Original Article

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 ^{111}In-(DTPA)$_n$-trastuzumab-(IRDye 800)$_m$.

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 ^{111}In-(DTPA)$_2$-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) 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).

Here, we describe a method for dual-labeling of an antibody with a NIR fluorophore, IRDye 800CW, and diethylenetriaminepentaacetic acid (DTPA) for the chelation of ^{111}In. The stearic effect of conjugation of a biomodal imaging tag to trastuzumab was investigated by altering the molar ratios of chelator/near-infrared dye to protein. The stability, binding affinity and targeting property of the dual-labeled imaging agent were assessed both *in vitro* and *in vivo*.

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). $^{111}InCl_3$ 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% CO_2.

Preparation of trastuzumab-based conjugate

Conjugation of DTPA to trastuzumab

Trastuzumab was dissolved in 50 mmol/L $NaHCO_3$ (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

$(DTPA)_2$-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

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each gel band and quantification of fluorescent intensity levels were performed.

Flow cytometry

In brief, 0.3×10^6 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 (FACS-Calibur, Becton Dickinson). As a control for specific binding, trastuzumab was tested at equivalent molar concentrations to provide the measurement of $MFI_{positive}$, whereas cells treated with phosphate-buffered saline (PBS) served as a negative control for the measurement of $MFI_{negative}$. The percentage of immunoreactivity was calculated as follows: $[(MFI_{sample}–MFI_{negative})/(MFI_{positive}–MFI_{negative})]\times100\%$.

^{111}In labeling and serum stability

The ^{111}In labeling procedure was similar to that described by Lub-de Hooge *et al.* (16). In brief, $^{111}InCl_3$ (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 ^{111}In. The resulting ^{111}In-DTPA was removed by ultrafiltration. ^{111}In radiolabeling yields were monitored by silica gel instant thin-layer chromatography (SG-ITLC, 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 ^{111}In-$(DTPA)_2$-trastuzumab-$(IRDye\ 800)_m$ 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, respectively.

Lindmo assay

The binding affinity of ^{111}In-$(DTPA)_2$-trastuzumab-$(IRDye\ 800)_m$ and ^{111}In-$(DTPA)_2$-transtuzumab was compared via the Lindmo assay as described previously (11). In brief, a fixed amount of trastuzumab-based conjugate (5 pmol) was added to increasing concentrations of SKBR-3 cells (from 0.1×10^6 to 2×10^6 in 0.5 mL) and incubated for 1 h. To determine non-specific cell binding, aliquots of cells were incubated with radiolabeled conjugates in the presence of 0.5 µmol unlabeled trastuzumab. The cell pellets were measured using a gamma counter. Specific binding was calculated as the ratio of the cell bound to the total radioactivity applied minus nonspecific binding. The immunoreactive fraction was calculated using OriginLab Microcal software.

Internalization studies

In brief, approximately 2.5 pmol of trastuzumab-based conjugate was added to pretreated SKBR-3 cells with an overexpression of HER2 and incubated (in triplicate) for 4h and 24 h at 37 °C. A 1,000-fold excess of each blocking agent was used to determine nonspecific internalization. At each time point, the internalization was stopped by the removal of the medium followed by washing the cells with ice-cold 0.01 mol/L PBS (pH 7.4). The cells were then treated for 5 min (three times) with ice-cold glycine buffer (0.05 mol/L glycine solution, and pH adjusted to 2.8 with 1 mol/L HCl) to distinguish between cell surface-bound (acid-releasable) and internalized (acid-resistant) radiolabeled antibodies. The internalized fluorescence intensity was measured using Odyssey infrared imaging system and the results were plotted with ImageJ software.

In vivo biodistribution of ^{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 ^{111}In-$(DTPA)_2$-trastuzumab-$(IRDye\ 800)_2$, ^{111}In-$(DTPA)_2$-trastuzumab-$(IRDye\ 800)_3$, and ^{111}In-$(DTPA)_2$-trastuzumab-$(IRDye\ 800)_5$ were compared to that of ^{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 ^{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,

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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 $(DTPA)_n$-trastuzumab and $(DTPA)_2$-trastuzumab-$(IRDye\ 800)_m$ were successfully prepared as described in this section. The chelator-to-trastuzumab ratios used for $(DTPA)_n$-trastuzumab were 1.03, 2.12, 3.05 and 5.18; the dye-to-protein ratios used for $(DTPA)_2$-trastuzumab-$(IRDye\ 800)_m$ were 1.06, 3.03 and 4.71.

Purity of $(DTPA)_2$-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 $(DTPA)_2$-trastuzumab-$(IRDye\ 800)_1$, $(DTPA)_2$-trastuzumab-$(IRDye\ 800)_3$ and $(DTPA)_2$-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}In-$(DTPA)_2$-trastuzumab-$(IRDye\ 800)_m$ in PBS and human serum was demonstrated using a LI-COR Odyssey near-infrared imaging system. The IRDye 800

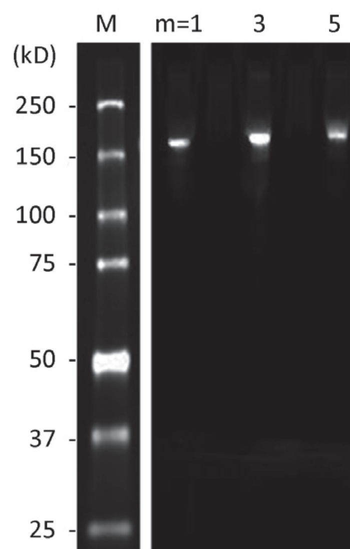

Figure 1 $(DTPA)_2$-trastuzumab-$(IRDye\ 800)_m$ with varying dye to protein ratios for purified conjugates. Fluorescence intensity of SDS-PAGE gels was imaged using a LI-COR Odyssey infrared imaging system. The gel to the left is a molecular weight ladder in kD. After purification, the band for each dual-labeled conjugate was found in approximately 145 kD. No free dye bands are observed in the purified conjugates. M, protein molecular weight ladder.

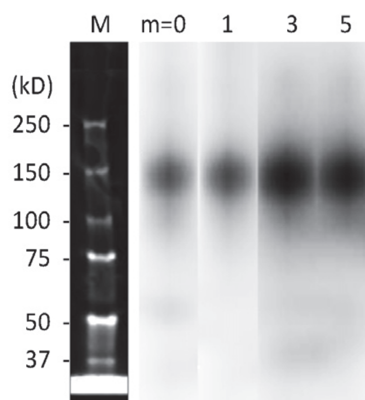

Figure 2 Radioactive purity of ^{111}In-$(DTPA)_2$-trastuzumab-$(IRDye\ 800)_m$ with varying dye to protein ratios. The gel to the left represents the molecular weight ladder in kD. The band for each dual-labeled conjugate was found in approximately 145 kD. No radioactive band was found in the final dual-labeled conjugates. M, protein molecular weight ladder.

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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*).

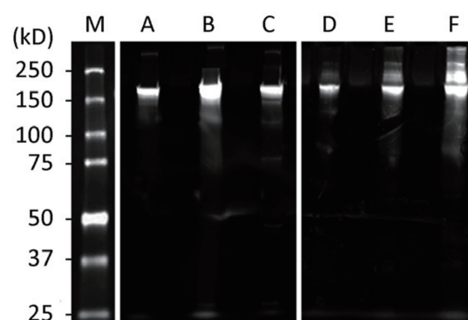

Figure 3 SDS-PAGE/Odysse analysis of ^{111}In-$(DTPA)_2$-trastuzumab-$(IRDye\ 800)_m$, m=1, 3 and 5, at 24 h after incubation in PBS (A–C) and human serum (D–F) at 37 °C. The ladder on the right represents the molecular weight. M, protein molecular weight ladder; (A, D) ^{111}In-$(DTPA)_2$-trastuzumab-$(IRDye\ 800)_1$; (B, E) ^{111}In-$(DTPA)_2$-trastuzumab-$(IRDye\ 800)_3$; (C, F) ^{111}In-$(DTPA)_2$-trastuzumab-$(IRDye\ 800)_5$.

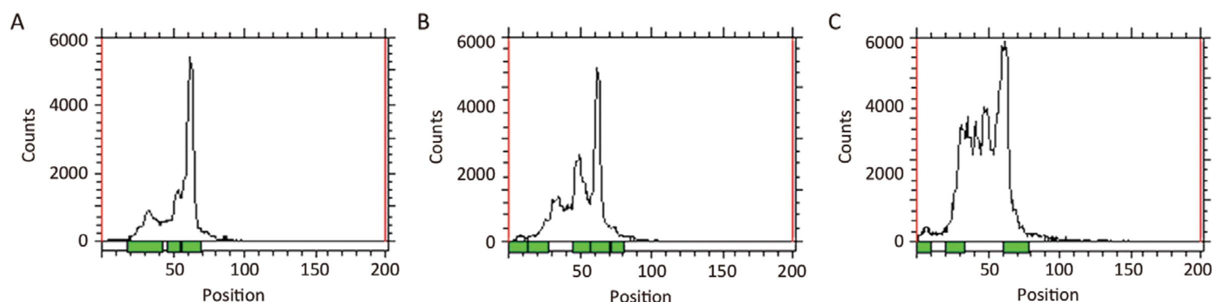

Figure 4 Representative histograms indicating radioactivity of ^{111}In-$(DTPA)_2$-trastuzumab-$(IRDye\ 800)_m$ at different m values (0, 1, 3 and 5), after incubation with human serum at 37 °C for 24 h. The larger the dye-to-protein ratio, the greater the amount of >150 kD proteins that were found in the conjugate solution. (A) ^{111}In-$(DTPA)_2$-trastuzumab-$(IRDye\ 800)_1$; (B) ^{111}In-$(DTPA)_2$-trastuzumab-$(IRDye\ 800)_3$; (C) ^{111}In-$(DTPA)_2$-trastuzumab-$(IRDye\ 800)_5$.

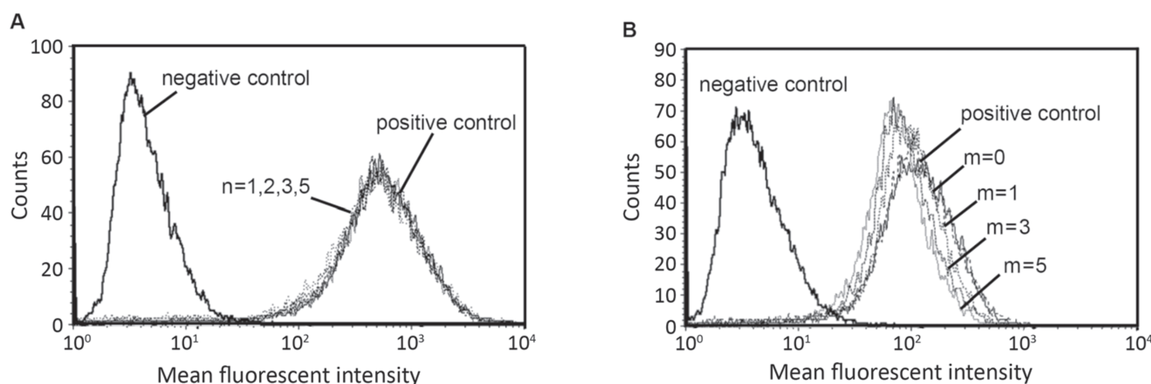

Figure 5 FACS histogram of trastuzumab-based conjugates showing counts *vs*. the mean fluorescent intensity using anti-human IgG as a secondary antibody. (A) The histogram to the left represents SKBR-3 cells incubated without trastuzumab. The overlaying peaks represent FACS histograms of SKBR-3 cells 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, $(DTPA)_2$-trastuzumab-$(IRDye\ 800)_1$, $(DTPA)_2$-trastuzumab-$(IRDye\ 800)_3$ and $(DTPA)_2$-trastuzumab-$(IRDye\ 800)_5$.

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However, the FACS histogram of $(DTPA)_2$-trastuzumab-$(IRDye\ 800)_1$ 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-$(IRDye\ 800)_5$ was moderately impaired, as evidenced in the clear shift of the histogram toward the negative control population of SKBR-3 cells. The immunoreactivity of $(DTPA)_2$-trastuzumab-$(IRDye\ 800)_3$ 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 ^{111}In-$(DTPA)_2$-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 ^{111}In-$(DTPA)_2$-trastuzumab-$(IRDye\ 800)_m$ into SKBR-3 cells, the NIR fluorescence signals associated with internalized IRDye 800 were detected on 12-well plates. No fluorescence was observed in the case of SKBR-3 cells pretreated with ^{111}In-$(DTPA)_2$-trastuzumab. The three-dimensional surface plot showed that the total fluorescence intensity of ^{111}In-$(DTPA)_2$-trastuzumab-$(IRDye\ 800)_5$ was higher than that of the other two. However, the percentages of internalization corresponding to "m" values of 1, 3 and 5 after the normalization of the total fluorescence intensities of dual-labeled conjugates by the dye-to-protein ratio were 24.88%±2.10%, 19.99%±0.59% and 17.47%±1.26% at 4 h, respectively.

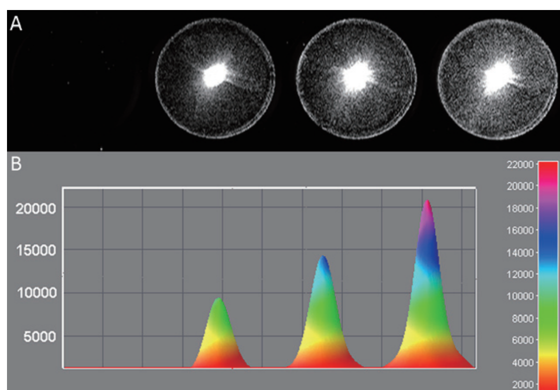

Figure 6 Internalization of ^{111}In-$(DTPA)_2$-trastuzumab-$(IRDye\ 800)_m$, m=1, 3 and 5, into SKBR-3 cells after incubation for 4 h at 37 °C. (A) SKBR-3 cells in wells were scanned with LI-COR Odyssey near-infrared imaging system; (B) Fluorescence intensity surface plot of SKBR-3 cells with radioconjugate uptake at 4 h.

The intensity of internalized radioactivity corresponding to "m" values of 0, 1, 3 and 5 was comparable to the results presented above, i.e., 26.34%±0.03%, 23.87%±0.02%, 21.07%±0.01% and 20.45%±0.01% at 4 h, respectively.

Biodistribution study

The biodistribution of ^{111}In-$(DTPA)_2$-trastuzumab-$(IRDye\ 800)_m$ 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 ^{111}In-$(DTPA)_2$-trastuzumab-$(IRDye\ 800)_5$ (6.77±1.73 %ID/g) was much less than that of ^{111}In-$(DTPA)_2$-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 ^{111}In-$(DTPA)_2$-trastuzumab-$(IRDye\ 800)_3$ and 8.84±1.85 %ID/g for ^{111}In-$(DTPA)_2$-trastuzumab $(IRDye\ 800)_2$, respectively. The uptake was specific to HER2(+) tumors, which was demonstrated by the lack of tumor retention in mice pre-injected with unlabeled trastuzumab.

Table 1 Biodistribution of ^{111}In-labeled trastuzumab-based agents in SKBR-3 tumor-bearing nude mice at 48 h after injection

Tissue	Biodistribution ($\bar{x}$±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 ^{111}In-$(DTPA)_2$-trastuzumab-$(IRDye\ 800)_5$ was 28.37±4.55 %ID/g, which is much higher than that of ^{111}In-$(DTPA)_2$-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

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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 ^{111}In, 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 $(DOTA)_4$-rituximab immunoconjugate 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 amount of ^{111}In (e.g., approximately 370-740 mBq per mg of antibody). Based on radiolabeling yields, specific radioactivity, solubility and immunoreactivity, we employed a molar DTPA-to-trastuzumab coupling ratio of 2:1 in this study.

As with radionuclide conjugation, dye-to-protein ratio is also liable to impact *in vivo* signal levels. A low dye-to-antibody ratio will reduce fluorescence intensity, while over-conjugation on non-specific sites of the protein may cause self-quenching of the dye as well as the loss of biological activity. Gee *et al.* synthesized $(Cy5.5)_m$-trastuzumab with molar ratios of the dye to protein ranging from 0.5 to 2.0 (20). A molecular dye to antibody ratio of 1.1 was found to be optimal based on the binding affinity. The use of higher dye-to-antibody ratios was found to decrease immunoreactivity, while a probe containing a lower dye-to-antibody ratio (0.5) was associated with reduced fluorescence intensity.

We synthesized $(DTPA)_2$-trastuzumab, $(DTPA)_2$-trastuzumab-$(IRDye\ 800)_1$, $(DTPA)_2$-trastuzumab-$(IRDye\ 800)_3$ and $(DTPA)_2$-trastuzumab-$(IRDye\ 800)_5$ to investigate the influence of dye-to-protein ratios on the affinity, stability and the *in vivo* targeting potential for near-infrared fluorescence imaging. The binding affinity of the immunoconjugates was tested in HER2-overexpressing SKBR-3 tumor cells and immunoreactivity assessed using FACS. The representative histogram shows a decrease in MFI corresponding to an increase in the dye-to-protein ratios. The successive percentage of immunoreactivity fell from 100% (without any dye on the antibody) to 83%, at a molar dye-to-protein ratio of 1:1, which demonstrates a high affinity of the dual-labeled conjugate for binding to HER2 on breast cancer cells. At molar dye-to-protein ratios of 5:1, a 42% loss of immunoreactivity was observed. Furthermore, the formation of large proteins (>150 kD) was observed with all three dual-labeled conjugates after incubation with human serum for 24 h. The larger the dye-to-protein ratio, the greater the amount of >150 kD proteins found in the conjugate solution.

To the best of our knowledge, the relationship between the dye-to-protein ratio and specific binding affinity has only been investigated *in vitro*, because quantitative measurement of tissue fluorescence is liable to be affected by variability in tissue absorption properties and scattering of fluorescent signals. Li *et al.* compared the biodistribution of ^{111}In-labeled c (KRGDf) peptide and dual-labeled c (KRGDf) peptide, both of which targeted $\alpha\nu\beta3$ integrin in melanoma xenografts, with a 1:1 molar ratio of chelator and fluorophore, using tissue radioactivity measurements (21). We evaluated the relationship of the dye-to-protein ratio with specific targeting using nuclear medicine techniques.

As compared to the biodistribution of ^{111}In-$(DTPA)_2$-trastuzumab, the percentage of HER2(+) tumor accumulation also 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 ^{111}In-$(DTPA)_2$-trastuzumab to 6.77±1.73 %ID/g for ^{111}In-$(DTPA)_2$-trastuzumab-$(IRDye\ 800)_5$, which represents an approximate 57% reduction. Furthermore, the renal uptake increased from 5.43±2.42 %ID/g for ^{111}In-$(DTPA)_2$-trastuzumab to 28.37±4.55 %ID/g for ^{111}In-$(DTPA)_2$-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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