Dual-Luminophore-Labeled Gold Nanoparticles with Completely Resolved Emission for the Simultaneous Imaging of MMP-2 and MMP-7 in Living Cells under Single Wavelength Excitation

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Abstract: Although considerable effort has been devoted to the design of various nanoprobes for the fluorescent detection of multiple biomarkers in a single assay, they often suffer from emission-overlapping, owing to small Stokes shifts and wide emission spectra, which results in cross-talk and inaccurate quantification. Herein, we report the design and synthesis of a new nanoprobe for multienzyme detection with completely resolved emission peaks under single-wavelength excitation. The probe was assembled by attaching a cleavable peptide spacer, which was comprised from a matrix metalloproteinase-2 (MMP-2) substrate and a MMP-7 substrate, onto the surface of gold nanoparticles (AuNPs) through cysteine residues. A lanthanide complex, BCTOT-EuIII (BCTOT = 1,10-bis(5-chorosulfo-thiophene-2-yl)-4,4,5,5,6,6,7,7-octafluorodecane-1,3,8,10-tetraone), and 7-amino-4-methylcoumarin (AMC) were attached to the N terminus and the C terminus of the peptide, respectively. In the presence of one or both targeting enzymes, the substrate was cleaved and fluorescence resonance energy transfer (FRET) between the dyes and AuNPs was prohibited, thereby resulting in the dramatic fluorescence emission of dyes. Importantly, there was no emission cross-talk between the two dyes, thereby ensuring accurate detection of each enzyme. Based on this, the simultaneous fluorescence image of MMP-2 and MMP-7 was accomplished in living cells under single wavelength excitation. The apparent differences in the fluorescence imaging indicated distinct differences between the expression levels of MMPs between the human normal liver cells and the human hepatoma cells.

Introduction

Simultaneous fluorescence monitoring of multiple biomarkers in a single assay facilitates the mapping and understanding of the different functions of biomarkers in pathological and physiological fields. [1–3] Moreover, it helps to decrease the number of “false positives” in clinical diagnosis. [4] Towards this goal, various hybrid bio-nanoassemblies or bio-nanostructures have been elaborately designed and fabricated for multiplex analysis based on the unique photochemical properties of nanomaterials and on the superior biochemical properties of biomolecules. [1,4–20] Fluorescent nanoprobes can typically be divided into three types according to their assembly patterns and spectroscopic characteristics: First, nanosensor solutions that are simply mixtures of different nanoparticles commonly show either single excitation and different emission wavelengths or sets of excitation and emission wavelengths. [12,13] Second, dye-labeled biomolecules are conjugated with, or embedded into, nanoparticles that have two or more sets of excitation- and emission wavelengths. [4,14–16] Third, the biomolecule-conjugated nanoparticles exhibit a single excitation wavelength and different emission wavelengths, but with overlapping emission signals. [17–20] Although these nanoprobes have shown significantly improved sensing performance in a variety of optical biosystems, there are also some limitations in their application in accurate and efficient analysis, especially in in vitro or in vivo imaging. [21,22] First, simple mixing of nanoparticles in one system needs two or more sets of excitation and emission wavelengths be monitored simultaneously, which necessitates a more-complex instrumental setup and decreases the continuity of data collection. Second, although a single excitation is observed, the emission overlap decreases the spatial resolution of the spectra and shields the signal of the component with low concentration, thereby preventing its accurate detection or imaging in complex samples. Third, the activity of the cells is influenced by repeated

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signal collection by using different excitation and emission wavelengths. Therefore, an ideal nanoprobe with excellent characteristics, including multireporter assembly, single excitation, and, most importantly, completely resolved emission peaks, is urgently needed for the simultaneous imaging of multiple targets in living cells.

Tumor metastasis involves intricate interactions between the invading cancer cells and the surrounding stromal cells. Such interactions promote degradation of the extracellular matrix (ECM) by specialized proteolytic enzymes, which are produced by both the cancer cells and the stromal cells. Among these enzymes, a variety of matrix metalloproteinases (MMPs) play key roles in multiple physiological and pathological processes that are related to extracellular matrix turnover. There is considerable evidence, both in vivo and in vitro, that enhanced MMP activity is associated with the malignant state. In hepatocellular carcinoma cells, matrix-metalloproteinase-2 (MMP-2) and matrix-metalloproteinase-7 (MMP-7) are expressed at statistically higher levels than in normal liver cells, thus highlighting their potential use as dual markers of neoplastic inception. Therefore, it is critical to develop strategies that, in the diseased state of the cell, allow for the simultaneous monitoring of multiple MMPs, which could effectively avoid false positive outcomes in cancer diagnosis. Previously, the fluorescent detection of MMPs has been achieved by using probes that were based on activatable cell-penetrating peptides, fluorescently labeled co-polymers, Cy5.5-substrate/AuNP assemblies, genetically encoded FRET sensors, and membrane-bound FRET probes. However, most of these systems are single-analyte assay approaches that could not accomplish the simultaneous detection of two or more MMPs in a single assay, especially for cell-imaging.

Herein, we report the design and synthesis of a new nanoprobe and its application in double enzyme sensing and imaging in living cells. To synthesize such a probe, we attached a cleavable peptide spacer that was comprised of an MMP-2 substrate of Gly-Pro-Leu-Gly-Val-Arg-Gly[29,33] and an MMP-7 substrate of Val-Pro-Leu-Ser-Leu-Thr-Met-Gly [34] onto the surface of AuNPs through cysteine residues. A lanthanide complex, BCTOT-EuIII (BCTOT = 1,10-bis(5-chlorosulfo-thiophene-2-yl)-4,4,5,6,7,7-octafluorodecane-1,3,8,10-tetraone), was attached onto the N terminus of the peptide, and AMC was attached onto the C terminus of the peptide (Scheme 1A). FRET between the labeled dyes and the AuNPs resulted in a well-quenched nanoprobe that readily resumed emission in the presence of one or both of the MMPs (Scheme 1B).

Results and Discussion

Probe design and characterization: For the preparation of the nanoprobe, BTOT and BCTOT were first synthesized according to literature procedures[39] and BCTOT-EuIII had the same excitation wavelength (350 nm), whilst their emission wavelengths (449 and 613 nm, respectively) were completely resolved (Figure 1); this is different from previously reported nanoparticles, which show single excitation and overlapped emission.[17–20] This result was attributed to the large Stokes shift and to the sharp emission of lanthanide chelates,[35,36] as well as to appropriate matching in the excitation wavelength of AMC and BCTOT-EuIII, thereby creating ideal conditions for multiple detection without any spectroscopic distortion. Although the assembly of lanthanide complexes that are anchored on AuNPs has been reported,[37,38] their application in multiple imaging processes has not yet been exploited. Furthermore, the double-labeled peptide used herein ensures an immobilization ratio of 1:1 of AMC and BCTOT-EuIII on the surface of the AuNPs. Finally, a simultaneous image of MMP-2 and MMP-7 in living cells under single-wavelength excitation was accomplished. The apparent difference in fluorescence imaging indicated a distinct difference in the expression levels of the MMPs between human normal liver cells and human hepatoma cells, which provides a new opportunity for understanding the physiological and pathological functions of MMPs.
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Fluorescence response of the probe to MMP-2 and MMP-7: As shown in Figure 1, a mixed solution of AMC and BCTOT-EuIII showed the same excitation wavelength (350 nm), whilst the emission wavelengths (449 nm and 613 nm, respectively) did not overlap but rather were completely separated, thus paving a way for multitarget detection without disturbing the spectra. Because AMC and BCTOT-EuIII were linked to the C- and N terminus of the peptide, respectively, the fluorescence emission at 449 and 613 nm corresponded to the activation of MMP-2 and MMP-7, respectively. Next, we tested whether the probe could simultaneously detect MMP-2 and MMP-7. As shown in Figure 2, there was low background fluorescence before the addition of MMP-2 and MMP-7, owing to the excellent quenching effect of the AuNPs. After the addition of different concentrations of MMP-2 and MMP-7, the emission of AMC and BCTOT-EuIII increased rapidly as the peptide was enzymatically cleaved by MMP-2 and MMP-7 and the fluorophore diffused away from the AuNPs. Therefore, the probe could detect MMPs in terms of a dose-dependent increase in fluorescence. As determined from a calibration curve of the fluorescence versus the concentration of MMPs, the increase in fluorescence was proportional to the concentration of MMP-2 in the range 0.076–0.76 µg mL−1 and to the concentration of MMP-7 in the range 0.04–0.4 µg mL−1 (Figure 3). According to C = 3S/k, a concentration of MMP-2 as low as 12.5 ng mL−1 and MMP-7 as low as 1.1 ng mL−1 was readily detected.

Investigation of the specificity of the probe: Specific response to MMP-2 and MMP-7: To test the specificity of the peptide sequences to MMP-2 and MMP-7, the two enzymes were added separately into the probe solution and the fluorescence responses were recorded (see the Supporting Information, Figures S4 and S5). These results showed that, in the presence of MMP-2 alone, the fluorescence emission at 449 nm increased proportionally with MMP-2 concentration, whilst the emission at 613 nm remained unchanged. Whereas, in the presence of MMP-7 alone, the emission at 613 nm increased with MMP-7 concentration, whilst the emission at 449 nm remained unchanged.
ions did not affect the fluorescence intensity. A C H T U N G T R E N N U N G: The influence of bio-
logically relevant substances, thereby constituting the basis for specifically assaying the two enzyme activity in biological samples.

**Optimal experimental conditions for sensing MMPs:** To obtain the sensitive response of MMP assays, we monitored the change in the relative fluorescence intensity with changing pH value (in the range 6.6–8.1), volume of the sensing solution (25–300 µL), and reaction time (3–40 min). The experiments were operated according to the same procedure for fluorescent detection. The optimal detection conditions were pH 7.4, a reaction time of 20 min, and a probe solution of 200 µL (see the Supporting Information, Figures S6–S8).

**Cytotoxicity of the nanoprobe:** To evaluate the cytotoxicity of the nanoprobe, an MTT assay in human HepG2 cells was performed with nanoprobe volumes in the range 100–400 µL (Figure 4). According to the experimental observations, the cell viabilities were all more than 70% compared to the control experiments. Whilst in the fluorescence detection of MPP-2 and MMP-7, 200 µL of the probe solution was used. Therefore, the nanoparticles appeared to show low toxicity on the cells and have good potential in biological applications.

**Cellular-uptake investigation:** To confirm that the nanoprobe was cell-permeable, the uptake of the nanoprobe by HL-7702 cells was quantified by ICP-AES analysis. The cellular concentration of the nanoprobe was 400 ng mL⁻¹ under the experimental conditions, thus indicating good cellular membrane permeability of the nanoprobe.

**Confocal imaging:** The nanoprobe was then applied to cell-imaging to estimate its potential effectiveness in bioassays. The probe was used to differentiate the levels of MMP-2 and MMP-7 in HepG2 and HL7702 cells because of the statistically higher levels of these two enzymes in hepatocellular carcinoma cells than in normal liver cells. When HepG2 cells were incubated with the probe for 1 h at 37°C, two strong fluorescence signals were observed (Figure 5 a–c). However, when HL7702 cells were incubated under the same conditions, only faint fluorescence was observed (Figure 5 d–f). To demonstrate that the fluorescence signal obtained from HepG2 cells was really the result of the cleavage of the nanoprobe by MMP-2 and MMP-7, commercial MMP inhibitor IV was preincubated with HepG2 cells for 30 min, and then incubated with the probe for a further 1 h

![Figure 3. Linear plot of the fluorescence intensity as a function of the concentrations of MMP-2 and MMP-7: R²(MMP-2) = 0.9962, R²(MMP-7) = 0.9910. The experiment was repeated three times and the data are shown as mean ± S.D.)](image1)

![Figure 4. MTT assay of human HepG2 cells in the presence of different volumes of the nanoprobe.](image2)
Crimination of analytes without any cross-talk, which is very solved emission peaks, thereby facilitating the selective discrimination of emissions, this system, which contains a EuIII in contrast to the nanoparticles that have multiple—but overlapping—emissions, this design has several advantages: First, the apparent difference in the image MMP-2 and MMP-7 between normal and carcinoma cells.

Conclusion

We have synthesized a nanoprobe that can simultaneously image MMP-2 and MMP-7 in living cells at a single excitation wavelength. This design has several advantages: First, in contrast to the nanoparticles that have multiple—but overlapping—emissions, this system, which contains a EuIII complex and a coumarin derivative, exhibited completely resolved emission peaks, thereby facilitating the selective discrimination of analytes without any cross-talk, which is very important in the accurate detection of low amounts of components in a complex system. Second, the quencher gold nanoparticle offered a good platform for the wide selection of suitable fluorophores as sensing sites for various analytes, including enzymes, proteins, and complementary ligand/receptor pairs. Third, unlike the methods that use mixed-nanoparticle solutions with different excitation and emission wavelengths, nanoparticles with single excitation and multiple emission wavelengths avoid complex performance and guarantee the continuity of data collection. Finally, our probe exhibited high specificity towards MMP-2 and MMP-7 in the presence of biologically relevant substances and good membrane permeability, as well as low cytotoxicity. Therefore, we anticipated that this multifunction nanoprobe may enable more informative and accurate imaging of tumor markers in cancer cells and effectively avoids false-positive outcomes in cancer cell diagnosis.

Experimental Section

Materials: Hydrogen tetrachloroaurate(III) (HAuCl₄·3H₂O) and trisodium citrate were purchased from the China Medicine Group Shanghai Chemical Reagent Corporation (Shanghai, China). Dimethyl octafluoroor- dinate was obtained from Shenzhen Meryer Chemical Technology Co., Ltd (Shenzhen, China). 2-Acetylthiophene was obtained from Pacific Chemical Co., Ltd (Zhengzhou China). EuCl₃ was purchased from Sigma–Aldrich. All other reagents were analytical reagent grade. 3-(4,5- Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was ob- tained from Sigma–Aldrich. Water was purified with a Mill-Q (18.2 MΩ·cm⁻¹) water system. A 100k Nanosep filter (Pall Corporation, USA) was used for ultra-purification.

MMP-2 and MMP-7 were purchased from Genetimmes Technology, Inc. (Shanghai, China). MMP inhibitor IV was purchased from Merck (Merck China). MMP activator p-amino phenyl mercuric acid (APMA) was pur- chased from Genmed Scientifics Inc., USA. The MMPs were activated by incubation with 1.0 mmolL⁻¹ of APMA for 1 h at 37°C prior to use. The peptides were purchased from GL Biochem (Shanghai, China) Ltd. Peptides were purified by reverse-phase HPLC. The peptide sequence with AMC at the C-terminal was: Gly-Val-Pro-Leu-Ser-Leu-Thr-Met-Gly-Cys-Gly-Gly-Val-Ary-Gly-AMC. This peptide comprised an MMP-2 substrate of Gly-Val-Pro-Leu-Gly-Val-Gly-Gly-Cys-Val-Met-Gly-Cys-Gly-Gly-Val-Arg-Gly-AMC. This peptide was obtained from GL Biochem (Shanghai, China) Ltd.

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Preparation of BTOT and BTOT: [36] For BTOT the choice of the chelator was key to the probe preparation. CH₂Ons (4 g) was added over 10 min in several batches to a flask that contained dimethyl octafluoroor- dinate (5 g) and 2-acetylthiophene (4 g) in dry Et₂O (50 mL). After stir- ring at 25°C for 48 h, the mixture was poured into 15% H₂SO₄ solution.

Figure 5. Confocal fluorescence images of HepG2 and HL7702 cells; the probe (200 μL) was incubated with the fixed cells for 1 h at 37°C and then examined under a confocal microscope. a)–c) Fluorescence images of HepG2 cells; d)–f) fluorescence images of HL7702 cells; g)–i) fluorescence images of HepG2 cells that were preincubated with commercial MMP inhibitor IV for 30 min, and then incubated with the probe for a further 1 h. a), d), g) Fluorescence image of MMP-2 (blue); b), e), h) fluorescence image of MMP-7 (red); c) overlay of (a) and (b); f) overlay of the bright-field image with (d) and (e); i) overlay of the bright-field image with (g) and (h); λₑ = 405 nm; scale bar = 25 μm.
(200 mL). The Et₂O phase was separated and removed under reduced pressure. The solid was recrystallized in anhydrous EtOH, filtered off, and dried under vacuum in the presence of P₂O₅. The NMR spectrum (CDCl₃; see the Supporting Information, Figure S1) showed that BCTOT existed predominantly in its enolic form. For the preparation of BCTOT, BTOT (1.6 g) was added to a flask that contained ClSO₃H (10 mL) under stirring. After heating the mixture at 40°C for 3 h, the solution was added dropwise to ice-cold water. The product was extracted with Et₂O. The Et₂O extract was concentrated to 0.5 mL and the precipitate was removed by filtration and recrystallized in anhydrous Et₂O. BCTOT was obtained as a gray powder and stored in small glass bottles in a desiccator at −20°C.

Peptide labeling with BCTOT: For convenience, a chlorosulfonyl group (SO₂Cl) was introduced into BTOH and BTOT and used as the functional group for labeling NH₂-containing compounds. The labeling reaction of BCTOT with peptides involved the formation of a covalent bond between the SO₂Cl group of BCTOT and the amino groups of the peptide, thereby producing SO₂-NH₂-containing groups on the peptides. BCTOT was coupled to the N terminus of the synthesized peptide by incubating peptide-AMC with BCTOT-peptide-AMC (1.6 g) in a molar ratio of 4:1 (BCTOT:peptide) in carbonate buffer (0.1 mol L⁻¹, pH 9.0). The reaction was allowed to proceed for 1 h at 25°C.

Chelation of Eu³⁺ ions with the peptide-AMC-labeled BCTOT: Because BCTOT contains four oxygen atoms, owing to the two SO₂Cl group of BCTOT and the amino groups of the peptide, thereby producing SO₂-NH₂-containing groups on the peptides, BCTOT was coupled to the N terminus of the synthesized peptide by incubating peptide-AMC with BCTOT-peptide-AMC (1.6 g) in a molar ratio of 4:1 (BCTOT:peptide) in carbonate buffer (0.1 mol L⁻¹, pH 9.0). The reaction was allowed to proceed for 1 h at 25°C.

Attachment of fluorescent peptides onto AuNPs: Near-monodisperse AuNPs (diameter: 13 nm) were prepared by using the classical citrate-reduction route pioneered by Frens. All of the glassware was cleaned in aqua regia (HCl/HNO₃, 3:1 v/v), rinsed with Sartorius water, and oven dried prior to use. Briefly, trisodium citrate (1%, 5.25 mL) was added rapidly to an aliquot of 0.004 g HACl₃ (150 mL) that was heated to reflux under stirring. This mixture was heated at reflux for an additional 15 min, during which time the color changed to deep red. Then, the solution was allowed to cool to RT to afford AuNPs with a net negative charge—from the citrate ions—that stabilized the particles. The size distribution and quality of the resulting particles with an average diameter of 13(±2) nm were determined by TEM (see the Supporting Information, Figure S3a). The concentration of the as-prepared AuNPs was 1.2×10⁹ particles mL⁻¹ (about 2 nm) with a high molar extinction coefficient (at 519 nm) of ε = 6.1×10⁴ mol⁻¹ cm⁻¹.⁵²

Attachment of fluorescent peptides to AuNPs to obtain the probe: AuNPs were covalently linked to the sulfhydryl group on the cysteine residue of the Eu³⁺-BCTOT-peptide-AMC conjugate in the ratio 3000:1 peptide/AuNPs. After continuous stirring for 48 h, excess Eu³⁺-BCTOT-peptide-AMC was removed by repeated centrifugation (Eppendorf 5417R Centrifuge, 12000 rpm, ×2) with pure water. The obtained red precipitate was suspended in fresh 10 mM PBS buffer (20 mL, pH 7.4).

Fluorescence detection: PBS buffer (10 mM, pH 7.4) that contained 200 µL of the probe solution was incubated with MMP-2 and MMP-7 at 37°C for an equilibration period of 20 min, then the fluorescence spectra was collected. The fluorescence intensity was measured with excitation at 350 nm and emission at 449 nm/613 nm. Care was taken to keep the pH value and the ionic strength of the sample solution the same for all measurements. Three independent experiments were used to check the reproducibility of these data.

Cell culture: A human normal liver cell line (HL-7702) and a human hepatoma cell line (HepG2) were maintained by following procedures that were provided by the American Type Tissue Culture Collection (Manassas, VA). Cells were seeded at a density of 1×10⁴ cells mL⁻¹ in RPMI 1640 medium that was supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin/streptomycin, 100 U mL⁻¹) for confocal imaging. Cultures were incubated at 37°C in a 5% CO₂/95% air humidified incubator (MCO-15AC, SANYO).

MTT assay: To investigate the cytotoxicity of the nanoprobe, MTT assays were carried out. Human HepG2 cells were seeded on a 96-well microtitrifer plates to a total volume of 100 µL/well. The plates were maintained at 37°C in a 5% CO₂ incubator for 24 h. Different volumes of the nanoprobe were loaded into each well, with six duplicates for each concentration of probe. No probe was added to the control cells. After incubation for 24 h, the supernatant was removed and the cells were washed with PBS three times. To evaluate cell viability, MTT solution (100 µL, 0.5 mg mL⁻¹ in PBS) was added to each well and the mixture was incubated at 37°C for 4 h. After incubation, the remaining MTT solution was removed, and DMSO (100 µL) was added to each well to dissolve the formazan crystals. The optical absorbance was measured at 490 nm on a microplate reader.

Cellular uptake of the nanoprobe: For the cellular-uptake studies, HL-7702 cells (1×10³ mL⁻¹) were incubated with the nanoprobe (200 µL) in RPMI 1640 medium that was supplemented with 10% fetal bovine serum for 1 h. At the end of incubation period, the cells were washed three times with PBS buffer and trypsinized to remove them from the bottom for quantification purposes. The cells were processed with HNO₃ at 200°C in an oil bath for ICP-AES (Thermo, IRIS Advantage, 242.795 nm) analysis to quantify the amount of AuNPs that had been internalized into the cells.

Confocal imaging: The cells were grown on glass coverslips that had been placed at the bottom of a culture dish. After 1 day, the media was replaced by media that contained the probe (200 µL). After 1 h incubation, the media was replaced. The coverslips were removed, washed three times with PBS buffer, and fixed into a chamber that was filled with PBS that was mounted on a glass slide. Florescence images were acquired on a confocal laser scanning microscope (Leica TCS SPE, Germany) with an immersion objective lens (×60). The excitation source was a 405 nm laser. Blue (400–430 nm) and red emissions (600–650 nm) were collected simultaneously. In the case of MMP inhibitor IV, the cells were preincubated with an MMP inhibitor IV (100 µg mL⁻¹) for 30 min, and then incubated with the nanoprobe for a further 1 h before imaging.

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