Influence of chelator and near-infrared dye labeling on biocharacteristics of dual-labeled trastuzumab-based imaging agents

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Abstract

Objective: To investigate the effect of fluorescent dye labeling on the targeting capabilities of ¹¹¹In-(DTPA),-trastuzumab-(IRDye 800)ₙ.

Methods: Trastuzumab-based conjugates were synthesized and conjugated with diethylenetriaminepentaacetic acid (DTPA) at molar ratios of 1, 2, 3 and 5 and with a fluorescent dye (IRDye 800CW) at molar ratios of 1, 3 and 5. Immunoreactivity and internalization were assessed on SKBR-3 cells, overexpressing human epidermal growth factor receptor 2. The stability in human serum and phosphate-buffered saline (PBS) was evaluated. The biodistribution of dual-labeled conjugates was compared with that of ¹¹¹In-(DTPA),-trastuzumab in a SKBR-3 xenograft model to evaluate the effect of dye-to-protein ratio.

Results: All trastuzumab-based conjugates exhibited a high level of chemical and optical purity. Flow cytometry results showed that increasing dye-to-protein ratios were associated with decreased immunoreactivity. Stability studies revealed that the conjugate was stable in PBS, while in human serum, increased degradation and protein precipitation were observed with increasing dye-to-protein ratios. At 4 h, the percentages of internalization of dual-labeled conjugates normalized by dye-to-protein ratio (m) were 24.88%±2.10%, 19.99%±0.59%, and 17.47%±1.26% for “m” equal to 1, 3, and 5, respectively. A biodistribution study revealed a progressive decrease in tumor uptake with an increase in the dye-to-protein ratios. The liver, spleen and kidney showed a marked uptake with increased dye-to-protein ratios, particularly in the latter.

Conclusions: With non-specific-site conjugation of the fluorescent dye with a protein based on imaging agent, the increase in dye-to-protein ratios negatively impacted the immunoreactivity and stability, indicating a reduced tumor uptake.

Keywords: Influence; near infrared dye; trastuzumab; human epidermal growth factor receptor 2; dual-labeled

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Introduction

Precision medicine, selecting appropriate and optimal therapies based on the context of a patient’s genetic content or other molecular analysis, has become increasingly important in the treatment strategies of cancer (1). Nuclear medicine and optical fluorescence imaging techniques provide powerful non-invasive imaging capability for the assessment of biomarker over-expression in tumor cells, including tissues that may not be amenable to detect with conventional imaging modalities (2,3). However, the low spatial resolution at the cellular level limits the ability of nuclear medicine techniques to detect microscopic lesions. Furthermore, low penetration depth is a key limitation of optical imaging modalities (4). The use of dual-modality imaging probes confers an unprecedented leverage by harnessing the complementary advantages and outweighing the respective limitations of these techniques (5,6).

Trastuzumab is a humanized anti-human epidermal growth factor receptor 2 (HER2) antibody that is approved for the treatment of breast cancer (7,8). HER2 overexpression, reported in 25%–30% of all breast cancers, is associated with a poor prognosis and aggressive tumor attributes (9). The reported conservation and upregulation of HER2 make trastuzumab a promising therapeutic modality for targeting metastatic disease in patients with HER2(+) breast cancer (10).

Recent studies have evaluated dual-labeling of trastuzumab for nuclear imaging using positron emission tomography (PET) or single photon emission computed tomography (SPECT) radionuclides as well as for near-infrared (NIR) fluorescence imaging at excitation wave lengths >750 nm (11-13). A comparison of the nuclear and NIR fluorescence images of xenografts following the administration of dual-labeled peptide and antibody showed comparable target-to-background ratios (indicative of comparable sensitivities and stable dual-labeling) but a higher signal-to-noise ratio (SNR), which is consistent with the increased photon count rates possible with fluorescence. However, the synthetic complexity and the effect of the fluorescent dye on the biodistribution of the imaging agent are two major challenges to the use of dual-modality imaging (14).

Here, we describe a method for dual-labeling of trastuzumab for nuclear imaging using positron emission tomography (PET) or single photon emission computed tomography (SPECT) radionuclides as well as for near-infrared (NIR) fluorescence imaging at excitation wave lengths >750 nm (11-13). A comparison of the nuclear and NIR fluorescence images of xenografts following the administration of dual-labeled peptide and antibody showed comparable target-to-background ratios (indicative of comparable sensitivities and stable dual-labeling) but a higher signal-to-noise ratio (SNR) of NIR fluorescence, which is consistent with the increased photon count rates possible with fluorescence. However, the synthetic complexity and the effect of the fluorescent dye on the biodistribution of the imaging agent are two major challenges to the use of dual-modality imaging (14).

Materials and methods

Materials

Trastuzumab (Herceptin™, Genentech South, CA) was purchased from clinical pharmacy for research purposes. IRDye 800CW was purchased from LI-COR Bioscience (Lincoln, NE) and p-SCN-Bn-DTPA (Dallas, TX). 111InCl3 was obtained from Perkin-Elmer Life Sciences (Waltham, MA).

Cell culture

Human breast cancer SKBR-3 cells with a stable overexpression of HER2 (American Type Culture Collection) were cultured in Dulbecco’s minimal essential medium (DMEM)/F-12 (Invitrogen) with 10% fetal bovine serum (HyClone) in a humidified incubator at 37 °C with 5% CO2.

Preparation of trastuzumab-based conjugate

Conjugation of DTPA to trastuzumab

Trastuzumab was dissolved in 50 mmol/L NaHCO3 (pH 8.0) at a concentration of 20 mg/mL and incubated with p-SCN-Bn-DTPA at room temperature (RT) for 1 h in varying DTPA-to-trastuzumab molar ratios (1:1 to 10:1). The final molar ratio of DTPA to protein, “n”, was determined as described by Hnatowich et al. (15). Unbound DTPA was removed using ultrafiltration (molecular weight cut-off 50 kD, Millipore). Final protein concentrations were determined by ultraviolet absorbance at 280 nm.

Conjugation of IRDye 800CW to DTPA-trastuzumab (DTPA)_n-trastuzumab was conjugated with IRDye 800CW in varying IRDye 800CW-to-antibody molar ratios of 1:1 to 10:1 as per the instructions provided by LI-COR Biosciences. The number of final dye molecules per protein molecule (dye-to-protein ratio, “m”) and protein concentration were calculated by measuring absorbance with a UV-Vis spectrophotometer (Beckman Coulter) at 280 nm and 780 nm (11).

Assessment of purity of (DTPA)_n-trastuzumab-(IRDye 800)_m

Aliquots of 0.1–0.2 ng conjugates mixed with Laemmli sample buffer (without beta-mercaptoethanol) were loaded onto 4%–12% Tris-glycine acrylamide gels (Bio-Rad). Gels were run until the bromophenol blue reached approximately 10 mm from the bottom of gels, and subsequently imaged using a LI-COR Odyssey infrared imaging system, and 16-bit tif files were analyzed using ImageJ software (NIH, MD). The identification of the region of interest (ROI) of a pre-defined pixel size for
each gel band and quantification of fluorescent intensity levels were performed.

Flow cytometry

In brief, 0.3×10⁶ SKBR-3 cells were incubated in 100 µL of culture medium that contained 5 µg of trastuzumab conjugate. After 30 min on ice, the cells were washed once with 4 mL of medium and incubated for an additional 30 min with fluorescein isothiocyanate (FITC)-conjugated mouse anti-human IgG antibody (BD Bioscience) on ice. After a final wash, the cells were resuspended in 2.5 µg/mL of 7-amino-actinomycin D solution. The mean fluorescence intensity (MFI) was assessed with flow cytometry (FACSCalibur, Becton Dickinson). As a control for specific binding, trastuzumab was tested at equivalent molar concentrations to provide the measurement of MFIpositive whereas cells treated with phosphate-buffered saline (PBS) served as a negative control for the measurement of MFInegative. The percentage of immunoreactivity was calculated as follows: \(\frac{[\text{MFI}_{\text{sample}} - \text{MFInegative}] - [\text{MFI}_{\text{positive}} - \text{MFInegative}]}{\times 100\%}\).

\(\text{^{111}In labeling and serum stability}\)

The \(\text{^{111}In}\) labeling procedure was similar to that described by Lub-de Hooge et al. (16). In brief, \(\text{^{111}InCl}_3\) (0.1 mol/L sodium acetate, pH 7.0) was incubated with conjugate solution for 30 min at RT; 20 mmol/L DTPA in 0.1 mol/L sodium acetate was added to quench free \(\text{^{111}In}\). The resulting \(\text{^{111}In-DTPA}\) was removed by ultrafiltration. \(\text{^{111}In}\) radiolabeling yields were monitored by silica gel instant thin-layer chromatography (SGITLC, EMD Chemicals) using a Bioscan System 200 Imaging Scanner. The radiolabeling efficiency was calculated using WinScan software.

Stability analysis was performed by adding 1 mCi \(\text{^{111}In-(DTPA)}_2\)-trastuzumab-(IRDye 800) to 1 mL of PBS or human serum (Millipore). The mixtures were then incubated at 37 °C for 24 h. Aliquots at each time point were run on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) system described above. The radiochemical and fluorescent purity were analyzed using a Bioscan System 200 Imaging Scanner and a LI-COR Odyssey infrared imaging system and the results were plotted with ImageJ software.

\(\text{^{111}In vivo biodistribution of \(\text{^{111}In labeled trastuzumab-based conjugates}\)}\)

All protocols followed for animal experiments were approved by the Institutional Animal Care and Use Committees of Peking University Cancer Hospital & Institute and University of Texas Health Science Center at Houston. Female nude BALB/c (nu/nu) mice (age, 4–5 weeks) were subcutaneously implanted with 10 million SKBR-3 cells, and the tumor was allowed to grow to a size of approximately 5 mm diameter.

The biodistribution profiles of \(\text{^{111}In-(DTPA)}_2\)-trastuzumab-(IRDye 800), \(\text{^{111}In-(DTPA)}_2\)-trastuzumab-(IRDye 800), and \(\text{^{111}In-(DTPA)}_2\)-trastuzumab-(IRDye 800) were compared to that of \(\text{^{111}In-(DTPA)}_2\)-trastuzumab. Groups of 3–4 athymic female nude mice bearing SKBR-3 cell tumors were used for all experiments. The mice were administered 20 µg of \(\text{^{111}In}\)-labeled conjugates (approximately 1.48 mBq) in 0.1 mL of 0.9% NaCl via the lateral tail vein. For the determination of nonspecific uptake in the tumor tissue, another group of 3 mice were pre-injected with 1 mg of trastuzumab dissolved in 0.1 mL of 0.9% NaCl solution, 1 h prior to the administration of the imaging agent. At 48 h after the administration of the imaging agent,
mice were anesthetized with isoflurane (2.5%, 0.4 L/min oxygen) and subsequently euthanized using carbon dioxide. The organs of interest were collected, excess blood was rinsed off, weighed, and assessed on a gamma-counter together with injection standards. The percentage of injected per gram (%ID/g) was calculated for each tissue. The tumor-to-tissue ratios were also determined.

Data analysis
All statistical analyses were performed using SPSS version 18.0 (SPSS Inc., Chicago, IL, USA) software. The data are expressed as \( \bar{x} \pm s \). Between-group differences were evaluated using Student’s \( t \) test, and \( P<0.05 \) was considered statistically significant.

Results

Preparation of dual-labeled imaging conjugate
A series of \((\text{DTPA})_n\)-trastuzumab and \((\text{DTPA})_2\)-trastuzumab-(IRDye 800)\(_m\) were successfully prepared as described in this section. The chelator-to-trastuzumab ratios used for \((\text{DTPA})_n\)-trastuzumab were 1.03, 2.12, 3.05 and 5.18; the dye-to-protein ratios used for \((\text{DTPA})_2\)-trastuzumab-(IRDye 800)\(_m\) were 1.06, 3.03 and 4.71.

Purity of \((\text{DTPA})_2\)-trastuzumab-(IRDye 800)\(_m\)
The relative amount of unconjugated IRDye 800CW was determined via SDS-PAGE and fluorescence imaging system. The location of free IRDye 800CW in the gel (approximately 1 kD) was similar to that of bromophenol blue (approximately 0.5 kD). The purity of \((\text{DTPA})_2\)-trastuzumab-(IRDye 800)\(_n\), \((\text{DTPA})_2\)-trastuzumab-(IRDye 800)\(_3\) and \((\text{DTPA})_2\)-trastuzumab-(IRDye 800)\(_5\) is presented in Figure 1, no free dye band was observed in the purified conjugates, and <3% of the fluorescent signal was associated with larger molecules (>250 kD).

Radiolabeling and stability
The radiolabeling yield of trastuzumab-based radiotracers ranged between 85% and 100% at a specific activity of approximately 370–740 mBq per mg protein as demonstrated by SG-ITLC analysis. After the purification of radiotracers, no free radioactive band was detected on gel scanning with Bioscan System (Figure 2), and a main radioactive band (approximately 150 kD) was displayed for each radiolabeled trastuzumab-based conjugate.

The optical stability of \(^{111}\text{In}-(\text{DTPA})_2\)-trastuzumab-(IRDye 800)\(_m\) in PBS and human serum was demonstrated using a LI-COR Odyssey near-infrared imaging system. The IRDye 800
signal remained constant in PBS after 24 h but not in human serum (Figure 3). After 24 h, large proteins (>150 kD) were found on the gel for all three conjugates (Figure 3). Similar results were obtained on scanning of SDS-PAGE gels with the Bioscan system (Figure 4). The extra radioactive peak, which corresponded to the higher molecular weight proteins, can be observed in the histograms of dual-labeled conjugates. The larger the dye-to-protein ratio, the greater the amount of >150 kD proteins were found in the conjugate solution.

**Immunoreactivity assay**

The FACS histogram for SKBR-3 cells incubated with (DTPA)$_1$-trastuzumab, (DTPA)$_2$-trastuzumab, (DTPA)$_3$-trastuzumab and (DTPA)$_5$-trastuzumab at the same concentration was comparable to that of trastuzumab, which suggests that the DTPA-to-trastuzumab ratios of 1 to 5 had minimal effect on immunoreactivity (Figure 5).

![Figure 3](image1.png)  
**Figure 3** SDS-PAGE/Odyssey analysis of $^{111}$In-(DTPA)$_m$-trastuzumab-(IRDye 800)$_m$, $m=1, 3$ and 5, at 24 h after incubation in PBS (A–C) and human serum (D–F) at 37°C. The extra radioactive peak, which corresponded to the higher molecular weight proteins, can be observed in the histograms of dual-labeled conjugates. The larger the dye-to-protein ratio, the greater the amount of >150 kD proteins were found in the conjugate solution.

![Figure 4](image2.png)  
**Figure 4** Representative histograms indicating radioactivity of $^{111}$In-(DTPA)$_m$-trastuzumab-(IRDye 800)$_m$ at different $m$ values (0, 1, 3 and 5), after incubation with human serum at 37°C for 24 h. The larger the dye-to-protein ratio, the greater the amount of >150 kD proteins that were found in the conjugate solution. (A) $^{111}$In-(DTPA)$_1$-trastuzumab-(IRDye 800)$_1$; (B) $^{111}$In-(DTPA)$_3$-trastuzumab-(IRDye 800)$_3$; (C) $^{111}$In-(DTPA)$_5$-trastuzumab-(IRDye 800)$_5$.

![Figure 5](image3.png)  
**Figure 5** FACS histogram of trastuzumab-based conjugates showing counts vs. the mean fluorescent intensity using anti-human IgG as a secondary antibody. (A) The histogram to the left represents SKBR-3 cells incubated without trastuzumab. The overlying peaks represent FACS histograms of SKBR-3 cells incubated with 5 µg of trastuzumab (DTPA)$_n$-trastuzumab with $n=1, 2, 3$, and 5; (B) Cytofluorimetric comparison of cultured SKBR-3 cells without trastuzumab and after treatment with 5 µg of (DTPA)$_2$-trastuzumab, (DTPA)$_3$-trastuzumab-(IRDye 800)$_m$, (DTPA)$_5$-trastuzumab-(IRDye 800)$_m$, and (DTPA)$_5$-trastuzumab-(IRDye 800)$_m$. 

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However, the FACS histogram of (DTPA)₂-trastuzumab-(IRDye 800), showed a slight reduction in fluorescent intensity, which indicates reduced immunoreactivity as compared to that of (DTPA)₂-trastuzumab. In contrast, the immunoreactivity of (DTPA)₂-trastuzumab-(IRDye 800), was in between those of the other two conjugates. Immunoreactive fractions of trastuzumab conjugates corresponding to “m” values of 0, 1, 3 and 5 were 99.74±4.56%, 83.14±1.43%, 63.96±4.00% and 57.71±4.41%, respectively.

The Lindmo assay was performed to determine the immunoreactivity fraction of ¹¹¹In-(DTPA)₂-trastuzumab-(IRDye 800) m. A linear relationship between TA/SB and 1/cells was established. The average fraction of immunoreactivity of the radiolabeled conjugates corresponding to “m” values of 0, 1, 3 and 5 was 90.72±3.77%, 80.42±2.28%, 60.74±5.41% and 50.71±1.45%, respectively.

**Internalization study**

Figure 6 illustrates the specific internalization of ¹¹¹In-(DTPA)₂-trastuzumab-(IRDye 800) m, m=1, 3 and 5, into SKBR-3 cells after incubation for 4 h at 37 °C. (A) SKBR-3 cells in wells were scanned with LI-COR Odyssey near-infrared imaging system; (B) Fluorescence intensity surface plot of SKBR-3 cells with radioconjugate uptake at 4 h.

![Figure 6](image)

**Biodistribution study**

The biodistribution of ¹¹¹In-(DTPA)₂-trastuzumab-(IRDye 800), in SKBR-3 tumor-bearing nude mice was assessed at 48 h, and the data are summarized in Table 1 as %ID/g tissue. Variable tumor uptake was observed with respect to the different “m” values. Tumor accumulation of ¹¹¹In-(DTPA)₂-trastuzumab-(IRDye 800), (6.77±1.73 %ID/g) was much less than that of ¹¹¹In-(DTPA)₂-trastuzumab (15.76±2.61 %ID/g) and also less than the other two conjugates with lower “m” values, i.e., 9.96±1.05 %ID/g for ¹¹¹In-(DTPA)₂-trastuzumab-(IRDye 800), and 8.84±1.85 %ID/g for ¹¹¹In-(DTPA)₂-trastuzumab (IRDye 800), respectively. The uptake was specific to HER2(+) tumors, which was demonstrated by the lack of tumor retention in mice pre-injected with unlabeled trastuzumab.

| Tissue   | Biodistribution (±s, %ID/g) |
|----------|-----------------------------|
|          | m=0                         | m=2                         | m=3                         | m=5                         |
| Blood    | 0.93±0.33                   | 0.78±0.27                   | 0.92±0.28                   | 1.00±0.18                   |
| Liver    | 8.33±4.09                   | 7.41±1.24                   | 8.09±1.39                   | 8.84±1.97                   |
| Kidney   | 5.43±2.42                   | 15.63±1.28                  | 25.99±1.52                  | 28.37±4.55                  |
| Muscle   | 0.98±0.54                   | 0.81±0.33                   | 1.18±0.14                   | 1.27±0.35                   |
| Bone     | 2.92±0.39                   | 0.91±0.51                   | 6.54±2.52                   | 6.02±1.26                   |
| Tumor    | 15.76±2.61                  | 9.96±1.05                   | 8.84±1.85                   | 6.77±1.73                   |

The excretion of dual-labeled conjugates was largely via the kidney and liver, particularly the former. The renal uptake of ¹¹¹In-(DTPA)₂-trastuzumab-(IRDye 800), was 28.37±4.55 %ID/g, which is much higher than that of ¹¹¹In-(DTPA)₂-trastuzumab (5.43±2.42 %ID/g), and the liver uptake of both conjugates was comparable (approximately 8 %ID/g at 48 h post-injection).

**Discussion**

Dual-labeling of targeting moieties with nuclear and
fluorescent entities provides a non-invasive modality for the localization of lesions with the use of highly penetrative, short-lived radionuclides and then uses the fluorescence signals from optical molecular probes allowing for disease monitoring over prolonged periods of time to guide surgical resection (5,13). Furthermore, the use of dual-labeled targeting tracer effectively avoids issues related to the use of agents with differing pharmacokinetic and pharmacodynamic properties that may otherwise affect image fusion and its interpretation (17). However, the development of dual-labeled targeting pharmaceuticals is a multistep and technically more challenging technique that requires complex design considerations such as careful selection of nuclear and optical tracers to minimize physical-chemical interference between various molecular components. Thus, the modification of the moiety to the maximum extent possibly whilst ensuring that the modification does not compromise bioactivity is of considerable interest (15,18). In our studies, we have used trastuzumab as a targeting moiety that is covalently linked to IRDye 800CW, a NIR fluorescent dye, and DTPA, a chelator of 111In, to develop a dual-labeled imaging agent to trace HER-positive primary breast lesions and metastases.

Previous studies have demonstrated dimer and polymer formation with an increase in the DTPA anhydride to antibody ratio, which resulted in a decreased biological affinity of the conjugate relative to the unlabeled antibody (15). However, Forrer et al. found no significant difference in the immunoreactivity between (DOTA)$_2$-rituximab immunonojugate and a commercially available rituximab on lymphoma cell line LVB1 (19). Our FACS results showed no significant difference in immunoreactivity between trastuzumab and the singly labeled DTPA conjugates up to a chelator-to-protein ratio of 5:1. Furthermore, a 1:1 molar ratio of DTPA to protein was not sufficient for prompt labeling with the needed protein ratio of 5:1. Furthermore, a 1:1 molar ratio of DTPA to trastuzumab, the percentage of HER2(+) tumor accumulation decreased significantly (P<0.05) with increasing dye-to-protein molar ratios, which is consistent with our in vitro findings. Tumor uptake decreased from 15.76±2.61 %ID/g for 111In-(DTPA)$_2$-trastuzumab to 6.77±1.73 %ID/g for 111In-(DTPA)$_2$-trastuzumab-(IRDye 800)$_5$, which represents an approximate 57% reduction. Furthermore, the renal uptake increased from 5.43±2.42 %ID/g for 111In-(DTPA)$_2$-trastuzumab to 28.37±4.55 %ID/g for 111In-(DTPA)$_2$-trastuzumab-(IRDye 800)$_5$, which is partly attributable to the decreased conjugate stability.

Conclusions

Dual-labeled trastuzumab conjugates were successfully coupled

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with DTPA and IRDye 800CW. Increasing dye-to-protein ratios decreased immunoreactivity and serum stability, and lowered tumor accumulation.

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**Footnote**

**Conflicts of Interest**: The authors have no conflicts of interest to declare.

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