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# *In vivo* fluorescence imaging for cancer diagnosis using receptor-targeted epidermal growth factor-based nanoprobe

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ABSTRACT

Receptor-targeted imaging is emerging as a promising strategy for diagnosis of human cancer. Herein, we developed an epidermal growth factor-based nanoprobe (EGF-NP) for *in vivo* optical imaging of epidermal growth factor receptor (EGFR), an important target for cancer imaging. The self-quenched EGF-NP is fabricated by sequentially conjugating a near-infrared (NIR) fluorophore (Cy5.5) and a quencher (BHQ-3) to EGF, a low-molecular weight polypeptide (6.2 kDa), compared to EGFR antibody (150 kDa). The self-quenched EGF-NP presented great specificity to EGFR, and rapidly internalized into the cells, as monitored by time-lapse imaging. Importantly, the self-quenched EGF-NP boosted strong fluorescence signals upon EGFR-targeted uptake into EGFR-expressing cells, followed by lysosomal degradation, as confirmed by lysosomal marker cell imaging. Consistent with cellular results, intravenous injection of EGF-NP into tumor-bearing mice induced strong NIR fluorescence intensity in the target tumor tissue with high specificity against EGFR-expressing cancer cells. Signal accumulation of EGF-NP in tumor was much faster than that of EGFR monoclonal antibody (Cetuximab)-Cy5.5 conjugates due to the rapid clearance from the body and tissue permeability of low-molecular weight EGF. This self-quenched, EGF-based imaging probe can be applied for diagnosis of various cancers.



## 1. Introduction

Receptor-targeted imaging is emerging as a promising strategy for the diagnosis of human cancer [1,2]. The advantage of specific receptor targeting is obvious—the use of imaging probes with higher specificity towards the target receptor allows highly sensitive and selective imaging with a low dose of the probe [3–5]. Furthermore, receptor-targeted imaging could be helpful in selecting patients for receptor-targeted therapy before the course of treatment [4]. During treatment, receptor-targeted imaging can also monitor effects of the receptor-targeted therapy, allowing clinicians to identify tumor responsiveness before evaluation of traditional, late-stage markers of treatment efficacy, such as a change in tumor size.

Epidermal growth factor receptor (EGFR) is an important target receptor in many types of cancers, including non-small cell lung cancer, skin cancer, breast cancer, small cell carcinoma of head and neck, and prostate cancer [6–8]. Overexpression of EGFR is shown to be correlated with poor prognosis and increased metastatic potential [9,10]. The current standard method to image EGFR has utilized EGFR antibodies, due to their exquisite specificity towards EGFR in tumor cells. When modified with radioisotopes or magnetic or fluorescent probes, EGFR antibodies are used to image various tumors by nuclear, magnetic resonance (MRI), and fluorescence imaging, respectively [11–13]. However, despite numerous studies using EGFR antibody-based imaging probes, they are still limited and insufficient, due to the large size of EGFR antibodies (150 kDa) that cause some drawbacks in receptor-targeted imaging, such as long circulation time, poor tissue permeability, and slow clearance from the body (few days to weeks), etc [14,15].

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Recently, epidermal growth factor (EGF) has shown great potential for EGFR-targeted cancer imaging [16–18]. EGF is the natural ligand for EGFR and is comprised of 53 amino acids with a molecular weight of ~6 kDa. EGF offers unique advantages for targeting EGFR over EGFR antibody: the binding affinity is typically stronger, cell penetration occurs in deeper regions of the tumor, and clearance rates are more rapid because of a smaller molecular weight compared to EGFR antibody [19,20]. For targeted EGFR imaging, EGF molecules have been labeled with various radionuclides and organic dyes for cell and small animal imaging [11–13]. Owing to its small size and high affinities, EGF-based imaging probe generally show fast tumor targeting (within a half hour), high tumor uptake, and quick clearance from normal tissues [16]. Therefore, EGF-based imaging probes have been proven to be a good platform for developing imaging probes for EGFR.

Herein, for specific and sensitive EGFR fluorescence imaging, EGF was labeled with a near-infrared (NIR) fluorophore and a quencher, producing a fluorescently quenched EGF-based nanoprobe (EGF-NP). A fluorescently quenched imaging probe can reduce background signals and boost strong fluorescence intensity, as in the case of EGF-NP for targeted EGFR imaging. In previous studies, we developed various protease-targeted activatable nanoprobes for *in vivo* fluorescence imaging, which have a strongly dual-quenched (dye-dark quencher and dye–dye self-quenching mechanism) system [21–25]. They showed highly efficient quenching and responsive amplified fluorescent signals upon exposure of the probes to targeted protease *in vitro* and *in vivo*.

We show that EGF-NP can be intensely activated in high EGFR-expressing cells and high EGFR-expressing tumor-bearing mice, compared to low EGFR-expressing cells and low EGFR-expressing tumor-bearing mice. Cellular imaging of EGF-NP in tumor cells was captured using live cell imaging microscopy with a video imaging system. In addition, cellular imaging and whole body imaging of EGF-NP treatment was compared to EGFR antibody (Cetuximab)-Cy5.5 conjugates.

## 2. Materials and methods

### 2.1. Materials

EGF consisting of 53 amino acid with a molecular weight of 6215 Da was obtained from Sigma (St. Louis, MO). Cy5.5-NHS (excitation/emission; 675/695 nm) was purchased from GE Healthcare (Piscataway, NJ) and BHQ3-NHS (maximum absorbance: 650 nm) was from Biosearch Technologies, Inc. (Novato, CA). PD-10 disposable columns and gradient gel were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden) and Elpisbio (Taejeon, Korea), respectively. EGFR antibody was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) and horseradish peroxidase-conjugated anti-mouse antibody was purchased from Waters (Milford, MA). Lipofectamine was purchased from Invitrogen (Carlsbad, CA). DMEM and RPMI 1640 were purchased from Gibco (Grand Island, NY). CCK-8 and a human EGF ELISA kit were purchased from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan) and RayBiotech, Inc. (Norcross, GA), respectively.

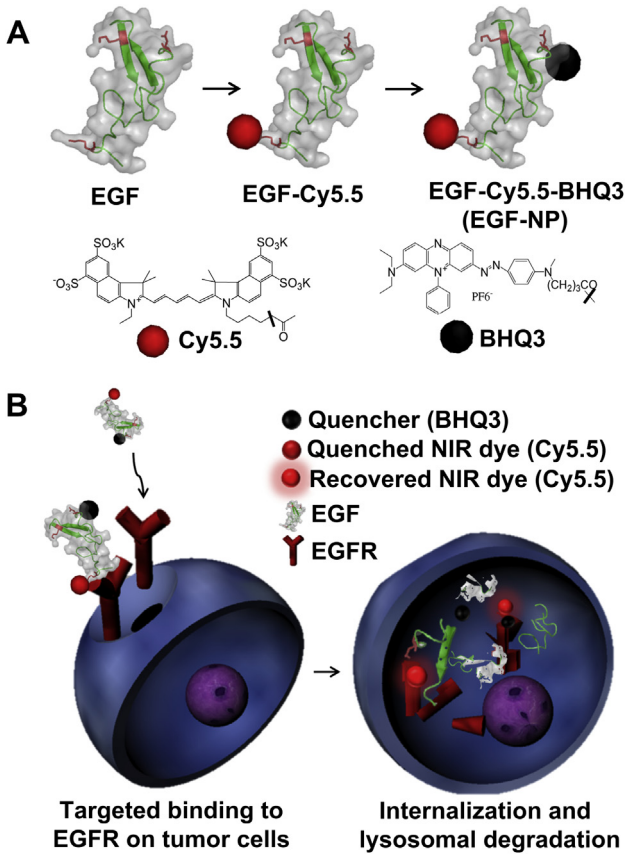
### 2.2. Synthesis and characterization of EGF-NP

An aqueous solution of recombinant human EGF (0.32 μmol, 1 ml sodium bicarbonate buffer, 0.1 M, pH 9.5) was incubated with Cy5.5-NHS (0.32 μmol) in 500 μl dimethyl sulfoxide (DMSO) at RT for 30 min, and non-reacted Cy5.5 was removed by gel filtration using PD-10 column. Then, BHQ3-NHS (0.32 μmol) in 500 μl DMSO was added to the aqueous solution of EGF-Cy5.5, which was reacted at RT for 30 min. The reaction mixture was purified with PD-10 column. The collected fractions, EGF-Cy5.5-BHQ3 conjugates (EGF-NP), were stored at 4 °C for further use.

EGF-NP was characterized using SDS-PAGE, spectrofluorometry, analytical reverse phase-HPLC equipped with a ZORBAX 300SB-C18 column, and matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF). For SDS-PAGE, EGF, EGF-Cy5.5 and EGF-NP were mixed with an equal volume of sample buffer (0.25 M Tris [pH 6.8] containing 0.1% [w/v] bromphenol blue), respectively. The mixed products were loaded on a 15% gradient polyacrylamide gel. The gel was washed twice with 2.5% (v/v) Triton X-100 solution (30 min and 1 h each) and incubated in 50 mM Tris HCl (pH 7.5), 10 mM $CaCl_2$ and 0.2 M NaCl at 37 °C for 24 h. The gel was stained with 3% (w/v) Coomassie blue, dried and scanned. Fluorescence signal of gels was imaged using a Kodak Image Station (4000 MM; Kodak, New Haven, CT). Kodak Image Station is equipped with the excitation filter for Cy5.5 and halogen lamp. Fluorescence images were obtained with an emission filter (680–720 nm; Omega Optical, Brattleboro, VT) and 12-bit CCD camera equipped with C-mount lens. In addition, fluorescence intensities of EGF-Cy5.5 and EGF-NP were measured using a fluorescence spectrophotometer (F-7000, Hitach, Tokyo, Japan). MALDI-TOF mass spectrometry was used to measure the molecular weight of EGF, EGF-Cy5.5 and EGF-NP using a MALDI-TOF system (Microflex LRF20, Bruker Daltonics, Bremen, Germany). Cy5.5, EGF, EGF-Cy5.5 and EGF-NP were characterized by analytical reverse phase-HPLC; 5%–90% acetonitrile containing 0.1% trifluoroacetic acid (TFA) versus distilled water containing 0.1% TFA over 20 min at a flow rate of 1.0 ml/min. To evaluate the de-quenching ability, EGF-NP was incubated in phosphate-buffered saline (PBS, pH 5.0) with or without lysozyme and 5% SDS for 20 min, and then imaged with Kodak Image Station. As control, EGFR monoclonal antibody, Cetuximab (3.3 nmol) was incubated with Cy5.5-NHS (40 nmol) in 0.1 M $Na_2HPO_4$ (pH 8.5) at RT for 1.5 h. Then, the reaction mixture was purified with a PD-10 column to remove unbound Cy5.5.

### 2.3. Cytotoxicity

Human breast cancer cell lines, MDA-MB-468 (high EGFR-expressing) and MDA-MB-436 cells (low EGFR-expressing) were obtained from the American Type Culture Collection (Manassas, VA) [26]. MDA-MB-468 cells were cultured in DMEM containing 10% fetal bovine serum (FBS) and 100 U/ml penicillin and MDA-MB-436 cells were cultured in RPMI 1640 containing 10% FBS and 100 U/ml penicillin. Cytotoxicity was evaluated using CCK-8. In brief, MDA-MB-468 cells and MDA-MB-436 cells, respectively, were seeded in a 96-well microplate and incubated with various concentrations of the EGF-NP (0–50 μg/ml) for 24 h before incubation with CCK-8




**Scheme 1.** Preparation of EGFR-targeted fluorescence activatable nanoprobes (EGF-NP). (A) Illustration of chemical reactions used to conjugate Cy5.5 and BHQ3 onto EGF and chemical structures of Cy5.5 and BHQ3. Each EGF has three amine groups (N-terminal and 2 lysine) available for reaction with Cy5.5 or BHQ3. Crystal structure of EGF was drawn by the program Pymol (DeLano, W.L. The PyMOL Molecular Graphics System (2002) on World Wide Web [http://www.pymol.org]). (B) Schematic presentation for activation of EGF-NP. EGF labeled with a NIR fluorophore and a quencher (EGF-NP) binds to EGFR, which leads to cellular internalization and intracellular degradation of EGF-NP in the lysosome. Then, lysosomal degradation of EGF-NP causes separation of the fluorophore from the quencher, allowing recovered fluorescence only within the EGFR-positive cells.