Fluorogenic Disassembly of Self-Quenched Near-Infrared Nanoparticles Enables Matrix Metalloproteinase Detection

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There are few reports on the enzyme-controlled disassembly of self-quenched near-infrared (NIR) fluorescent nanoparticles that turn fluorescence on for specific detection/imaging of their activity in vitro and in vivo. Herein, we report the rational design of a new NIR probe 1 whose fluorescence signal was self-quenched upon reduction-controlled condensation and subsequent assembly of its nanoparticles (i.e., 1-NP). Subsequent disassembly of 1-NP by matrix metalloproteinase 2 (MMP-2) turned the fluorescence on. Employing this enzymatic strategy, we successfully applied 1-NP for NIR detection of MMP-2 in vitro and NIR imaging of MMP-2 activity in living cells. Moreover, we also applied 1-NP for specific NIR imaging of MMP-2 activity in MC38 tumors in nude mice. We envision that this NIR probe 1 might be further developed for tumor-targeted imaging in routine preclinical studies or even in patients in the future.

KEYWORDS: Self-Quenched, Disassembly, NIR, MMP-2, Tumor.

INTRODUCTION

For in vivo imaging, near-infrared (NIR) fluorescence probes have shown great potential in recent years due to their low biological autofluorescence and the better penetration of wavelengths in the NIR range (650–900 nm) through tissues.1–3 Compared to inorganic NIR probes, organic NIR fluorescent probes are accessible for large-scale chemical syntheses and have better photophysical properties; thus they are emerging as very attractive candidates for in vivo imaging.4,5 Furthermore, modifying these organic dyes with functional groups to allow the facile conjugation to biomolecules, such as DNA, peptides, proteins, or antibodies, enables molecular imaging of their corresponding biomarkers in vitro, in cells or in vivo.7–9

Compared with the probes whose fluorescence is “Turn-Off” or “Always On,” fluorescent “Turn-On” probes have a lower background signal and thus higher sensitivity (or signal-to-noise ratio) for in vivo imaging.10–12 For most “Turn-On” probes, fluorescence resonance energy transfer (FRET) is commonly used to quench their fluorescence before the probe turns “On” at the targeting site.13–16 Recently, a few studies have adopted aggregation-induced emission (AIE) to turn “On” fluorescence for imaging.17–20 In general, the utilization of a “Turn-On” mechanism has been limited until now. In our previous work, employing a “Turn-On” mechanism, we developed two probes to detect intracellular proteases and image tumors.21 However, there has been no report of using a “Turn-On” probe to image extracellular enzymes secreted in multiple physiological or pathological conditions.

Matrix metalloproteinases (MMPs) have long been associated with various stages and types of cancer and have been believed to be critical for basement-membrane penetration during metastasis.23 Consequently, MMPs were thought to be promising targets for fighting against late-stage diseases for a long time. A common extracellular MMP, MMP-2 has been considered a potential therapeutic target for more than 35 years, based on its association with highly invasive cells, its high level of expression in...
Figure 1. Detailed schematic illustration of reduction-controlled self-assembly of 1-NP to turn NIR signal "Off" and extracellular protease-controlled disassembly of 1-NP to turn NIR signal "On."

many human tumor samples, and its capacity to degrade local type IV collagen. MMP-2 preferentially cleaves Pro-Leu-Gly ↓ Leu-Ala-Gly (PLG ↓ LAG) peptide substrates (↓ indicates the cleavage site), which has been exploited to rationally design substrates for MMP-2 cleavage. So far, based on this specific substrate, many probes for imaging MMP-2 activity (including optical imaging, photoacoustic imaging, and magnetic resonance imaging) have been reported.

On the basis of the literature described above, as shown in Figure 1, we rationally designed a new NIR compound 1 whose fluorescence is self-quenched upon reduction-controlled condensation and self-assembly of its nanoparticles (i.e., 1-NP). When 1-NP were subjected to MMP-2 cleavage and disassembly, the fluorescence was recovered, and we successfully applied 1-NP for NIR imaging of MMP-2 activity in cells. Moreover, we also injected 1-NP into nude mice xenografted with MC38 tumors for discriminative NIR imaging of MC38 tumors in vivo.

EXPERIMENTAL REAGENTS AND INSTRUMENTS
Materials
All the starting materials were obtained from Adams or BaoMan Inc. (Shanghai). Commercially available reagents were used without further purification, unless noted otherwise. All chemicals were reagent grade or better. Recombinant Human MMP-2 was purchased from PeproTech Inc. MMP-2 activity was measured by its ability to cleave a chromogenic peptide MMP-2 substrate at room temperature. At a MMP-2 concentration of 2.5 μg/mL, 50% cleavage was achieved at an incubation time of approximately 25 minutes.

General Methods
Matrix-assisted laser desorption (MALDI) ionization-time of flight (TOF)/TOF and ESI mass spectra were obtained on a time-of-flight Ultraflex II mass spectrometer (Bruker Daltonics, USA) and on a Finnigan LCQ Advantage ion trap mass spectrometer (Thermo Fisher Corporation) equipped with a standard ESI source, respectively. High-performance liquid chromatography (HPLC) purification was performed on a Shimazu UFLC system equipped with two LC-20AP pumps, an SPD-20A UV/vis detector, and a Shimazu PRC-ODS column. HPLC analyses were performed on an Agilent 1200 system equipped with a G1322A pump, in-line diode array UV detector, and an Agilent Zorbax 300SD-C18 RP column, with CH₃CN (0.1% of TFA) and water (0.1% of TFA) as the eluent. Fluorescence spectra were recorded on an
F-4600 fluorescence spectrophotometer (Hitachi High-Technologies Corporation, Japan) with the excitation wavelength set to 675 nm. UV-vis absorption spectra were recorded on a Perkin-Elmer Lambda 25 spectrophotometer. Transmission electron microscopy (TEM) images were obtained on a JEM-2100F field emission transmission electron microscope operated at an acceleration voltage of 200 kV. MC38, a mouse colon adenocarcinoma cell line and B16F10, a mouse melanoma cell line, were kindly provided by Dr. Yangxin Fu (University of Science and Technology of China (USTC)), respectively. Nude mice were purchased from the Nanjing BioMedical Research Institute of Nanjing University (NBRI). Cell and animal images were obtained on a Perkin-Elmer IVIS Spectrum in vivo Imaging System. All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of USTC and approved by the Animal Ethics of USTC Animal Care and Use Committee.

Synthesis and characterization of 1 (Cys(StBu)-Pro-Leu-Gly-Leu-Ala-Gly-Lys(Cy5.5)-CBT). 2-Cyano-6-aminobenzothiazole (CBT) was synthesized following a method described in the literature. The synthetic route for 1 is shown in Figure 2(a).

**Figure 2.** (a) The synthetic route for 1. (b) HR-ESI/MS spectrum (left) and $^1$H NMR spectrum (right) of 1.

Synthesis of B: Compound Fmoc-Pro-Leu-Gly-Leu-Ala-Gly-Lys(Boc)-OH (A) was synthesized by solid phase peptide synthesis (SPPS). Isobutyl chloroformate (IBCF, 269 μL, 1.92 mmol) was added to a mixture of compound A (954 mg, 0.72 mmol) and 4-methylmorpholine (MMP, 269 μL, 1.92 mmol) in THF (5.00 mL) at 0 °C under N₂. The reaction mixture was stirred for 1 h. The solution of 2-cyano-6-aminobenzothiazole (CBT, 169 mg, 0.96 mmol) was added to the reaction mixture and further stirred at 0 °C for 1 h. Then, the mixture was stirred overnight at room temperature. Compound B (479 mg, yield: 50%) was purified by HPLC using water-acetonitrile with 0.1% TFA as the eluent. MS: calculated for B [(M+H)$^+$]: 1325.59; obsvd ESI-MS: m/z 1325.08.

Synthesis of C: The Boc protecting groups of compound B were removed with dichloromethane (DCM, 1 mL) and triisopropylsilane (TIPS, 200 μL) in TFA (20 mL) for 3 h. Compound C (366 mg, yield: 83%) was obtained after HPLC purification using water-acetonitrile with 0.1% TFA as the eluent. MS: calculated for C [(M+H)$^+$]: 1225.54; obsvd. ESI/MS: m/z 1225.38.

Synthesis of D: Cy5.5 NHS ester (7.3 mg, 0.01 mmol) was well-dispersed in 500 μL DMF, after which compound C (26.8 mg, 0.02 mmol) and N,N-diisopropylethylamine (DIPEA, 10 μL, 0.06 mmol) were
added into the mixture and further stirred for 3 h at room temperature to yield compound D. Compound D (15.1 mg, yield: 84%) was purified with HPLC using water-acetonitrile with 0.1% TFA as the eluent. MS: calculated for D [(M + H)⁺]: 1790.86; obsvd. ESI-MS: m/z 1790.72.

Synthesis of 1: The Fmoc protecting group of compound D was cleaved with 10% piperidine in DMF (4 mL) at 0 °C for 5 min, after which 500 μL TFA was added to neutralize the alkaline, yielding I (8.8 mg, yield: 67%) after HPLC purification with water-acetonitrile as the eluent. MS: calculated for I [(M + H)⁺]: 1568.78933; [(M + 2H)⁺]/2: 784.89958; obsvd. HR-ESI/MS [(M + H)⁺]: m/z 1567.78894; [(M + 2H)⁺]/2: m/z 784.89929 (Fig. 2(b)).

1H NMR of 1 (d₆-DMSO, 300 MHz, Fig. 2(b)) δ (ppm): 8.71 (s, 1H), 8.56–7.97 (m, 16H), 7.96–7.58 (m, 7H), 7.49 (s, 2H), 6.71–6.24 (m, 3H), 5.30 (s, 1H), 4.30 (d, J = 62.7 Hz, 7H), 2.95 (t, J = 23.0 Hz, 4H), 1.94 (s, 12H), 1.69 (s, 4H), 1.50 (d, J = 7.6 Hz, 4H), 1.42 (s, 4H), 1.29 (s, 12H), 1.22 (s, 12H), 0.88–0.74 (m, 12H).

MTT Assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to measure the cytotoxicity of 1-NP in MC38 tumor cells. Cells were seeded into 96-well cell-culture plates at 5 x 10⁴ cells/well. The cells were incubated at 37 °C under 5% CO₂. A solution of 1-NP diluted in DMEM (100 μL/well) was added to each well. After 4 h of incubation, the culture media was aspirated, and a solution of DMSO (150 μL/well) was added to dissolve the formazan with additional shaking for 10-minutes. The data were obtained using an ELISA reader (Varioskan Flash) to detect the absorption of the solution at 490 nm. Each of the experiments was performed at least three times.

RESULTS AND DISCUSSION

Rationale of the Design

We designed our probe with the following components, as shown in Figure 1: a disulfide cysteine (Cys) motif, a 2-cyanobenzothiazole (CBT) motif, a PLGLAG substrate for MMP-2 specific cleavage, and a Cy5.5 motif conjugated to the side chain of a lysine (Lys) motif to generate the NIR signal. In the presence of a reducing agent (e.g., tris(2-carboxyethyl)-phosphine, TCEP) and at neutral pH, the disulfide bond of 1 was reduced and the N-terminal Cys motif condensed with the cyano group of another monomer 1 to yield cyclized dimer (i.e., 1-Dimer, Fig. 1) which self-assembled into the self-quenched nanoparticles (i.e., 1-NP), as previously demonstrated. After incubation with MMP-2 or MMP-2-overexpressing tumor cells,
incubated with 10-fold TCEP at 37 °C (PB, 0.02 M) containing 20% DMSO (v/v) (pH 7.4) was applied to MMP-2 in vitro. The NIR fluorophore Cy5.5 in 1-NP was specifically and highly sensitively imaged by turning “On.” By this means, MMP-2-overexpressing tumor cells were specifically and highly sensitively imaged by the NIR fluorophore Cy5.5 in 1-NP.

**In Vitro NIR Fluorescence Detection of MMP-2 Activity with 1-NP**

After characterizing the pure compound 1 (Fig. 2), we first used TCEP to trigger condensation of 1 to assemble 1-NP and applied 1-NP for NIR fluorescence detection of MMP-2 in vitro. Compound 1 (10 μM) in phosphate buffer (PB, 0.02 M) containing 20% DMSO (v/v) (pH 7.4) was incubated with 10-fold TCEP at 37 °C for 1 h. The reaction mixture was then centrifuged, and the 1-NPs obtained by centrifugation were redispersed in same volume of PB for fluorescence measurements. In comparison with the fluorescence emission at 710 nm of 1, the NIR fluorescence emission of 1-NP dropped by over 6-fold (Fig. 3(a)), suggesting efficient self-quenching of fluorescence after nanoparticle formation. After the 1-NP dispersion was incubated with MMP-2 at 37 °C for 6 h, the fluorescence emission at 710 nm of 1-NP increased 2.5-fold, suggesting disassembly of the nanoparticles by the enzyme and recovery of the fluorescence (Fig. 3(a)). We also used the conditioned media from B16F10 and MC38 tumor cell lines, both of which highly express and secrete MMP-2.30,31 for incubation with 1-NP for further validation. As shown in Figure 4, media from both cell lines could augment the fluorescence intensity within 4 h by 2.0- and 4.0-fold in B16F10 and MC38 media, respectively. Furthermore, HPLC traces clearly indicated that 1-NP was composed of the condensation product of 1 (i.e., 1-Dimer) (blue trace in Fig. 3(b)) and that MMP-2 efficiently digested the substrate 1-Dimer to yield 1-D-cleaved (red trace in Fig. 3(b)). Additionally, UV-vis spectroscopy indicated that after successful condensation of 1, the absorbance peak at 320 nm for 1 shifted to 350 nm for 1-Dimer (Fig. 3(c)), which further confirmed the formation of 1-Dimer and was consistent with our previous work.32 Moreover, the absorbance peak of 1-D-Cleaved showed an obvious collapse at 320 nm (Fig. 3(c)), which further suggested the successful condensation. Transmission electron microscopy (TEM) images clearly showed the TCEP-controlled self-assembly and subsequent MMP-2-controlled disassembly of 1-NP (Figs. 3(d and f)). Statistical analyses indicated that as-formed nanoparticles (i.e., 1-NP) have an average diameter of 29.54 ± 6.5 nm (Fig. 3(e)).

**NIR Imaging of MMP-2-Like Activity in Cells**

After confirming that 1-NP could be efficiently digested by MMP-2 to turn the fluorescence “On,” we applied it for MMP-2 detection in living cells. The cytotoxicity of 1-NP up to concentration of 10 μM and an
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0.2h 0.5h 1h 1.5h 2h 2.5h 3h

1-NP

SB-3CT + 1-NP

Figure 6. (a) Top row, fluorescence images of MMP-2-high secretion MC38 cells after incubation with 0.5 μM 1-NP at 37 °C for 0.2 h, 0.5 h, 1 h, 1.5 h, 2 h, 2.5 h, and 3 h. Bottom row, fluorescence images of MC38 cells after preincubation with 1 μg/mL SB-3CT for 0.5 h, then incubation with 0.5 μM 1-NP at 37 °C for 0.2 h, 0.5 h, 1 h, 1.5 h, 2 h, 2.5 h, and 3 h. Excitation: 675 nm. (b) Statistic curves indicate the total fluorescence (total flux) in (a). 1-NP (red), SB-3CT + 1-NP (blue).

incubation time of 48 h with MC38 cells was investigated; the results indicated that, up to 48 h at 10 μM, 1-NP did not induce obvious cytotoxicity in the cells (Fig. 5). As MMP-2 is an extracellular protease, we employed a living imaging system to take images for detecting its activity. As shown in Figure 6(a), 5 × 10^4 MC38 cells were incubated with 0.5 μM 1-NP (top) or 1 μg/mL MMP-2 specific inhibitor SB-3CT and 0.5 μM 1-NP (bottom) at

(a) 0h 6h 12h 24h 48h 72h

1-NP

SB-3CT + 1-NP

Figure 7. (a) Top row, time-course fluorescence imaging of nude mice xenografted with MC38 tumor cells after tumor-direct injection of 25 μL 1-NP suspension at 10 μM at 0 h, 2 h, 6 h, 12 h, 24 h, 48 h, and 72 h. Bottom row, time-course fluorescence imaging of nude mice xenografted with MC38 tumor cells after tumor-direct injection of 10 μL, 10 μg/mL SB-3CT for 0.5 h, followed by tumor-direct injection of 25 μL 1-NP suspension at 10 μM at 0 h, 2 h, 6 h, 12 h, 24 h, 48 h, and 72 h. (b) Quantified total photon output for mice images in (a).
37 °C for time course analyses. The fluorescence intensity of 1-NP group increased significantly within 1 h of incubation and achieved peak fluorescence after 2.5 h. The addition of an MMP-2 inhibitor significantly suppressed the fluorescence recovery in the incubation system, which convincingly supports the specificity of 1-NP to MMP-2. Additionally, we measured the intensity of the total fluorescence (total flux) in the culture plate wells, which indicates that after continuously increasing for 2.5 h, the total flux began to decrease (Fig. 6(b)). Although the total flux of the inhibitor addition group showed a mild increase, it was still significantly lower than that of 1-NP group. These findings are fully consistent with our hypothesis that 1-NP could actively target the MMP-2-high secretion tumor cells via the specific enzyme digestion. They also suggest that the MMP-2 extracellular protease is a good biomarker for tumor cell-targeted imaging.

NIR Imaging of MMP-2-Like Activity in Tumor-Bearing Mice

We then applied 1-NP for tumor-targeted imaging using nude mice xenografted with MC38 tumors near the right thighs. After the tumor sizes grew to within 5–10 mm in diameter, the nude mice were randomly divided into 2 groups (n = 3 for each group). For first group, 25 µL of 10 µM 1-NP suspension at pH 7.4 was injected into each of these tumor-bearing nude mice through tumor-direct injection. For the other group, 10 µL of 10 µg/mL SB-3CT was injected into tumors in order to first inhibit MMP-2 activity. After 0.5 h, 25 µL of a 10 µM 1-NP suspension was injected via tumor-direct injection. All the mice were imaged for 72 h in a living imaging system. As shown in Figure 7(a), the fluorescence signals from the tumors of these two groups all gradually increased from 2 h to 48 h. However, the fluorescence in the 1-NP group increased by 1.8-fold (Fig. 7(b)). These results clearly indicated that 1-NP could be efficiently digested by the extracellular protease MMP-2 secreted from tumor cells, enabling tumor-targeted NIR imaging in vivo.

To further validate that the strong NIR-fluorescence in 1-NP-treated mice was actually emitted from MC38 tumors, we sacrificed the mice after in vivo imaging at 72 h and removed the tumors and organs to conduct ex vivo imaging. As shown in Figure 8, MC38 tumors in the experimental group showed stronger NIR-fluorescence than those in control group, which was consistent with the fluorescence imaging of tumors in Figure 7. Interestingly, fluorescence signals from stomachs and colons of both groups were higher than those of other organs except tumors (Fig. 8), which was probably due to the sodium-dependent multivitamin transporter (SMVT) on the intestinal walls that transfers essential nutrients to the gastrointestinal organs.

CONCLUSIONS

In conclusion, we rationally designed the NIR probe 1 to facilely prepare the self-quenched nanoparticle 1-NP for MMP-2 detection in vitro, in cells, and in vivo. Reduction-controlled self-assembly and MMP-2-controlled disassembly of 1-NP, along with a fluorescence switch from “Off” to “On,” were validated by in vitro characterizations. Upon enzymatic cleavage by extracellular MMP-2 secreted from tumor cells, 1-NP was disassembled efficiently and accompanied by fluorescence “Turn-On,” which was confirmed with an MMP-2 specific inhibition control. With the fluorescence “Turn-On” property, 1-NP was successfully applied to image MMP-2 activity in cells and in tumors. We envision that our nanoparticles could be applied for MMP-2 specific detection and for fluorescence-guided tumor surgery in the near future.

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