed for detection of caspase-3 in our work, in which $[Ru(bpy)_3]^{2+}$-encapsulated silica nanoparticles (SiNPs) were used as fluorescence energy donors and gold nanoparticles (AuNPs) were used as energy acceptors.[33] However, further development of a high sensitive luminescent probe for caspase-3 sensor in analytical- and bio-systems is still a challenge.

To further increase the sensitivity, an FRET system with strong fluorescence coumarin 343 (CM343) as energy donors and AuNPs as energy acceptors has been designed and applied for caspase-3 detection. Because the fluorescence of the coumarin 343 has a large overlap with the absorption band of AuNPs, the fluorescence quenching property of gold nanoparticles was utilized to efficiently quench the fluorescence of coumarin 343. Another advantage of coumarin 343 as an emission donor is the much high quantum yield compared to luminescent transition metal-based complexes, although the lifetime is shorter.[34] Two kinds of CM343-peptide@AuNPs probes were designed and synthesized by two different lengths of peptide chains linking CM343 and AuNPs to tune the distance between the donor and the acceptor. The peptide chains contain recognition sequence DEVD

specific for active caspase-3 cleavage.[35] A difference in distance between the two building blocks resulted in a difference in FRET efficiency on account of different length of peptide chains. The synthesis and principle of the sensor is shown in Scheme 1. The fluorescence was quenched in physiological conditions due to the quenching effect of AuNPs, when caspase-3 triggers the cleavage of the linkers and the system releases coumarin 343 from the Au nanoparticles, the quenched fluorescence was recovered and the fluorescence efficiency increased. Based on this FRET mechanism, caspase-3 could be detected in a simple and sensitive way, which might be significant in bioanalysis and biodection.



Scheme 1. Schematic diagram of synthesis and principle of functionalized AuNPs.

Experiments

Materials and chemicals

Coumarin 343 was synthesized in our group according to the previous report.[36] Peptide (95%, target sequence 1: Gly-Asp-Glu-Val-Asp-Cys; target sequence 2: Gly-Gly-Ala-Asp-Glu-Val-Asp-Gly-Cys) were purchased from Sangon Biotech (Shanghai) Co., Ltd. Caspase-3 (3000pmol/min/μg) were purchased from R&D system. Chlorauric acid ($HAuCl_4$) and sodium citrate were purchased from Sigma-Aldrich. Other chemicals were analytical reagent grade and used as received.

Experimental apparatus

Fluorescent and UV−vis spectra were obtained on a HITACHI F-4600 spectrofluorometer and a PERKIN ELMER LAMBDA 750 UV−vis spectrometer, respectively. Raman spectrometer was obtained on a RENISHAW INVIA Raman spectrometer. Transmission electron microscopy (TEM) images were obtained using a TECNAI G2F20 High-Resolution Transmission Electron Microscope (Manufacturer:

FEI, Ltd., America). Dynamic light scattering (DLS) experiment was carried out on Malvern Zetasizer NanoZS90.

Synthesis of AuNPs

Aqueous dispersions of citrate-stabilized AuNPs were prepared by the citrate reduction of chloroauric acid as described in detail elsewhere.[37] Briefly, a 50 ml aqueous solution of $HAuCl_4$ (0.25 mM) is heated to boiling under vigorous stirring, followed by quickly adding 0.5 ml of trisodium citrate solution (1%). The reaction was allowed to continue for another 20 min. The prepared citrate-stabilized AuNPs generally appeared red color. Then, the solution was cooled to room temperature and stored at 4℃ in the refrigerator for further use. The AuNPs were characterized by TEM and DLS experiments.

Functionalization of AuNPs

Peptide (target sequence 1: Gly-Asp-Glu-Val-Asp-Cys; target sequence 2: Gly-Gly-Ala-Asp-Glu-Val-Asp-Gly-Cys) conjugated to AuNPs via the Au-S bond as described in previous literature by a slight modification.[38] Briefly, 375 μl of 15.7 mM peptides solution were added to 20 ml of 5.9 nM AuNPs, followed by incubation for 4 h. Excess peptides were then removed by centrifugation at 12000 rpm for 30 min. The resultant peptide-modified AuNPs (peptide@AuNPs) were washed with DI water and redispersed in phosphate buffer solution (PBS, 10 mM, pH 7.2). The peptide-modified gold colloids appeared wine red after being centrifuged, suggesting sufficient steric stabilization.

Carbodiimide chemistry was employed to functionalize AuNPs with CM343 by modification of a literature method.[39] Briefly, 180 μl of 13 mM CM343 was suspended in 10 ml of 0.05 M 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, pH 7.2) buffer containing 1.20 mM N-hydroxysuccinimide (NHS) and 1.20 mM 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide (EDC) to activate carboxylic groups. After 1 h, 20 ml of 5.9 nM peptide@AuNPs were added to the mixture, and it was stirred for 12 h at room temperature. Excess CM343 was then removed by centrifugation at 12000 rpm for 30 min. The AuNPs mondified by CM343 (CM343-peptdie@AuNPs) were washed with 10 mM PBS (pH 7.2) and then

resupended in 10 mM PBS (pH 7.2). At last, the AuNPs mondified by CM343 (CM343-peptdie@AuNPs) showed deep red and were stored at 4°C in the refrigerator for further use. Then the functionalized AuNPs were characterized by Rama, Uv-vis absorption and fluorescent spectra.

Sensing application

For caspase-3 sensing, the functionalized CM343-peptide-AuNPs (5.9nmol, 2ml) were incubated with caspase-3(final concentrations: 0.004-1.8 ng/ml) in assay buffer (total volume 2.5ml, pH 7.4, 50 mM HEPES, 100 mM NaCl, 1 mM EDTA, 10% v/v glycerol and 0.1% v/v CHAPS) for 0-150min at 37°C. Then the emission intensity was recorded on the F-4600 spectrofluorometer. The optimum response time was about 30min in our experiments. The control experiment was also carried out for CM343-peptide-AuNPs system in the absence of caspase-3. To evaluate the selectivity of the functionalized AuNPs toward caspase-3, a series of proteases including trypsin, chymotrypsin, esterase, and the ions including K^+, Na^+, Cl were examined for their possible interference in caspase-3 detection.

Caspase-3 determination with the nanohybrid sensor was also conducted in cell extracts.[40] Hela299 cells were obtained from the Type Culture Collection of the Chinese Academy of Sciences (CAS, Shanghai, China), and were cultured in DMEM medium (supplemented with 15% fetal bovine serum) at 37°C in a humidified 5% CO_2 atmosphere. 1.0×10^5 cells were collected in the exponential phase of growth, and were then dispensed in an EP tube. After washed twice with ice-cold PBS, cells were resuspended in 200 μL of ice-cold CHAPS buffer. The suspension was subjected to sonication in an ice bath (KQ3200DB sonicator amplitude set at 70%) for 30 min and centrifuged at 12000 rpm for 20 min. The supernatant was carefully transferred to a new EP tube, and was used immediately for caspase-3 detection after 6-fold dilution with assay buffer or stored at −70 °C. 250μL diluted cell extracts incubated with the functionalized CM343-peptide-AuNPs (5.9nmol, 2ml) in buffer solution (total volume 2.5ml) for 30min at 37°C were used to determine the caspase-3 in cell extracts. In the standard addition experiment, 250μL diluted cell extracts and 10μL standard caspase-3 solution (0.2μg/mL) incubated with the functionalized

CM343-peptide-AuNPs (5.9nmol, 2ml) in buffer solution (total volume 2.5ml) for 30min at 37°C were used to determine the total concentration of caspase-3.

Results and discussion

Synthesis and functionalization of AuNPs

AuNPs were obtained by the citrate reduction of chloroauric acid. [37] Fig. 1A displays a typical TEM image of AuNPs, where AuNPs are regular, monodisperse, and spherical in shape. The dynamic light scattering (DLS) experiment shows a diameter of ～13 nm (Fig.1B) and the Zeta potential is -39.33mV. Peptides were functionalized onto AuNPs via the Au−S chains (peptide@AuNPs), and the peptide-modified AuNPs generally appeared wine red suggesting sufficient steric protection of peptide. The further functionalization of peptide@AuNPs with CM343 were carried out using carbodiimide chemistry, where cross-linking reactions between free carboxylic groups of coumarin 343 and amine groups on peptide were initiated by adding a solution of EDC and NHS.[41] The Fig. 2 shows the Raman spectra of CM343 and AuNPs funtionalized by two kinds of peptides. The CM343-peptide@AuNPs probes show main features in Raman spectra relative to CM343 similar to previous report.[42] The phenyl ring breathing motions are at 633-785 cm^{-1}, the CH_2 rock motions and C-C stretching vibrations are at 1200 cm^{-1}, C＝C、C－N and C＝C stretching vibrations are at 1370-1574 cm^{-1}, and the 1654 and 1728 cm^{-1} are C=O stretching modes. The results demonstrated that the coumarin 343 had been successfully modified on the peptide of gold nanoparticles surface by amide bond.

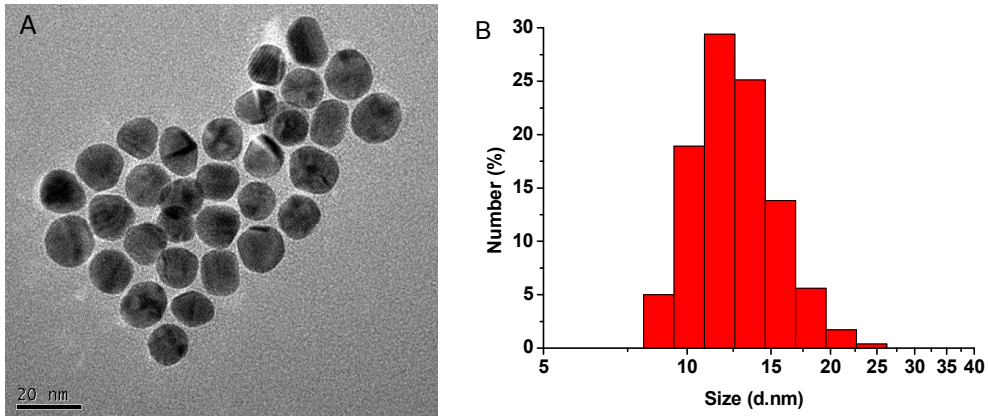


Fig.1. TEM images of AuNPs (A) and the particle size distribution from DLS (B).

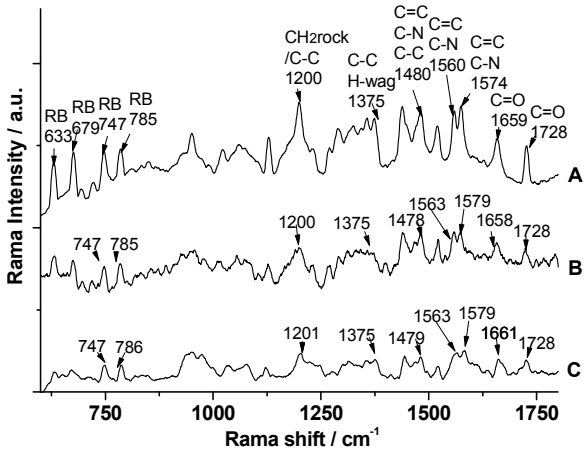


Fig. 2. Raman spectra of CM343 (A); CM343-peptide1@AuNPs (B): and CM343-peptide2@AuNPs (C).

The optical properties of AuNPs were examined by UV-Vis absorption spectra. As shown in Fig. 3, a characteristic surface plasmon resonance (SPR) peak is located at 520 nm. Compared to AuNPs, the CM343-peptide1@AuNPs and CM343-peptide2@AuNPs showed a slight red shift of ca. 5 nm in the UV−vis absorption bands, which demonstrated that the conjugation of CM343 on AuNP surfaces by polypeptide chains had a tiny effect on the aggregation of AuNPs. The functionalized gold colloids generally appeared deep red after being centrifuged, which suggested sufficient steric stabilization after it was protected by peptide.

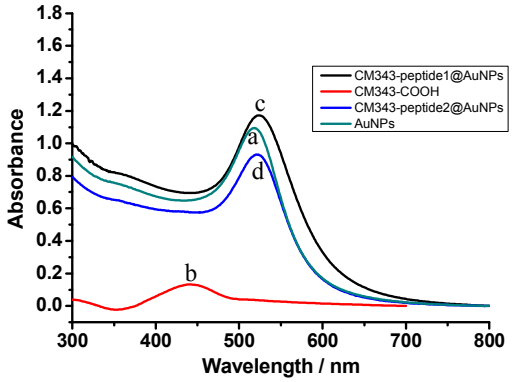


Fig. 3. UV-vis absorption spectra of bare AuNPs (a); CM343 (b); CM343-peptide1@AuNPs (c); CM343-peptide2@AuNPs (d).

The emission spectra of the CM343-peptide1@AuNPs, CM343-peptide2@AuNPs and CM343 in aqueous solution are also shown in Fig. 4. The CM343 gave rise to a strong emission at ca. 478 nm, and CM343-peptide1@AuNPs and CM343-peptide2@AuNPs showed a weak emission at about 490 nm with a small red shift of ca. 12 nm in the emission wavelength. The excess and adsorption CM343 on AuNP surface were removed by washing and centrifuge during the preparation and the partial CM343 was conjugated on AuNP surface. The fluorescence of conjugated CM343 was efficiently quenched by AuNPs due to the large overlap between the fluorescence of CM343 and the absorption band of the functionalized AuNPs based on the FRET process from CM343 to AuNPs. The above results further suggested that CM343 have successfully modified on the peptide@AuNPs.

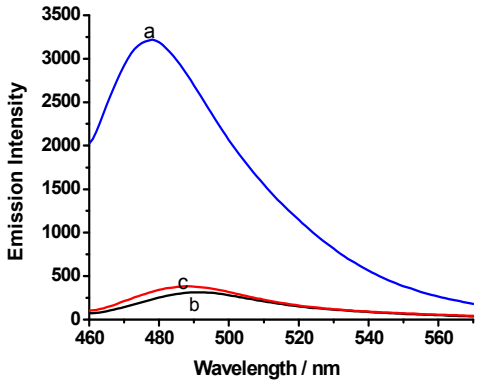


Fig. 4. Fluorescent spectra of CM343 (a); CM343-peptide1@AuNPs (b); CM343-peptide2@AuNPs (c).

Sensing application of the CM343-peptide@AuNPs

Except for the overlap of the emission spectra of a donor and the absorption band of an acceptor, the FRET process also depends on the distance between the donor and the acceptor. In our assembled CM343-peptide@AuNPs nanohybrid, the distance between AuNPs and CM343 can be tuned through engineering the bridging molecules. This property can be utilized for developing new sensing strategies through employing FRET technology which is a distance-dependent energy transfer phenomenon. A change in distance between AuNPs and CM343 will result in a change in FRET efficiency and cause a ratiometric change in emission. Because protease can recognize and cleave a specific substrate peptide (DEVD) to produce two separated fragments and thus change the distance between the two ends of the peptide, it is possible to explore the biological sensing ability of caspase-3 and compare the sensing abilities to caspase-3 by two peptides link of functionalized gold nanoparticles, in which both contain a specific sequence of DEVD. As shown in Fig. 5, when caspase-3 was introduced into these probe systems, the dramatic fluorescence recovery of the CM343-peptide@AuNPs system was observed within 30 min, although a small fluorescence enhancement was detected for the CM343-peptide@AuNPs systems without caspase-3 due to the gold nanopaticles' electrostatic incorporation. Upon addition of caspase-3, the emission increased in intensity and reached saturation at about 1.6 ng/ml and 4.0 ng/ml of caspase-3 for CM343-peptide1@AuNPs and CM343-peptide2@AuNPs, respectively. And the emission intensity was enhanced about 1-fold and 0.4-fold for CM343-peptide1@AuNPs and CM343-peptide2@AuNPs, respectively, after addition of caspase-3 in 30 min. The results showed that the shorter linker between the fluorophore and AuNP quencher would result in a higher FRET efficiency and the recovery of emission would be more obvious after the linkage was cleaved by caspase-3.

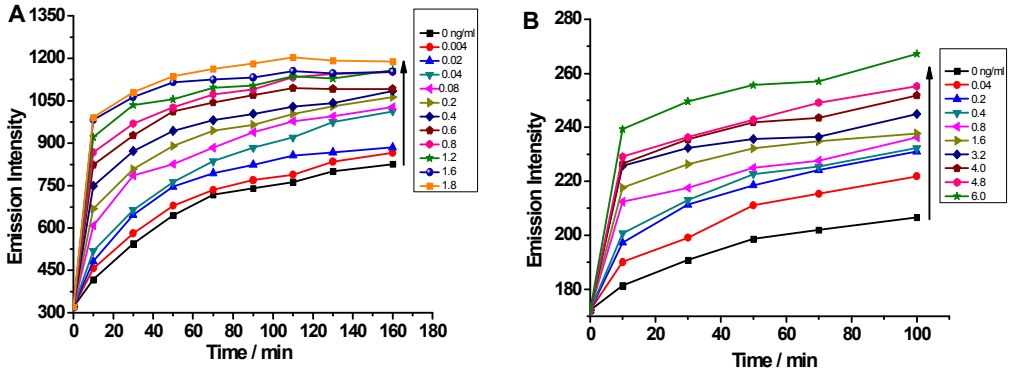


Fig. 5. (A) Fluorescence intensity of CM343-peptide1@AuNPs in the presence of various concentrations of caspase-3 (0, 0.004, 0.02, 0.04, 0.08, 0.2, 0.4, 0.6, 0.8, 1.2, 1.6, 1.8 ng/ml) as function of incubation time. (B) Fluorescence intensity of CM343-peptide2@AuNPs in the presence of various concentrations of caspase-3(0, 0.04, 0.2, 0.4, 0.8, 1.6, 3.2, 4.0, 4.8, 6.0 ng/ml) as function of incubation time.

After addition of caspase-3 to the CM343-peptide1@AuNPs probes in buffer solution, the fluorescent intensity increased significantly in 30 min. As shown in Fig. 6A, the fluorescence recovery of the CM343-peptide1@AuNPs system was observed after only 0.004 ng/ml (～1.2 pM) of caspase-3 was added, and the fluorescence intensity increased gradually with the increase of the caspase-3 concentration from 0.004 to 1.8 ng/ml. The fluorescence recovery linearly correlated to the concentration of caspase-3 over the range of 0.08-1.2 ng/ml (inset of Fig. 7A). For the CM343-peptide2@AuNPs probes, the fluorescence recovery of the CM343-peptide2@AuNPs system was obtained after addition of 0.04 ng/ml (～12pM) of caspase-3, and the fluorescence intensity increased gradually with the increase of the caspase-3 concentration from 0.04 to 6ng/ml (Fig. 6B). The fluorescence recovery linearly correlated to the concentration of caspase-3 over the range of 0. 20-3.2 ng/ml (Fig.7B). The results further demonstrated that the shorter linkage between the donor and the acceptor would result in a higher FRET efficiency, which would cause more significant changes in the emission spectra after addition of caspase-3 and show a higher sensitivity toward caspase-3.

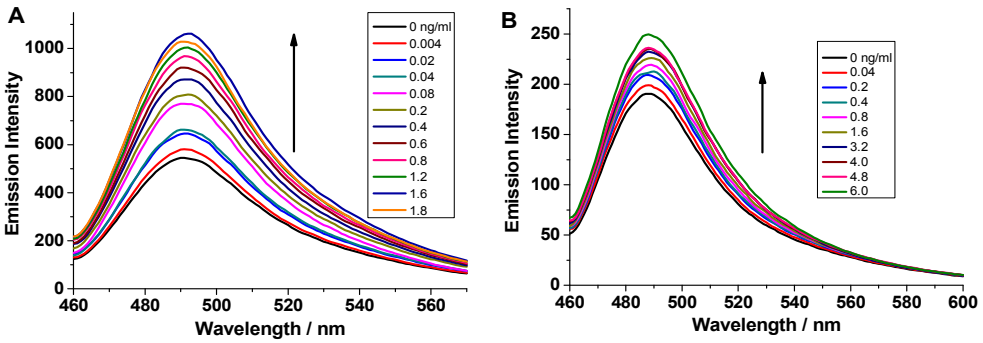


Fig. 6. (A) Fluorescence spectra of CM343-peptide1@AuNPs in the presence of various concentrations of caspase-3(0, 0.004, 0.02, 0.04, 0.0.08, 0.2, 0.4, 0.6, 0.8, 1.2, 1.6, 1.8 ng/ml) after incubation for 30 min. (B) Fluorescence spectra of CM343-peptide2@AuNPs in the presence of various concentrations of caspase-3(0, 0.04, 0.2, 0.4, 0.8, 1.6, 3.2, 4.0, 4.8, 6.0 ng/ml) after incubation for 30 min.

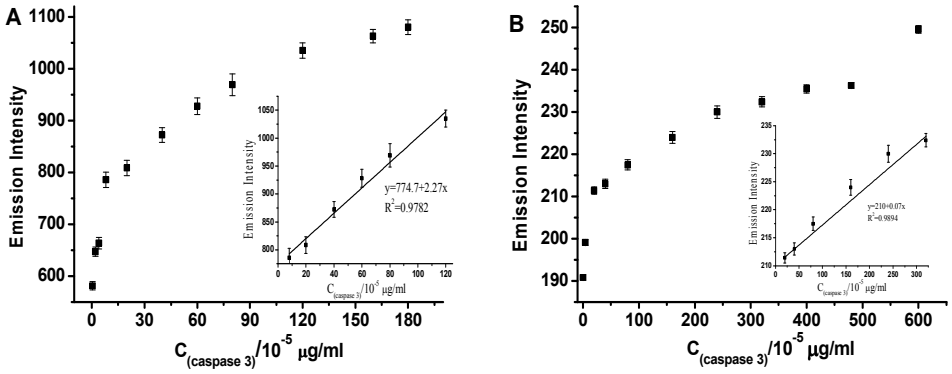


Fig. 7. (A) Fluorescence intensity of CM343-peptide1@AuNPs as a function of caspase-3 concentration. (B) Fluorescence intensity of CM343-peptide2@AuNPs as a function of caspase-3 concentration.

The results show that the CM343-peptide1@AuNPs system has a higher sensitivity toward caspase-3 by monitoring the fluorescence change of peptide-bridged nanohybrid system. The limit of detection (LOD) of the CM343-peptide1@AuNPs system toward caspase-3 was about 4 pg/ml, which is more sensitive than previous reports,[32,33,43,44] probably due to a large overlap between the emission of coumarin-343 and the absorption of AuNPs which resulted in

a high efficient energy transfer process between the donor and the acceptor in our system. The results showed that it will be potentially suitable for the DNA sensing in bioanalytical application

Selectivity

To evaluate the selectivity of the functionalized AuNPs toward caspase-3, a series of proteases and ions were examined for their possible interference in caspase-3 detection. In this study, typical proteases including trypsin, chymotrypsin, esterase, and the ions including K^+, Na^+, Cl^-, have been evaluated for their interference to caspase-3 detection. As shown in Fig. 8, no obvious fluorescence recovery was observed for any inspected substances except for caspase-3. All the results indicated that the peptide linker containing DEVD sequence shows a good specificity for caspase-3 cleavage, and suggested that the CM343-peptide@AuNPs system demonstrated a high selectivity toward caspase-3 detection.

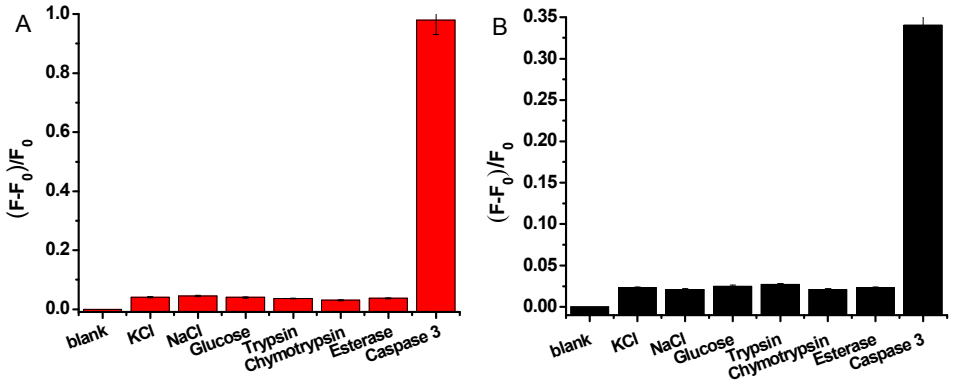


Fig. 8. Specificity test for caspase-3, where F_0 represents the fluorescence intensity of the CM343-peptide1@AuNPs (A) and CM343-peptide2@AuNPs (B) and F is the fluorescence intensity of the fuctionalized AuNPs plus inspected species.

Determination of caspase-3 in sample

Caspase-3 determination with the functionalized AuNP sensor was also conducted in cell extracts. The CM343-peptide1@AuNPs probe was used in this determination. Using the calibration curve obtained in the aqueous solution, the diluted caspase-3 concentration in cell extracts was determined as 0.26 ng ml^{-1}. The standard addition experiment was also carried out, and the recovery was found to be 106.6% with RSD

around 1% (Table 1). The results demonstrated the sensing capabilities of the functionalized AuNP FRET system in complex biological environment and the potential of this kind of nano sensor in bioanalysis and biodection, which might be significant in disease diagnosis in the future.

Table 1 Analytical results of the determination of Caspase-3 in the cell extract sample

Sample	Standard caspase-3 spiked(ng ml^{-1})	Caspase-3 measured (ng ml^{-1})	Recovery (%)	RSD (%) (n =3)
Cell extracts	0	0.26±0.45		6.8
	0.8	1.13±0.18	106.6	1.1

Conclusion

In summary, the CM343-peptide@AuNPs probes have been fabricated through peptide-bridged assembly in a controllable way, and the functionalized AuNP system has been successfully employed for caspase-3 detection by tuning the FRET efficiency between CM343 and AuNPs. A peptide containing recognition sequence DEVD specific for active caspase-3 cleavage was utilized to compose a kind of funcitionalized AuNP biosensor based on FRET process. Caspase-3 cleaves the peptide-bridge and releases CM343 from AuNPs, which forms the basis of caspase-3 recognition. The CM343 funcitionalized AuNP biosensor by the proper linkage is high sensitive and specific towards caspase-3 determination, which might be significant in bioanalysis and biodection and potential application in disease diagnosis in the future.

Acknowledgement

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