Click Chemistry in the Design and Production of Hybrid Tracers

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Supporting Information

ABSTRACT: Hybrid tracers containing both fluorescent and radioactive imaging labels have demonstrated clinical potential during sentinel lymph node procedures. To combine these two labels on a single targeting vector that allows tumor-targeted imaging, end-labeling strategies are often applied. For αβ3-integrin-targeting hybrid tracers, providing an excellent model for evaluating tracer development strategies, end-labeling-based synthesis provides a rather cumbersome synthesis strategy. Hence, the aim of this study was to investigate the use of heterobifunctional cyanine dyes in a click-chemistry-based synthesis strategy evaluating tracer development strategies, end-labeling-based synthesis provides a rather cumbersome synthesis strategy. Hence, the aim of this study was to investigate the use of heterobifunctional cyanine dyes in a click-chemistry-based synthesis strategy for RGD-based hybrid tracers. The triazole-based hybrid tracers DTPA.DBCO.N3(SO3)-Cy5-c[RGDyK] and DTPA.BCN.N3(SO3)-Cy5-c[RGDyK] were obtained in fewer steps than DTPA-Lys(Cy5(SO3)methyl)-Cys-c[RGDyK] and had partition coefficients of log P(o/w) = −2.55 ± 0.10, −1.45 ± 0.03, and −2.67 ± 0.12, respectively. Both tracers were chemically stable, and the brightnesses of DTPA.DBCO.N3(SO3)-Cy5-c[RGDyK] and DTPA.BCN.N3(SO3)-Cy5-c[RGDyK] were, respectively, 23 ± 10 and 40 ± 10 % ID/g; lower than that of the reference tracer DTPA-Lys(Cy5(SO3)methyl)-Cys-c[RGDyK] (50 ± 10 % ID/g cm⁻³). Assessment of serum protein binding revealed no statistically significant difference (44 ± 2 and 40 ± 2% bound for DTPA.DBCO.N3(SO3)-Cy5-c[RGDyK] and DTPA.BCN.N3(SO3)-Cy5-c[RGDyK], respectively; 36 ± 5% bound for DTPA-Lys(Cy5(SO3)methyl)-Cys-c[RGDyK]; p > 0.05). DTPA.DBCO.N3(SO3)-Cy5-c[RGDyK] (Kd = 17.5 ± 6.0) had a statistically significantly higher affinity than the reference compound DTPA-Lys(Cy5(SO3)methyl)-Cys-c[RGDyK] (Kd = 30.3 ± 5.7; p < 0.0001), but DTPA.BCN.N3(SO3)-Cy5-c[RGDyK] had a statistically significantly lower affinity (Kd = 76.5 ± 18.3 nM; p < 0.0001). Both [111In]DTPA.DBCO.N3(SO3)-Cy5-c[RGDyK] and [111In]-DTPA.BCN.N3(SO3)-Cy5-c[RGDyK] enabled in vivo visualization of the 4T1 tumor via fluorescence and single-photon emission computed tomography (SPECT) imaging. Biodistribution data (% ID/g) revealed a significant increase in nonspecific uptake in the kidney, liver, and muscle for both [111In]DTPA.DBCO.N3(SO3)-Cy5-c[RGDyK] and [111In]-DTPA.BCN.N3(SO3)-Cy5-c[RGDyK]. As a result of the higher background activity, the tumor-to-background ratio of the click-labeled RGD analogues was twofold lower compared to the end-labeled reference compound. The use of click chemistry labeling did not yield a pronounced negative effect on serum protein binding, in vitro stability, and receptor affinity; and tumors could still be visualized using SPECT and fluorescence imaging. However, quantitative in vivo biodistribution data suggest that the triazole and strained cyclooctyne moieties associated with this type of click chemistry negatively influence the pharmacokinetics of RGD peptides. Nevertheless, the design might still hold promise for other targets/targeting moieties.

INTRODUCTION

Fluorescence has a broad application as a modality for in vitro and in vivo imaging.1,2 In the clinical field, an increasing interest
in fluorescence imaging has been triggered by the introduction of fluorescence camera systems that can be applied during surgery.\textsuperscript{3–5} In this context, real-time fluorescence imaging is utilized to visualize a distinct (e.g., diseased) tissue type within the surgical field. The value of fluorescence for surgical guidance is limited by signal attenuation, and therefore the technique only provides accurate guidance in superficial applications (<1 cm) and does not allow for preoperative imaging.\textsuperscript{6,7} On the other hand, \( \gamma \) rays produced by isotopes such as \(^{99m}\)Tc and \(^{111}\)In are not held back by this characteristic and can penetrate human tissue without limitations, which makes them suited for noninvasive (preoperative) imaging.\textsuperscript{8}

To combine these features, tracers that contain both a fluorescent and radioactive imaging label can be designed. In these so-called “dual-labeled”, “bimodal”, or rather “hybrid” tracers, the fluorescent and radioactive signatures complement each other, creating a “best-of-both-worlds” scenario in clinical image-guided surgery applications.\textsuperscript{8–10} Today, hybrid tracers containing cyanine dyes (Scheme 1A, left) are constructed by conjugating branched linkers (in white) bearing both the radioactive label (in red; mostly a chelate) and the fluorescent dye (in blue) to the targeting vector (in green), i.e., containing end-conjugated dyes as introduced by Josephson et al.\textsuperscript{11,12} In practice, however, such end-conjugated cyanine-dye-based hybrid tracers make use of redundant spacers and suffer from a low synthetic yield due to the larger number of synthetic steps with concomitantly low yields.

As an alternative to end-labeling strategies, we hypothesized that it may be possible to improve the ease of synthesis by using click chemistry, which has been on the rise ever since the investigation of the 1,3-dipolar cycloaddition by Huisgen in the 1960s. After about four decades, Sharpless and Meldal independently discovered the copper-catalyzed click reaction in which 1,4-disubstituted 1,2,3-triazoles were formed from azides and terminal alkynes. The toxicity of copper prompted Sletten and Bertozzi to explore copper-free click chemistry using strain-promoted azide–alkyne cycloaddition (SPAAC). SPAAC has been of great interest in the field of molecular imaging where radio- and fluorescence-labeling strategies have been independently studied.\textsuperscript{2,13} The required ligands (e.g., dibenzocyclooctyne (DBCO) or bicyclo[6.1.0]nonyne (BCN) groups) are commercially available, and this reaction is one of the most-studied in the field of bioorthogonal chemistry. Despite the fact that click chemistry for in vivo fluorescence imaging has already been applied,\textsuperscript{14,15} the synthesis of click-chemistry-based hybrid tracers is, to date, only described using antibodies and not...
peptides. A design wherein the fluorophore also fulfills the role as linker (Scheme 1, right) has to the best of our knowledge, only been researched using 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene (BODIPY) dyes. Use of a small-molecule, copper-free click-based design incorporating cyanine dyes as a linker has not yet been described.

The aim of this study was therefore to evaluate the use of a click-chemistry-based labeling strategy for peptides to improve the ease of synthesis and synthetic yield of peptide-based hybrid tracers while creating a conjugated tracer with a more compact structure (Scheme 1). To study the influence of chemically introduced alterations in the context of earlier end-conjugated tracer developments (resulting in reference tracer DTPA-Lys(Cy5(SO3)methyl)-Cys-c[RGDyK] and DTPA.BCN.N3(SO3)-Cy5-c[RGDyK]; Scheme 1 C), the well-known αβ3-integrin-targeting RGD peptide (c[RGDyK]) was chosen as the targeting moiety. In the novel tracer design, the heterobifunctional dye functions as a linker that enables the coupling of both a chelator, e.g., diethylenetriaminepentaacetic acid (DTPA), and the targeting vector (resulting in reference tracer DTPA-Lys(Cy5(SO3)methyl)-Cys-c[RGDyK]; Scheme 1B), virtually no change in brightness,25,26 but since the BCN moiety is considerably less bulky than its DBCO counterpart, it seems that this heterobifunctional Cy5 dye functioned as a linker unit for further synthetic modifications. By using an adapted version of the Gabriel synthesis, the amine could easily be liberated. Subsequent diazotransfer24 with 1H-imidazole-sulfonyl azide-HCl could be performed, which resulted in N3(SO3)-Cy5-c[RGDyK]. This modification supported SPAAC with either DBCO–DTPA or BCN–DTPA, resulting in DTPA.BCN.N3(SO3)-Cy5-c[RGDyK] and DTPA.BCN.N3(SO3)-Cy5-c[RGDyK], respectively (Scheme 1).

**Photophysical and Chemical Properties.** To assess the influence that the chemical alterations exerted on the dyes’ and hybrid tracers’ photophysical properties, the molar extinction coefficient (ε) and quantum yield (Φ) were assessed to evaluate the brightness (ε · Φ).

Solvent polarity plays a large role in ε; the ε value of N3(SO3)-Cy5-COOH in dimethyl sulfoxide (DMSO) was reduced by 40% when changing to H2O (218 × 10^4 M−1 cm−1 in DMSO vs 131 × 10^4 M−1 cm−1 in H2O), and a 13% reduction was seen when phosphate-buffered saline (PBS) was used (189 × 10^4 M−1 cm−1), which is in line with previous results.22 The relative Φ of N3(SO3)-Cy5-COOH was similar to methyl-Cy5-COOH32 (precursor to DTPA-Lys(Cy5(SO3)methyl)-Cys-c[RGDyK]; Φ = 13% for both dyes in PBS).

The brightness of the precursor for the triazole-based hybrid tracers (N3(SO3)-Cy5-COOH) was in the same range as the precursor for the end-labeled design (methyl-Cy5-COOH; 29 × 10^4 vs 28 × 10^4 M−1 cm−1, respectively). When the click reaction incorporating DBCO was used to form DTPA.BCN.N3(SO3)-Cy5-c[RGDyK], virtually no change in brightness was observed compared to N3(SO3)-Cy5-COOH (23 × 10^4 vs 25 × 10^4 M−1 cm−1, respectively). The brightness was, however, enhanced to 40 × 10^4 M−1 cm−1 for DTPA.BCN.N3(SO3)-Cy5-c[RGDyK]. The reference tracer DTPA-Lys(Cy5(SO3)methyl)-Cy5-c[RGDyK] had an even higher brightness (50 × 10^5 M−1 cm−1). Cis–trans isomerization around the polyethylene bridge plays a large role in the differences in brightness,25,26 but since the BCN moiety is considerably less bulky than its DBCO counterpart, it seems that intra- or intermolecular interactions other than steric hindrance caused this effect.

By using the shake-flask method with radiolabeled hybrid tracer, the partition coefficients in octanol/H2O (log P_{o/w}) were determined for all three hybrid tracers as log P_{o/w} = 2.55 ± 0.10, −1.45 ± 0.03, and −2.67 ± 0.12 for [99mTc]DTPA.DBCO.N3(SO3)-Cy5-c[RGDyK], [99mTc]DTPA.BCN.N3(SO3)-Cy5-c[RGDyK], and [99mTc]DTPA.

### RESULTS AND DISCUSSION

**Chemistry.** As a general synthon for all compounds in this study, the Phth(SO3)-Cy5-COOH dye was prepared. Following its coupling to c[RGDyK] using standard NHS chemistry, this heterobifunctional Cy5 dye functioned as a linker unit for

| compound                        | λex/λem (Stokes shift, nm) | Molar absorbance coefficient (ε · Φ) (M−1 cm−1) | Φ (in PBS) | brightness (Φ · ε) (M−1 cm−1) | log P_{o/w} (99mTc-labeled, n = 6) |
|---------------------------------|-----------------------------|-----------------------------------------------|-----------|--------------------------------|----------------------------------|
| N3(SO3)-Cy5-COOH                | DMSO: 656/676 (20) H2O/PBS: 644/662 (18) | DMSO: 624/658 (16) H2O/PBS: 1.91/2.12 (18) | 2.18      | 1.31                           | 1.89                             | 2.5 ± 0.12                       |
| methyl-(SO3)Cy5-COOH            | DMSO: 653/672 (19) H2O/PBS: 642/658 (16) | DMSO: 638/658 (16) H2O/PBS: 1.76/2.12 (18) | 2.38      | 1.76                           | 1.22                             | 2 ± 0.12                        |
| DTPA-Lys(Cy5(SO3)methyl)-Cys-c[RGDyK] | DMSO: 654/675 (21) H2O/PBS: 644/663 (19) | DMSO: 638/658 (16) H2O/PBS: 1.76/2.12 (18) | 19        | 5.00                           | −2.67 ± 0.12                     |                                |
| DTPA.DBCO.N3(SO3)-Cy5-c[RGDyK]  | DMSO: 655/674 (19) H2O/PBS: 665/666 (19) | DMSO: 638/658 (16) H2O/PBS: 1.76/2.12 (18) | 12        | 2.37                           | −2.55 ± 0.10                     |                                |
| DTPA.BCN.N3(SO3)-Cy5-c[RGDyK]   | DMSO: 645/665 (20) H2O/PBS: 655/674 (19) | DMSO: 638/658 (16) H2O/PBS: 1.76/2.12 (18) | 21        | 4.00                           | −1.45 ± 0.03                     |                                |

Note that for the determination of log P_{o/w}, the following radiolabeled tracers were used: [99mTc]DTPA.Lys(Cy5(SO3)methyl)-Cys-c[RGDyK], [99mTc]DTPA.DBCO.N3(SO3)-Cy5-c[RGDyK], [99mTc]DTPA.BCN.N3(SO3)-Cy5-c[RGDyK].

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Lys(Cy5(SO3)methyl)-Cys-c[RGDyK], respectively (Table 1). These data indicated, surprisingly, that [99mTc]DTPA.Lys(Cy5(SO3)methyl)-Cys-c[RGDyK], [99mTc]DTPA.DBCO.N3(SO3)-Cy5-c[RGDyK], and the reference tracer [99mTc]DTPA.Lys(Cy5(SO3)methyl)-Cys-c[RGDyK] had similar lipophilicities.

**Serum Interaction and Chemical Stability.** Depending on their functionalization, cyanine dyes can form strong

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**Figure 1.** Plasma protein interactions portraying (A) no significant difference (p > 0.05) in the plasma protein binding of the heterobifunctional tracers compared to the alternative branched design and high stability over a 24 h timespan for both heterobifunctional tracers using (B) absorbance and (C) fluorescence measurements.

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**Figure 2.** In vitro evaluation of the hybrid tracers. Fluorescence confocal microscopy images of a mixed-cell culture of (A) αβ3-positive Geβ3 and αβ3-negative MDAMB231 X4 cells incubated with the reference tracer (i) DTPA.Lys(Cy5(SO3)methyl)-Cys-c[RGDyK], (ii) DTPA.DBCO.N3(SO3)-Cy5-c[RGDyK], (iii) DTPA.BCN.N3(SO3)-Cy5-c[RGDyK]. (B) The same cell lines after blocking with c[RGDyK] prior to incubation with one of the hybrid tracers. For additional reference, cell nuclei were stained with Hoechst (in blue), and the intrinsic membranous GFP expression of the MDAMB231 X4 cells was used to discriminate between the two cell lines (bright green). (C) Quantified evaluation of the percentage of signal decrease between (A) and (B) (blocked; checkered) p < 0.0001, n ≥ 13. (D) Combined saturation curves of the affinity of DTPA.Lys(Cy5(SO3)methyl)-Cys-c[RGDyK], DTPA.DBCO.N3(SO3)-Cy5-c[RGDyK], and DTPA.BCN.N3(SO3)-Cy5-c[RGDyK] (with the binding affinity (Kd) of the tracers in nanomolar), n = 3.
noncovalent bonds with serum proteins, e.g., albumin. In addition, the dyes can also be metabolically degraded,17,21,22,27,28 yielding circulation of tracer fragments in an in vivo setting. Such degradation underlines the pressing need for serum interaction assays during preclinical research. A serum binding assay for the two triazole-containing RGD-targeting hybrid tracers revealed no significant differences between the two click analogues (44 ± 2% bound for DTPA.DBCO.N₃(SO₃)₂-Cys-c[RGDyK] and 40 ± 2% bound for DTPA.BCN.N₃(SO₃)₂-Cys-c[RGDyK]; Figure 1A) and the reference tracer DTPA-Lys(Cys(SO₃)methyl)-Cys-c[RGDyK] (36 ± 5% bound; p > 0.05 for all comparisons).17 Hence, the difference in lipophilicity did not seem to affect the serum binding ability of the different hybrid tracers.

Dye stabilities of >94% were found after incubation with non-heat-inactivated serum (24 h; 37 °C), which was in line with earlier reports for Cy5 dyes.22,29 (Figure 1B,C).

**In Vitro Localization and Receptor Affinity.** Fluorescence confocal imaging in mixed-cell culture experiments revealed comparable uptake of the reference tracer DTPA-Lys(Cys(SO₃)methyl)-Cys-c[RGDyK] (Figure 2Ai) in αᵥβ₃-positive cells (Cy5-related uptake in red) compared to both

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Figure 3. In vivo imaging and biodistribution. In vivo (A) SPECT images and (B) accompanying fluorescence images of 4T1-tumor-bearing mice acquired at 24 h after administration of 1 nmol of the reference tracer [¹¹¹In]DTPA-Lys(Cys(SO₃)methyl)-Cys-c[RGDyK] (i), [¹¹¹In]DTPA.DBCO.N₃(SO₃)₂-Cys-c[RGDyK] (ii), and [¹¹¹In]DTPA.BCN.N₃(SO₃)₂-Cys-c[RGDyK] (iii). Tumor is encircled in red. (C) Quantified biodistribution (% ID/g) of [¹¹¹In]DTPA.DBCO.N₃(SO₃)₂-Cys-c[RGDyK] (purple) and [¹¹¹In]DTPA.BCN.N₃(SO₃)₂-Cys-c[RGDyK] (green; n = 6 per compound). For reference, biodistribution of [¹¹¹In]DTPA-Lys(Cys(SO₃)methyl)-Cys-c[RGDyK] (black) was included as previously presented by Bunschoten et al.17 Significance between the hybrid triazole-containing RGD conjugates and the reference annotated as: ****p ≤ 0.0001, ***p ≤ 0.001, **p ≤ 0.01, *p ≤ 0.05, and ns = p > 0.05.
DTPA.DBCO.N₃(SO₃)⁺-Cy5-c[RGDyK] (Figure 2Aii) and DTPA.BCN.N₃(SO₃)⁻-Cy5-c[RGDyK] (Figure 2Aiii). Uptake in αβ₁-negative cells present in the mixed-cell culture was not seen (Figure 2A,B, with green fluorescent protein (GFP)-tagged membranous reference in bright green). Blocking studies confirmed selective binding of the tracers to αβ₁ (Figure 2B); incubation with unlabeled c[RGDyK] resulted in a statistically significant decrease in uptake of the reference tracer DTPA-Lys(Cys(SO₃)methyl)-Cy5-c[RGDyK] (49 ± 6%) and both triazole-containing tracers DTPA.DBCO.N₃(SO₃)⁺-Cy5-c[RGDyK] (86 ± 8%) and DTPA.BCN.N₃(SO₃)⁻-Cy5-c[RGDyK] (83 ± 24%; Figure 2C; p < 0.0001).

Flow-cytometry-based affinity measurements showed that the affinity of DTPA.BCN.N₃(SO₃)⁻-Cy5-c[RGDyK] for the αβ₁ integrin (Kᵦ = 17.5 ± 6.0; Figure 2D) was significantly increased compared to the previously reported affinity of the reference tracer DTPA-Lys(Cys(SO₃)methyl)-Cy5-c[RGDyK] (K₀ = 30.3 ± 5.7, p > 0.0001; Figure 2D, black).

The receptor affinity of DTPA.BCN.N₃(SO₃)⁻-Cy5-c[RGDyK] was significantly lower (K₀ = 76.5 ± 18.3, p > 0.0001; Figure 2D, green) than that of the reference tracer DTPA-Lys(Cys(SO₃)methyl)-Cy5-c[RGDyK], but still in the lower nanomolar range.

In Vivo Imaging and Biodistribution. To assess the effect of implementation of triazole-based click chemistry on the in vivo targeting efficiency and pharmacokinetics of the RGD tracers, [ⁱ¹¹In]DTPA.DBCO.N₃(SO₃)⁺-Cy5-c[RGDyK] and [ⁱ¹¹In]DTPA.BCN.N₃(SO₃)⁻-Cy5-c[RGDyK] were directly compared to the end-conjugated reference tracer [ⁱ¹¹In]DTPA-Lys(Cys(SO₃)methyl)-Cy5-c[RGDyK]. To this end, single-photon emission computed tomography (SPECT) imaging (Figure 3A), fluorescence imaging (Figure 3B), and quantitative assessment of the in vivo biodistribution of [ⁱ¹¹In]DTPA.DBCO.N₃(SO₃)⁺-Cy5-c[RGDyK] and [ⁱ¹¹In]DTPA.BCN.N₃(SO₃)⁻-Cy5-c[RGDyK] (Figure 3C) were performed using identical experimental settings as used previously (i.e., at 24 h after intravenous administration of 1 nmol of the respective hybrid RGD tracer derivative).¹⁷,²¹ Both SPECT and fluorescence imaging of tumor-bearing mice resulted in tumor visualization after administration of [ⁱ¹¹In]DTPA-Lys(Cys(SO₃)methyl)-Cy5-c[RGDyK], [ⁱ¹¹In]DTPA.DBCO.N₃(SO₃)⁺-Cy5-c[RGDyK], or [ⁱ¹¹In]DTPA.BCN.N₃(SO₃)⁻-Cy5-c[RGDyK] (Figure 3AB, respectively, i and ii; tumor encircled in red). Besides tumor visualization, SPECT imaging also enabled visual assessment of the biodistribution of the hybrid tracers, revealing a dominant kidney accumulation for all compounds (Figure 3A) and a relatively high uptake in other nontargeted organs for [ⁱ¹¹In]DTPA.BCN.N₃(SO₃)⁻-Cy5-c[RGDyK] (Figure 3Aiii). It was suspected that the visibility of the tumor when using [ⁱ¹¹In]DTPA.BCN.N₃(SO₃)⁻-Cy5-c[RGDyK] was further impeded by the location of the tumor.

The ex vivo biodistribution data not only underlined the imaging observations but also revealed differences (Figure 3C), e.g., in tumor uptake: [ⁱ¹¹In]DTPA.DBCO.N₃(SO₃)⁺-Cy5-c[RGDyK] and [ⁱ¹¹In]DTPA.BCN.N₃(SO₃)⁻-Cy5-c[RGDyK] have tumor uptakes of 3.5 ± 1.7 and 2.9 ± 1.7% ID/g, respectively. [ⁱ¹¹In]DTPA-Lys(Cys(SO₃)methyl)-Cy5-c[RGDyK], with a tumor uptake of 1.6 ± 0.3% ID/g, is significantly different from [ⁱ¹¹In]DTPA.DBCO.N₃(SO₃)⁺-Cy5-c[RGDyK] (p = 0.0312), but is in the same range as [ⁱ¹¹In]DTPA.BCN.N₃(SO₃)⁻-Cy5-c[RGDyK] (p > 0.05). The tumor-to-muscle ratios (T/M) of both [ⁱ¹¹In]DTPA.DBCO.N₃(SO₃)⁺-Cy5-c[RGDyK] (4.8 ± 2.1) and [ⁱ¹¹In]DTPA.BCN.N₃(SO₃)⁻-Cy5-c[RGDyK] (5.1 ± 1.5) were, however, clearly negatively affected by nonspecific signal present in adjoining muscle tissue; compared to these two tracers, the T/M of the reference tracer [ⁱ¹¹In]DTPA-Lys(Cys(SO₃)methyl)-Cy5-c[RGDyK] was found to be superior (12.8 ± 1.8; p < 0.0001 for both comparisons). Nevertheless, the tumors could still be visualized in vivo with fluorescence imaging despite the lower T/M (Figure 3B).²⁸

Affinity values are generally used to explain T/M ratios.¹⁷ In this case, however, the measured affinities did not directly link to the tumor uptake or T/M ratios; both [ⁱ¹¹In]DTPA.DBCO.N₃(SO₃)⁺-Cy5-c[RGDyK] and [ⁱ¹¹In]DTPA.BCN.N₃(SO₃)⁻-Cy5-c[RGDyK] have an affinity in the same nanomolar range as [ⁱ¹¹In]DTPA-Lys(Cys(SO₃)methyl)-Cy5-c[RGDyK], but their T/M ratio is significantly reduced compared to the reference tracer [ⁱ¹¹In]DTPA-Lys(Cys(SO₃)methyl)-Cy5-c[RGDyK] (p < 0.0001). Other than affinity, differences in charge, lipophilicity, and/or serum binding also influence the T/M ratio. In this case, a trend (albeit nonsignificant) was observed for serum binding ([ⁱ¹¹In]DTPA.DBCO.N₃(SO₃)⁺-Cy5-c[RGDyK] > [ⁱ¹¹In]DTPA.BCN.N₃(SO₃)⁻-Cy5-c[RGDyK] > [ⁱ¹¹In]DTPA-Lys(Cys(SO₃)methyl)-Cy5-c[RGDyK]; see Figure 1A; n = 3), which seemed to translate to changes in the biodistribution values; in vivo, the trend became more pronounced and differences were statistically significant. The fact that this uptake pattern was similar for all of the evaluated organs (Figure 3C; n = 6) further suggested a generic effect; serum binding has e.g. been the culprit for such observations in previously reported results.²⁸

For all compounds, renal retention was by far the highest, and the uptake of [ⁱ¹¹In]DTPA.DBCO.N₃(SO₃)⁺-Cy5-c[RGDyK] and [ⁱ¹¹In]DTPA.BCN.N₃(SO₃)⁻-Cy5-c[RGDyK] was significantly higher compared to that of the reference tracer [ⁱ¹¹In]DTPA-Lys(Cys(SO₃)methyl)-Cy5-c[RGDyK] (14.7 ± 3.2, 22.0 ± 5.7, and 3.0 ± 1.4% ID/g, respectively; p ≤ 0.0001). As the αβ₁ integrin is ubiquitous in the kidney, some renal accumulation of the RGD-containing conjugates can be expected;³⁵,³¹ however, considering conditions between groups were identical, this explanation appeared inadequate. This effect could also not be explained based on the lipophilicity of the compounds (see above). Hence, it seems that the increase in renal uptake of the triazole-containing RGD hybrid tracers is attributable to the introduction of triazole-containing moieties formed by click chemistry. Literature indicates that some triazoles, e.g., Isavuconazole, are characterized by prolonged half-life time,³³,³⁴ and that 3-amino-1,2,3-triazole irreversibly binds to catalase proteins,³³,³⁴ thus implying that these moieties may induce this behavior. It is generally known that molecular alterations and their size have an undisputed effect on the biodistribution, but no prior research regarding the influence of the moieties resulting from copper-free click chemistry has been published to date to the best of our knowledge.

Besides SPAAC and its triazole product, other forms of bioorthogonal click chemistry such as the isocyanide [4 + 1] cycloaddition or inverse electron demand Diels–Alder (IEDDA) could also be employed in the design of a hybrid tracer. However, while IEDDA (k = 1–10 × 10⁷ M⁻¹s⁻¹) is considerably faster than SPAAC (k = 10⁻²–1 M⁻¹s⁻¹),²⁸ it has been used previously in pretargeting strategies,³⁶,³⁷ the effect of IEDDA on the in vivo characteristics of a tracer without an antibody has not yet been investigated. That being said, given the relatively large effects that the triazole-containing moieties...
have been shown to exert, it would be of great interest to compare the effect of both types of copper-free click chemistry in an in vivo setting.

As previously mentioned, the 1,2,3-triazole-containing moiety introduced by click chemistry seemed to have a negative effect on the pharmacokinetics of RGD-based hybrid tracers, but the utility of the heterobifunctional design regarding other targets and/or other targeting moieties should be considered in future studies considering e.g. receptor pockets can vary. The composition of the hybrid tracer plays a large role in the pharmacokinetics of small-molecule-based hybrid tracers, but the tumors could still be visualized. Thus, the hybrid tracer design has a negative effect on its pharmacokinetics, although the tumors could still be visualized. Thus, the design could hold promise for other targets and/or other targeting moieties.

**CONCLUSIONS**

Despite the tracer’s stability and good in vitro performance of triazole-based hybrid tracers, biodistribution data indicated that incorporation of the relatively bulky chemical modifications used for the copper-free click conjugation in RGD-targeting hybrid tracer design has a negative effect on its pharmacokinetics, although the tumors could still be visualized. Thus, the design could hold promise for other targets and/or targeting moieties.

**METHODS**

All chemicals were obtained from commercial sources and used without further purification. Solvents were obtained from Actu-All Chemicals (Oss, The Netherlands) in high-performance liquid chromatography (HPLC) or peptide grade and used without further purification. The reactions were monitored by thin-layer chromatography (TLC) and/or mass spectrometry using a Bruker microflex LRF matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF). HPLC was performed on a Waters (Etten-Leur, The Netherlands) HPLC system using a 1525 ESI pump and a 2489 UV/vis detector. For preparative HPLC, a Dr. Maisch GmbH (Ammerbuch, Germany) Reprosil-Pur 120 C18-AQ 10 μm (250 × 20 mm²) column was used (12 mL/min). For semipreparative HPLC, a Dr. Maisch GmbH Reprosil-Pur C18-AQ 10 μm (250 × 10 mm²) or a Waters XBridge BEH C8 10 μm (250 × 10 mm²) Prep OBD column was used (5 mL/min). For analytical HPLC, a Dr. Maisch GmbH Reprosil-Pur C18-AQ 5 μm (250 × 4.6 mm²) column was used. For all HPLC runs, a gradient of 0.1% trifluoroacetic acid (TFA) in H₂O/CH₃CN 95:5 to 0.1% TFA in H₂O/CH₃CN 5:95 in 40 min was used. Magnetic nuclear resonance (NMR) spectra were taken using a Bruker DPX-300 spectrometer (300 MHz ¹H NMR, 75 MHz ¹³C NMR), and chemical shifts (δ) are reported relative to TMS (δ = 0) and/or referenced to the solvent in which they were measured. Abbreviations used include singlet (s), doublet (d), doublet of doublets (dd), triplet (t), and unresolved multiplet (m). Full ¹H NMR spectra are provided in the Supporting Information available online.

**Synthesis.** (SO₃)Indole, Indole, and Indole-COOH. (SO₃)-Indole, Indole and Indole-COOH were synthesized according to earlier published procedures. All products were directly used without further purification.

(SO₃)Indole-AmineC₄Phth. (SO₃)Indole-AmineC₄Phth was synthesized inspired by adjusted synthesis methods. Briefly, (SO₃)Indole (25 mmol) and 1-(4-bromobutyl)-pyrrolidine-2,5-dione (75.0 mmol) were dissolved in sulfolane (40 mL) and left stirring at 90 °C for 24 h under nitrogen atmosphere. The resulting mixture was, after cooling down to room temperature (r.t.), precipitated by adding to CH₃OH. The product was washed twice with ethyl acetate and diethyl ether and dried in vacuo, resulting in a brownish powder (5.9 g), which was used without any further purification.

DTPA-NHS. DTPA(1Bu)₄ (100.0 mg, 161.9 μmol), dipyrrolidino(N-succinimidyl)-oxycarbonyl hexafluorophosphate (HSPyU) (73.0 mg, 178.1 μmol), and N,N-diisopropyldihydropyrilidylamine (DiPEA) (141.0 μL, 809.3 μmol) were dissolved in CH₂Cl₂ (5 mL, dried on 4 Å molecular sieves) and stirred for 15 h at r.t. The crude product was used without further purification in the following reaction steps. m/z [M + H]+ calcld. 715.9, found 715.5.

DTPA-Ethylene Diamine. DTPA-NHS (57.5 mg, 93.1 μmol), N-Boc-ethylenediamine (12.8 μL, 81.0 μmol), and DiPEA (28.2 μL, 160.9 μmol) in CH₂Cl₂ (2.5 mL) were stirred for 30 min until full conversion according to TLC and MALDI-TOF. The solvent was removed in vacuo and 95:5 TFA/H₂O (2 mL) was added followed by stirring for 2 h. The product was precipitated in diethyl ether (45 mL), centrifuged, and decanted. This was repeated twice, and the precipitate was desiccated and used without further purification. m/z [M + H]+ calcld. 436.4, found 436.0.

DTPA–DBCO. DTPA-ethylene diamine (23.0 μmol) and triethylamine (23.0 μmol) were dissolved in dimethylformamide (DMF) (120.0 μL) and stirred at 30 °C. DBCO-NHS (15.3 μmol) was dissolved in DMF (580 μL) and added to the DTPA-ethylene diamine solution before stirring at 30 °C for 7 h. Subsequently, DiPEA (115.0 μmol) was added and the mixture was stirred at 30 °C overnight before cooling down to r.t. and precipitating in ice-cold diethyl ether. The precipitate was collected, and residual solvents were removed in vacuo. The crude, white solid was purified by prep-HPLC to obtain a white solid (0.8 mg), which was used without any further purification.

DTPA–BCN. DTPA-ethylene diamine (121 μmol) was dissolved in DMSO (800 μL). BCN-NHS (81 μmol, purchased at Sigma-Aldrich Cat. No. 744867) and DiPEA (810 μmol) were dissolved in DMSO (200 μL) and added to the DTPA-ethylene diamine solution before stirring overnight at r.t. After rinsing the reaction vial with H₂O and CH₃CN, the solvents and DiPEA were removed by centrifugal evaporation at 50 °C and <0.128 mbar for 5 h. The remaining, slightly yellow oil was used without further purification.

c(RGDyK). This compound was synthesized as previously described. Phth-(SO₃)Cy5-COOH. The synthesis of the Cy5 dye was performed based on literature procedures. In short, Indole-COOH (1.1 g, 4.0 mmol) and N’-((2E,4E)-4-(phenylimino)but-2-en-1-yl)amine (1.2 g, 4.4 mmol) were dissolved in acetic anhydride (1:1, 30 mL) and left stirring at 60 °C; the progress of the reaction was monitored by absorption spectroscopy. After stirring at 60 °C overnight, the mixture was heated to 120 °C. The crude product was dissolved in DMF/CH₂Cl₂ (70 mL; 1:1) after precipitation in diethyl ether (600 mL). Merrifield resin (1.2 g, 2.0 mmol) was suspended in CH₂Cl₂ for 5 min and then left to bubble in 20% TFA in CH₂Cl₂ (50.0 mL) for 1 h and in 20% DiPEA (50.0 mL) in CH₂Cl₂ for 15 min. The dissolved hemicyanine was then added to the pre-conditioned resin. After bubbling through N₂ for 1 h, the resin was washed with DMF/CH₂Cl₂ (20 mL; 1:1) thrice. (SO₃)Indole-AmineC₄Phth (0.44 g, 1.0 mmol) was dissolved
in pyridine/acetic anhydride (40 mL; 3:1) and added to the resin. The resulting reaction mixture was shaken overnight at rt. Afterward, the liquids were collected and the resin was washed with DMF and/or CH2Cl2 until the washings were light blue. The crude reaction mixture was concentrated in vacuo and separated by column chromatography (CH2Cl2: CH3OH) and then further purified by prep-HPLC, yielding a blue solid after lyophilization (39.0 mg, 5%) with a purity of 99%. m/z [M + H]+ calcd. 750.9, found 751.1. 1H NMR (300 MHz, MeOD) δ 8.23 (dt, J = 13.0 Hz, 2H), 7.79–7.88 (m, 4H), 7.23–7.54 (m, 8H), 6.65 (t, J = 12.4 Hz, 1H), 6.31 (d, J = 13.6 Hz, 2H), 4.15–4.18 (m, 4H), 3.78 (m, 2H), 2.35 (t, J = 2.35 Hz, 2H), 1.82–1.90 (m, 6H), 1.72–1.75 (m, 14H), 1.53 (m, 2H) (Figure S11).

H2N(SO3)2Cy5-COOH. Phth-(SO3)2Cy5-COOH (55.0 mg, 73.2 μmol) was dissolved in CH3CN (25 mL). After stirring for 21 h, the reaction appeared to be complete. Residual CH3NH2 and ethanol were removed in vacuo before dividing into two portions. The crude product was dissolved in H2O (0.1% v/v TFA) (3 mL) and CH3CN (0.1% v/v TFA) (1 mL), purified by means of reversed-phase (RP) preparative (prep)-HPLC, and after removal of solvents in vacuo, the resulting mixture was stirred at rt under a N2 atmosphere for 3 h. Then, the solvent was removed in vacuo and the crude product was redissolved in CH3OH (10 mL) before basifying with K2CO3 (180.0 mg) and CH3NH2 dissolved in DMSO (400 μL) dried on 4 Å molecular sieves). After stirring for 30 min at rt, 100% conversion was complete. Residual CH3NH2 and ethanol were removed in vacuo before dividing into two portions. The crude product was redissolved in H2O (0.1% v/v TFA) (3 mL) and CH3CN (0.1% v/v TFA) (1 mL), purified by means of RP-HPLC. Solvents were removed in vacuo, yielding a blue solid (28.9 mg, 43%). m/z [M + H]+ calcd. 646.8, found 646.3. 1H NMR (300 MHz, DMSO-d6) δ 8.35 (td, J = 13.0, 5.7 Hz, 2H), 7.83 (d, J = 1.4 Hz, 1H), 7.68–7.64 (m, 1H), 7.64–7.60 (m, 3H), 7.43 (s, 2H), 7.35–7.23 (m, 3H), 6.58 (d, J = 12.1 Hz, 1H), 6.31 (t, J = 13.9 Hz, 2H), 4.13 (d, J = 6.6 Hz, 5H), 2.80 (s, 2H), 2.20 (t, J = 7.1 Hz, 2H), 1.71 (s, 6H), 1.68 (s, 6H), 1.58–1.51 (m, 4H), 1.41–1.34 (m, 2H), 1.23 (s, 1H) (Figure S12).

N2(SO3)2Cy5-COOH. H2N(SO3)2Cy5-COOH (43.1 mg, 66.7 μmol) was dissolved in CH3OH (10 mL) before basifying with K2CO3 (180.0 μmol). CuSO4 (cat., in H2O) and 1/16-imidazole-1-sulfonyl azide hydrochloride (80.0 μmol) was dissolved in CH3OH (10 mL) before basifying with DMF and/or CH2Cl2 until the washings were light blue. Afterward, the liquids were collected and the resin was washed twice with diethyl ether, and desiccated. Purification by RP-HPLC followed by lyophilization yielded a blue solid (490.0 μg, 24%). m/z [M + H]+ calcd. 1,352.6, found 1,351.8. 1H NMR (300 MHz, DMSO-d6) δ 2.20 (t, J = 7.1 Hz, 2H), 1.72–1.75 (m, 14H), 1.53 (m, 2H) (Figure S13).

Phth(SO3)2Cy5-COOH. Phth-(SO3)2Cy5-COOH (20.0 mg, 26.7 μmol), HSVPy (12.0 mg, 29.3 μmol) and DiPEA (23.0 μL, 133.3 μmol) were dissolved in DMSO (400 μL, dried on 4 Å molecular sieves). After stirring for 30 min at rt, 100% conversion was confirmed by TLC and MALDI-TOF. The compound was purified by RP-HPLC and lyophilized, yielding a blue solid (10.0 mg, 28%). m/z [M + H]+ calcd. 751.2, found 751.1. 1H NMR (300 MHz, DMSO-d6) δ 8.35 (td, J = 13.0, 5.7 Hz, 2H), 7.83 (d, J = 1.5 Hz, 1H), 7.69–7.56 (m, 2H), 7.41 (d, J = 4.1 Hz, 2H), 7.34 (d, J = 8.3 Hz, 1H), 7.25 (dt, J = 12.4, 4.2 Hz, 1H), 6.58 (t, J = 12.3 Hz, 1H), 6.32 (dd, J = 13.8, 9.2 Hz, 2H), 4.48 (s, 5H), 4.19 (br s, J = 22.5 Hz, 4H), 2.20 (t, J = 7.1 Hz, 2H), 1.69 (d, J = 3.2 Hz, 14H), 1.60–1.47 (m, 2H), 1.46–1.28 (m, 2H), 1.11 (s, 2H) (Figure S13).

Photophysical Properties. Absorption (ε), quantum yield (Φ), and maximum absorption/emission measurements of N1(SO3)2Cy5-COOH and its two conjugates (DTPA.DBCO.N3(SO3)-Cy5-c[RGDYk] and DTPA.BCN.N3(SO3)-Cy5-c[RGDKy]) and DTPA.BCN.N3(SO3)-Cy5-c[RGDKy]), serum binding analysis, and serum binding experiments were performed according to the previously described methods.11−12 The brightness of the hybrid tracers was based on their respective quantum yield and lipophilicity.

Lipophilicity. log P(aq/o/w) was calculated according to previously published methods.41 In short, to a solution of 0.5−1 MBq of 99mTc radiolabeled hybrid tracer in 500 μL of phosphate-buffered saline (PBS, pH 7.4), 500 μL of 1-octanol was added (n = 6). Vials were vigorously shaken on a IKA VXR basic Viborax orbital shaker (IKA-Werke GmbH & Co. KG, Staufen, Germany) for 3 min and subsequently centrifuged at 6000 rpm for 5 min in a Fisher Scientific MicroV centrifuge (Fisher Scientific, Hampton) to achieve quantitative phase separation. The activity concentrations in 100 μL samples of both the aqueous and organic phases were measured in a PerkinElmer Wizard2 γ-counter (PerkinElmer, Waltham).

In Vitro Tracer Specificity. A mix of αβ-integrin-expressing Ge3β cells and MDAMB231 X4 cells was seeded onto coverslips (635 mm; MatTek Corporation) and incubated at 37 °C in 2 mL of medium for 48 h. One hour prior to imaging, the cells were incubated at 37 °C with DTPA-Lys5(Cys(SO3)-methyl)-Cy5-c[RGDKy], DTPA.DBCO.N3(SO3)-Cy5-c[RGDKy], or DTPA.BCN.N3(SO3)-Cy5-c[RGDKy] (1 μM). For blocking experiments, 1 μM unlabeled c[RGDKy] was added 1 h prior to addition of DTPA.DBCO.N3(SO3)-Cy5-c[RGDKy]. The resulting reaction mixture was filtered through a 0.45 μm syringe filter and then analyzed by RP-HPLC, and after removal of solvents in vacuo, yielding a blue solid after lyophilization (394.0 mg, 5%). m/z [M + H]+ calcd. 1,351.8. 1H NMR (300 MHz, DMSO-d6) δ 2.20 (t, J = 7.1 Hz, 2H), 1.72–1.75 (m, 14H), 1.53 (m, 2H) (Figure S13).
DTPA.BCN.N3(SO3)-Cy5-c-[RGDyK] or DTPA.BCN.N2(SO3)-Cy5-c-[RGDyK]. Prior to imaging, the cells were washed three times with PBS. Confocal microscopy was performed on viable cells, using an SP8 WLL fluorescence confocal microscope (Leica Microsystems, Wetzlar). For additional reference, the nuclei of the cells were stained with Hoechst 33342 (1 mg/mL stock, Thermo Fisher). Sequential scanning settings were used to visualize the different fluorescent features: Hoechst (nuclear staining; λex 405 nm, λem 42–470 nm); intrinsic GFP (λex 500 nm, λem 500–550 nm; MDAMB231 X4 only), and Cy5 (λex 633 nm, λem 650–700 nm).

Quantification of cellular uptake was performed as previously described. In images depicting the Cy5-related signal intensity, regions of interest were placed around the whole cell (minimum of 13 cells per image) using Fuji software, whereafter the pixel intensity per cell could be determined. Unblocked and blocked conditions were evaluated for all three tracers (n = 3 experiments per condition). For the unblocked conditions, the percentage of fluorescence uptake (intensity per cell) was set at 100%. The percentage fluorescence uptake after blocking was determined relative to the unblocked condition.

Receptor Affinity. The affinity (Kd) of the tracers for Geβ3 cells was determined by saturation binding experiments. To this end, 3 × 10⁵ cells were incubated for 1 h at 4 °C with different concentrations of either DTPA.DBCO.N3(SO3)-Cy5-c-[RGDyK] or DTPA.BCN.N3(SO3)-Cy5-c-[RGDyK] ranging from 0 to 1500 nM. To accomplish this, Geβ3 cells were trypsinized, pelleted (4 min, 1200 rpm, 4 °C), and resuspended in Dulbecco’s modified Eagle’s medium (DMEM; enriched with 5% fetal bovine serum and 1% penicillin/streptomycin (1:1 v/v)) to obtain a concentration of 6 × 10⁵ cells/mL. Subsequently, 50 μL of the cell suspension (3 × 10⁵ cells) was added to 50 μL of the tracer in cold DMEM (0–300 nM). After 1 h incubation at 4 °C, the samples were washed three times with 0.1% fetal calf serum in cold PBS by pelleting (4 min, 1200 rpm, 4 °C) and subsequently resuspended in 150 μL of 0.1% fetal calf serum in cold PBS. The samples were then measured by flow cytometry using an LSR-II after excitation at 633 nm and collecting the signal in the APC-A channel. All experiments were performed in triplicate.

Normalized geometric means were fitted with equations in the GraphPad Prism 7 software. The Kd values were calculated using the “Binding—Saturation, One site—Total” nonlinear regression equation (eq 1), with the background value set at 0.

$$\log IC_{50} = \log \left( \frac{1 + [\text{Ref}]}{K_{D,\text{ref}}} \right)$$

where y = normalized fluorescence, $B_{\text{max}}$ = maximum specific binding in the units of the y-axis, $x$ = concentration of the hybrid labeled tracer, $K_d$ = equilibrium dissociation constant of the hybrid labeled tracer in nanomolar.

Radiolabeling. For logP experiments, 66 μL of 10 μM Tin(II)chloride dihydrate in sodium acetate buffer (250 mM, pH 4.0) and 6 MBq of 99mTc in 7–11 μL saline were added to 1 nmol compound dissolved in a NH₄OAc buffer (250 mM, pH 5.5). After shaking at room temperature for 5 min, the radiochemical purity was determined to be >90% using ITLC with PBS as eluent.

For biodistribution experiments, 4 nmol of either DTPA.DBCO.N3(SO3)-Cy5-c-[RGDyK] or DTPA.BCN.N3(SO3)-Cy5-c-[RGDyK] was dissolved in a NH₄OAc buffer (0.25 M; pH = 5.5) and mixed with 12.2 μL of an 111InCl₃ solution in 0.05 M HCl (0.33 MBq/μL, Mallinckrodt Medical, Petten, The Netherlands). The mixtures were shaken for 1 h at r.t. After addition of HCl (0.05 M; 232 μL), the mixtures were supplemented with isotonic PBS to obtain a volume of 600 μL, and 150 μL (1 nmol) per mouse was then injected.

For combined imaging and biodistribution experiments, 2 nmol of each compound, dissolved in a NH₄OAc buffer (0.25 M; pH = 5.5), was mixed with 121.9 μL of an 111InCl₃ solution in 0.05 M HCl (0.33 MBq/μL, Mallinckrodt Medical, Petten, The Netherlands). The mixtures were shaken for 1 h at r.t., whereafter the mixtures were supplemented with isotonic PBS to obtain an injection volume of 300 μL.

In Vivo Imaging and Biodistribution. The 4T1 tumor model was generated in BALB/c nude mice, as previously described. The animal studies were approved by the institutional Animal Ethics Committee of the Leiden University Medical Center.

Imaging and biodistribution were performed 24 h after intravenous tracer (1 nmol) administration (n = 6 animals for each tracer; n = 4 biodistribution only, 2 SPECT imaging/biodistribution). SPECT imaging was performed as previously described. Hereafter, fluorescence imaging was performed in vivo and ex vivo using an IVIS Spectrum imaging system (Caliper Life Science, Hopkinton, MA) according to previously described methods.

For combined imaging and biodistribution experiments, 2 nmol of each compound, dissolved in a NH₄OAc buffer (0.25 M; pH = 5.5), was mixed with 121.9 μL of an 111InCl₃ solution in 0.05 M HCl (0.33 MBq/μL, Mallinckrodt Medical, Petten, The Netherlands). The mixtures were shaken for 1 h at r.t., whereafter the mixtures were supplemented with isotonic PBS to obtain an injection volume of 300 μL.

1H NMR spectra of Phth-(SO3)Cy5-COOH, H₂N-(SO3)Cy5-COOH and N₃-(SO3)Cy5-COOH; mass spectra of DTPA-Lys(Cy5(SO₃)methyl)-Cys-c-[RGDyK], DTPA.DBCO.N₃(SO₃)-Cy5-c-[RGDyK], and DTPA.BCN.N₃(SO₃)-Cy5-c-[RGDyK]; and analytical HPLC images of DTPA.DBCO.N₃(SO₃)-Cy5-c-[RGDyK] and DTPA.BCN.N₃(SO₃)-Cy5-c-[RGDyK] (PDF)

ASSOCIATED CONTENT

Supporting Information

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Notes
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**ABBREVIATIONS**

(DTPA), 4, diethylenetriaminepentaacetate acid  
(DMF), 17, dimethylformamide  
(HSPyU), 16, dipyrrylidino(N-succinimidloyloxy)carbenium hexafluorophosphate  
(HPLC), 15, high-performance liquid chromatography  
(MALDI-TOF), 15, matrix-assisted laser desorption/ionization time-of-flight (e), 7, molar absorbance coefficient  
(DiPEA), 16, N,N-disopropylethylamine  
(NMR), 16, nuclear magnetic resonance (prep), 19, preparative  
(Φ), 7, quantum yield  
(RP), 19, reversed-phase  
(SPECT) imaging, 11, single-photon emission computed tomodgraphy (TFA), 16, trifluoroacetic acid

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