Published in final edited form as:

J Inorg Biochem. 2016 January ; 154: 60–66. doi:10.1016/j.jinorgbio.2015.10.012.

Transferrin Conjugates of Triazacyclononane-Based Bifunctional NE3TA Chelates for PET Imaging: Synthesis, Cu-64 Radiolabeling, and In vitro and In vivo evaluation

Chi Soo Kang¹, Ningjie Wu¹, Yunwei Chen¹, Xiang Sun¹, Nilantha Bandara², Dijie Liu⁴, Michael Lewis^{3,4}, Buck Rogers², and Hyun-Soon Chong^{1,*}

¹Department of Biological and Chemical Sciences, Illinois Institute of Technology, Chicago, IL

²Department of Radiation Oncology, Washington University in St. Louis, St. Louis, MO

³Research Service, Harry S. Truman Memorial Veterans' Hospital

⁴Department of Veterinary Medicine and Surgery, University of Missouri, Columbia, MO

Abstract

Three different polyaminocarboxylate-based bifunctional NE3TA (7-[2-[carboxymethyl]amino]ethyl)-1,4,7-triazacyclononane-1,4-diacetic acid) chelating agents were synthesized for potential use in copper 64-PET imaging applications. The bifunctional chelates were comparatively evaluated using transferrin (Tf) as a model targeting vector that binds to the transferrin receptor overexpressed in many different cancer cells. The transferrin conjugates of the NE3TA-based bifunctional chelates were evaluated for radiolabeling with ⁶⁴Cu. *In vitro* stability and cellular uptake of ⁶⁴Cu-radiolabeled conjugates were evaluated in human serum and prostate (PC-3) cancer cells, respectively. Among the three NE3TA-Tf conjugates tested, *N*-NE3TA-Tf was identified as the best conjugate for radiolabeling with ⁶⁴Cu. *N*-NE3TA-Tf rapidly bound to ⁶⁴Cu (>98% radiolabeling efficiency, 1 min, RT), and ⁶⁴Cu-*N*-NE3TA-Tf remained stable in human serum for 2 days and demonstrated high uptake in PC-3 cancer cells. ⁶⁴Cu-*N*-NE3TA-Tf was shown to have rapid blood clearance and increasing tumor uptake in PC-3 tumor bearing mice over a 24 h period. This bifunctional chelate presents highly efficient chelation chemistry with ⁶⁴Cu under mild condition that can be applied for radiolabeling of various tumor-specific biomolecules with ⁶⁴Cu for potential use in PET imaging applications.

Graphical Abstract

*Corresponding Author: Hyun-Soon Chong, Department of Chemistry, Illinois Institute of Technology, 3101 S. Dearborn St, LS 182, Chicago, IL, 60616, Chong@iit.edu, Fax: 312-567-3494.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

**^{64}Cu -Radiolabeled
NE3TA-Transferrin Conjugates**

Three different NE3TA (7-[2-[carboxymethyl]amino]ethyl]-1,4,7-triazacyclononane-1,4-diacetic acid)-based bifunctional chelating agents were comparatively evaluated for potential use in copper ^{64}PET imaging applications using transferrin as a model targeting vector. The biological data indicate that *N*-NE3TA more rapidly and stably bound with ^{64}Cu than the other NE3TA chelating agents.

Keywords

Copper 64; bifunctional chelator; transferrin; radiolabeling; serum stability; biodistribution

Introduction

Positron emission tomography (PET) is a non-invasive and sensitive nuclear imaging technique that uses two highly energetic photons (511 keV) produced from interaction of positrons emitted from decay of radionuclides and electrons. [1,2] Among the clinically available positron-emitting radionuclides, ^{18}F ($t_{1/2} = 110$ min) has been successfully applied for PET imaging, and ^{18}F -2-Fluoro-2-deoxy-glucose (^{18}F -FDG) is one of the most frequently used tracers for detection of tumors. [3] However, the short half-life of ^{18}F presents a limitation for its broad application in imaging of cancers, particularly for biomolecules with slow tumor accumulation such as antibodies. Copper-64 ($t_{1/2} = 12.7$ h) with a relatively longer half-life and useful decay property ($I_{\beta^+} = 17\%$, $I_{\beta^-} = 39\%$, $I_{\text{EC}} = 43\%$, $\beta_{\text{max}}^+ = 0.656$ MeV) is an attractive metallic radionuclide for PET imaging. [4] Research efforts have been made to develop clinically viable bifunctional chelating agents for radioimmuno-PET imaging applications of ^{64}Cu using tumor-specific antibodies or peptides. [4–6]

Macrocyclic polyaminocarboxylate-based chelating agents including DOTA (1,4,7,10-tetraazacyclododecane-*N,N',N'',N'''*-tetraacetic acid), TETA (1,4,8,11-tetraazacyclotetradecane-*N,N',N'',N'''*-tetraacetic acid), and CB-TE2A (1,4,8,11-tetraazabicyclo[6.6.2]hexadecane-4,11-diacetic acid) have been evaluated for development of ^{64}Cu -based PET imaging agents. [4–6] However, DOTA, TETA, and CB-TE2A are not optimal chelating agents of ^{64}Cu for PET applications due to *in vivo* dissociation of ^{64}Cu -radiolabeled complex or harsh radiolabeling conditions. [7–9]

Recently, we reported NE3TA (7-[2-(carboxymethyl)amino]ethyl]-1,4,7-triazacyclononane-1,4-diacetic acid) and bifunctional versions of NE3TA (*N*-NE3TA and *C*-NE3TA, Figure 1) as potential chelating agents of ^{64}Cu . [10–11] The heptadentate NE3TA analogues rapidly bound to ^{64}Cu , and ^{64}Cu -radiolabeled complexes of the chelates remained inert in serum. [11] Copper-64-radiolabeled complexes of *C*-NE3TA (7-[2-(carboxymethyl)amino]-3-(4-nitrophenyl)propyl]-1, 4,7-triazacyclononane-1,4-diacetic acid) and *N*-NE3TA (7-[2-[(carboxymethyl) amino]-2-(4-nitrophenyl)methyl]-1,4,7-triazacyclononane-1,4-diacetic acid) were shown to have lower uptake in normal organs and more rapid blood and muscle clearance and superior *in vivo* stability than the ^{64}Cu -*C*-DOTA complex. [10]

With the encouraging data on chelation chemistry of bifunctional NE3TA analogues with ^{64}Cu , we proceeded with evaluation of the bifunctional NE3TA-based chelates for targeted PET imaging using transferrin as a model tumor targeting vector. Transferrin (Tf) binds to ferric iron (Fe^{3+}) in circulation and transports the iron into cells and tissues. When the transferrin binds to transferrin receptor (TfR), the Tf-TfR complex is transported into the cell via endocytosis. TfR is highly expressed on some cancers including cervical, colon, prostate, and breast cancers. [12–13] Due to its elevated expression level in cancer cells, TfR has been used as a therapeutic target for cancer treatment and diagnosis. Particularly, holotransferrin (Holo-Tf) in the form of its iron complex has high binding affinity to TfR and has been used as a targeting moiety of therapeutics for cancers with high expression of TfR. [14]

We herein report preparation, characterization, and evaluation of transferrin conjugates of three bifunctional NE3TA analogues for targeted PET applications of ^{64}Cu . The NE3TA-transferrin conjugates were prepared, characterized, and evaluated for radiolabeling kinetics with ^{64}Cu . The corresponding ^{64}Cu -radiolabeled NE3TA-Tf conjugates were further evaluated for complex stability in human serum and binding affinity to TfR using PC-3 cancer cells, as well as *in vivo* biodistribution in PC-3 xenograft-bearing mice.

Experimental

Instruments and analyses

^1H and ^{13}C NMR spectra were obtained using a Bruker 300 instrument, and chemical shifts are reported in ppm on the δ scale relative to TMS or solvent. Fast atom bombardment (FAB) or Electrospray ionization (ESI) high resolution mass spectra (HRMS) were obtained on JEOL double sector JMS-AX505HA mass spectrometer (University of Notre Dame, IN). Size-exclusion HPLC (SE-HPLC) chromatograms were obtained on Agilent 1200 equipped

with a diode array detector and an in-line IN/US γ -Ram Model 2 radiodetector (Tampa, FL), fitted with BioSep-SEC S 3000 column (Phenomenex, Torrance, CA). All absorbance measurements for the protein concentration and ligand protein ratio were obtained on an Agilent 8453 diode array spectrophotometer equipped with an 8-cell transport system (designed for 1-cm cells). Arsenazo III (AIII, 2,2-(1,8-dihydroxy-3,6-disulfonaphthylene-2,7-bisazo) bis-benzenearsonic acid), copper atomic absorption standard solution, and diferric (holo) transferrin were purchased from Sigma-Aldrich (St. Louis, MO) and used as received. C-DOTA analogues were purchased from Macrocyclics (Dallas, TX).

2-([(4-aminophenyl)methyl]({2-[4,7-bis(carboxymethyl)-1,4,7-triazonan-1-yl]ethyl})-amino)acetic acid (2)

4M HCl/dioxane (6 mL) was added to **1** [15] (39 mg, 62.9 μ mol) in a flask was cooled with an ice bath. After the addition was complete, the reaction mixture was gradually warmed to room temperature and allowed to stir overnight at room temperature. Ethyl ether (50 mL) was added to the reaction mixture with vigorous stirring, and the resulting slurry was kept in the freezer for 2 h. The precipitate was collected and washed with ethyl ether, and immediately dissolved in water, and the resulting solution was lyophilized to provide pure salt **2** (34 mg, 85%) as a light yellow solid. ^1H NMR (D_2O , 300 MHz) δ 2.97 (m, 4H), 3.21 (m, 6H), 3.33 (s, 4H), 3.48 (t, J = 5.4 Hz, 2H), 3.93 (s, 4H), 3.99 (s, 2H), 4.46 (s, 2H), 7.40 (d, J = 6.4 Hz, 2H), 7.59 (d, J = 6.4 Hz, 2H). ^{13}C NMR (D_2O , 75 MHz) δ 48.4 (t), 48.7 (t), 49.8 (t), 49.9 (t), 51.1 (t), 51.3 (t), 53.9 (t), 56.1 (t), 58.5 (t), 124.1 (d), 129.5 (s), 131.8 (s), 133.2 (d), 168.7 (s), 171.2 (s); HRMS (Positive ion ESI) Calcd for $\text{C}_{14}\text{H}_{26}\text{N}_4\text{O}_6$ [$\text{M} - \text{BnNH}_2 + \text{H}$] $^+$ m/z 347.3874, Found: [$\text{M} - \text{BnNH}_2 + \text{H}$] $^+$ m/z 347.1958.

2-({2-[4,7-bis(carboxymethyl)-1,4,7-triazonan-1-yl]ethyl}[(4-isothiocyanatophenyl)-methyl]amino)acetic acid (3)

To a solution of **2** (20.1mg, 31.7 μ mol) in water (0.3 mL) was added CSCl_2 in CHCl_3 (39.4 μ L). The resulting mixture was stirred at room temperature for 4 h. The aqueous layer was transferred to a round bottom flask, and the CHCl_3 layer was washed with water (1 mL $\times$ 2). The combined aqueous layers were lyophilized to provide compound **3** (*N*-NE3TA-NCS) as a yellow solid (20 mg, 99%). ^1H NMR (D_2O , 300 MHz) δ 2.97 (m, 4H), 3.02–3.36 (m, 12H), 3.44 (t, J = 5.4 Hz, 2H), 3.93 (s, 4H), 3.98 (s, 2H), 4.36 (s, 2H), 7.29 (d, J = 6.1 Hz, 2H), 7.47 (d, J = 6.1 Hz, 2H). HRMS (Positive ion ESI) Calcd for $\text{C}_{22}\text{H}_{32}\text{N}_5\text{O}_6\text{S}$ [$\text{M} + \text{H}$] $^+$ m/z 494.2073, Found: [$\text{M} + \text{H}$] $^+$ m/z 494.2098.

2-([1-(4-aminophenyl)-3-[4,7-bis(carboxymethyl)-1,4,7-triazonan-1-yl]propan-2-yl](benzyl)amino)acetic acid (5)

To a solution of **4** [16] (14.5 mg, 20.2 μ mol) in ethanol (8.0 mL) was added dry 10% Pd/C (3.9 mg) under Argon gas. The reaction mixture was subjected to hydrogenolysis (20 psi) for 14 h. at room temperature. The resulting mixture was filtered via Celite® bed and washed thoroughly with ethanol. The filtrate was concentrated *in vacuo* to provide compound **5** as a light yellow solid (14 mg, 96%). ^1H NMR (D_2O , 300 MHz) δ 2.35–2.66 (m, 1H), 2.69–3.28 (m, 18H), 3.50 (s, 2H), 3.56–3.97 (m, 8H), 4.21 (s, 1H), 7.18–7.49 (m, 9H). ^{13}C NMR (D_2O , 75 MHz, DEPT-135) δ 45.0 (t), 48.5 (t), 50.1 (t), 50.9 (t), 51.9 (t),

55.9 (t), 57.6 (t), 61.0 (d), 62.3 (t), 123.5 (d), 123.7 (d), 129.4 (d), 130.0 (d), 130.1 (d), 130.6 (d), 130.8 (d). HRMS (Negative ion ESI) Calcd for $C_{28}H_{38}N_5O_6$ $[M - H]^+$ m/z 540.6312, Found: $[M - H]^+$ m/z 540.2823.

2-[benzyl({1-[4,7-bis(carboxymethyl)-1,4,7-triazonan-1-yl]-3-(4-isothiocyanatophenyl)propan-2-yl})amino]acetic acid (6)

To a solution of **5** (10 mg, 13.8 μ mol) in water (100 μ L) was added 1M $CSCl_2$ in $CHCl_3$ (16.6 μ L). The resulting mixture was stirred at room temperature for 4 h. The aqueous layer was transferred to a round bottom flask, and the $CHCl_3$ layer was washed with water (2×1 mL). The combined aqueous layers were lyophilized to provide compound **6** as a pale yellow solid (9.2 mg, 91%). 1H NMR (D_2O , 300 MHz) δ 2.35–2.66 (m, 1H), 2.69–3.28 (m, 18H), 3.50 (s, 2H), 3.56–3.97 (m, 8H), 4.21 (s, 1H), 7.18–7.49 (m, 9H). HRMS (Positive ion ESI) Calcd for $C_{29}H_{37}N_5O_6S$ $[M + H]^+$ m/z 583.6990, Found: $[M + H]^+$ m/z 584.2534.

Conjugation of NE3TA-based bifunctional chelating agents to transferrin and Spectroscopic Determination of Ligand to Protein (L/P) Ratio for transferrin conjugates

A stock solution of holotransferrin (holo-Tf) was freshly prepared. HEPES (N-(2-hydroethyl)-piperazine-N'-ethanesulfonic acid) buffer (50 mM HEPES, 150 mM NaCl, pH 8.6) was prepared and stored at 4 °C. Each Tf conjugate was prepared by reaction of a bifunctional chelating agent in the isothiocyanate form with holo-Tf. In brief, holo-Tf (40 mg) was dissolved in HEPES buffer (2.0 mL, 50 mM HEPES, 150 mM NaCl at pH 8.6). To a sterilized test tube containing the resulting solution, 10-fold molar excess of each chelating agent was added. The resulting solution was gently agitated at room temperature overnight. The following day, this solution was placed on an Amicon C-50 membrane (50,000 MWCO) and spun down to reduce volume. PBS (3×2 mL) was added to the remaining solution of the Tf conjugate, followed by centrifugation in order to remove unreacted ligand. The final volume of purified transferrin conjugates was brought to ~400 μ L using PBS. The protein concentration was determined by measuring absorbance of the solution at 280nm. L/P ratio of Tf conjugates was spectroscopically determined using Cu(II)-AAIII (0.15 M NH_4OAc , pH 7.0). For ligand concentration, an aliquot of a Tf conjugate was added to the cuvette containing the Cu(II)-AAIII reagent, and the absorbance at 610nm was monitored over time. The reaction was complete after 15 min, and the concentration of *N*-NE3TA, *C*-NE3TA or *C*-NE3TA-Bn in the conjugate solution was calculated. The L/P ratio of *N*-NE3TA-Tf, *C*-NE3TA-Tf, *C*-NE3TA-Bn-Tf, and *C*-DOTA-Tf conjugate was found to be 4.5, 3.1, 6.6, and 2.9, respectively.

Radiolabeling of NE3TA-Tf conjugates with ^{64}Cu

All HCl solutions were prepared from ultra-pure HCl. Ultra pure NH_4OAc was purchased from Aldrich and used to prepare all NH_4OAc buffer solutions (0.25 M, pH 5.5). The buffer solutions were chelexed, and filtered through 0.22 μ m filter (Corning) prior to use. ^{64}Cu was purchased from Washington University (St. Louis, MO). To a buffer solution (12.8–18.4 μ L, pH 5.5) in a capped microcentrifuge tube was sequentially added a solution of each conjugate (20 μ g) in PBS and ^{64}Cu (0.05 M HCl, 60 μ Ci). The final volume of the resulting solution was around 20 μ L, and the pH of the resulting reaction mixture was 5.5. The

reaction mixture was agitated on the thermomixer set at 1,000 rpm at room temperature for 30 min. The labeling efficiency (%) was determined by TLC (solvent: 20 mM EDTA/ 0.15 M NH₄OAc). The specific activity was calculated based on the activity of ⁶⁴Cu and the amount of conjugate used in radiolabeling reaction. The activity was corrected by radiolabeling efficiency (%) and divided by amount of conjugate (mg) to determine the specific activity (mCi/mg). A solution of radiolabeled mixture (2 µL) was withdrawn at the designated time points (1 min, 10 min and 30 min), and DTPA (diethylenetriamine pentaacetic acid) solution (1 mM, 0.6 µL) was added to the mixture, and the resulting mixture was left for at least 20 min at RT. In TLC analysis, peaks for bound and unbound radioisotope appeared around 30 mm and 50 mm from the origin, respectively.

For radiolabeling of each conjugate at higher activity, ⁶⁴Cu (30 µCi) was added to a solution of each conjugate (1 µg), and the final volume of the solution (pH 5.5) was adjusted to 20 µL. The reaction mixture was agitated on the thermomixer set at 1,000 rpm at room temperature for 1 h. The radiolabeling efficiency (%) was determined by TLC as described above.

A control experiment was conducted to check if ⁶⁴Cu was bound to holo-transferrin (Tf). Radiolabeling of naïve holo-Tf (without NE3TA chelate attached) with ⁶⁴Cu was repeated under the same reaction condition used for testing NE3TA-Tf conjugates. ⁶⁴Cu (60 µCi) was added to a solution of holo-Tf (20 µg), and the final volume of the solution (pH 5.5) was adjusted to 20 µL. The reaction mixture was agitated on the thermomixer set at 1,000 rpm at room temperature for 30 min. A solution of radiolabeled mixture (2 µL) was withdrawn at the designated time points (1 min, 10 min and 30 min), and DTPA solution (1 mM, 0.6 µL) was added to the mixture, and the resulting mixture was left for at least 20 min at RT. The labeling efficiency (%) was determined by both TLC (solvent: 20 mM EDTA/ 0.15 M NH₄OAc) and SE-HPLC (Phenomenex, BioSep-SEC S 3000, 7.8 × 30 cm, eluent: 0.05 M NaSO₄ / 0.02 M NaH₂PO₄ / 0.05% NaN₃, pH 6.8, flow rate: 1 mL/min).

***In vitro* stability of ⁶⁴Cu-radiolabeled NE3TA-Tf conjugates**

Each ⁶⁴Cu-radiolabeled NE3TA-Tf conjugate was prepared by reaction of a NE3TA-Tf conjugate with ⁶⁴Cu at RT. ⁶⁴Cu-C-DOTA-Tf conjugate was evaluated for comparison. The ⁶⁴Cu-radiolabeled transferrin conjugates were purified using Biospin 6 column, and the purity of the conjugates was confirmed by HPLC. The reaction mixture containing ⁶⁴Cu-radiolabeled Tf conjugate (7 µL, 15 µCi) was added into human serum (50 µL, Gemini Bioproducts, #100110) in a microcentrifuge tube. The stability of the conjugates was evaluated for 2 days. The serum stability of the radiolabeled complexes was assessed by measuring the transfer of the radionuclide from the complex to serum proteins using SE-HPLC (Phenomenex, BioSep-SEC S 3000, 7.8 × 30 cm, eluent: 0.05 M NaSO₄ / 0.02 M NaH₂PO₄ / 0.05% NaN₃, pH 6.8, flow rate: 1 mL/min). A solution of the radiolabeled complex in serum (2 µL) was withdrawn at the designated time point, treated with DTPA (1 mM, 0.6 µL), incubated at room temperature at least for 5 min and then diluted with the HPLC eluent (200 µL) prior to SE-HPLC analysis.

Cell uptake studies

PC3 human prostate cancer cell line was obtained from American Type Culture Collection (ATCC). The cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS) in a humidified atmosphere with 5% CO₂ at 37 °C. ⁶⁴Cu was purchased from Essential Isotopes (Columbia, MO). Hela human cervix adenocarcinoma, HT 29 human colorectal and PC3 human prostate cancer cell lines were obtained from American Type Culture Collection (ATCC) and maintained and grown in exponential growth phase in RPMI-1640 medium (Life Technology), supplemented with 10% FBS, at 37°C and 5% CO₂ incubator. Reaction buffer (0.25 M NH₄OAc, pH 5.5), *N*-NE3TA [10], *N*-NE3TA-CA (cholic acid) [15], or NE3TA-transferrin conjugate (20 µg/ 0.5 –2 µl) and ⁶⁴Cu (60 µCi/3 µl) were sequentially added into a reaction vial for final total volume of 20 µl. The reaction mixture was incubated at room temperature for 30 min with shaking at 1000 rpm, after which 1mM DTPA was added to quench the reaction. 2 µl of the reaction mixture was aliquoted and treated with DTPA (1mM, 0.6 µl) at room temperature for 20 min. Radio-TLC plates were developed with 20mM EDTA/0.15M NH₄OAc, and the radiochemical purity was determined using Bioscan AR-2000 TLC Imaging Scanner. At this reaction condition, the radio-labeling efficiency was 95%. ⁶⁴Cu-labeled Tf conjugate was diluted in RPMI-1640/0.2% bovine serum albumin (BSA) before addition to the cell suspension. An aliquot of ⁶⁴Cu-labeled complex (10 µCi) in 0.1 mL medium was added to 3.0 × 10⁶ PC3 cells in 5 mL of the medium. An aliquot of 0.2 mL of cell suspension was removed at various time points from 5 min to 90 min. Cells were centrifuged and rinsed twice with 2 × 1 ml ice-cold medium. The cell pellets were counted by Wallac Wizard 3" gamma counter to determine the percentage cellular uptake of ⁶⁴Cu-labeled Tf conjugate.

Biodistribution

All animal experiments were performed in compliance with the Guidelines for Care and Use of Research Animals established by the Division of Comparative Medicine and the Animal Studies Committee of Washington University School of Medicine. Female 3–4 weeks old SCID mice were purchased from Charles River Laboratories and housed under pathogen-free conditions in WUSM animal facility. Mice weighing 20–25 g were implanted subcutaneously on the rear right flank with 2.0 × 10⁶ PC-3 cells suspended in 100 µl of saline. The tumors were allowed to grow for 3 weeks after implantation at which time they were about 150–250 mg. *In vivo* biodistribution studies were conducted in the Washington University Small Animal Imaging facility. In tumor bearing mice (n=4 per group), ~8 µCi of ⁶⁴Cu-*N*-NE3TA-Tf conjugate was administered via tail vein. After each time point (1, 4, and 4 h), mice were anesthetized with 1–2% isofluorane/oxygen mixture and sacrificed by cervical dislocation. Tumors and select normal tissues were collected, weighted, and radioactivity counted on a Beckman Gamma 8000 counter containing a NaI crystal (Beckman Instruments, Inc., Irvine, CA). Tumor and normal-tissue uptake were analyzed and measured as percentage injected dose per gram (%ID/g).

Statistical Analysis

Quantitative data were expressed as mean $\pm$ SD. The Student *t*-test was used to determine significance at the 95% confidence level, *P* values of <0.05 were considered significantly different.

Results and Discussion

Synthesis of NE3TA-based bifunctional chelates and NE3TA-transferrin (Tf) conjugates

Introduction of a functional unit to the chelate backbone is known to affect stability and formation rate of metal complexes. [10,17–19] The three different bifunctional ligands *N*-NE3TA, *C*-NE3TA, and *C*-NE3TA-Bn (7-[2-[(carboxymethyl)amino]-2-(phenylmethyl)]-3-(4-nitrophenyl)propyl]-1,4,7-triazacyclononane-1,4-diacetic acid), containing the isothiocyanate (NCS) group for conjugation to transferrin, were prepared and evaluated in an effort to identify the best bifunctional version. *N*-NE3TA-NCS (**3**) and *C*-NE3TA-Bn-NCS (**6**) were synthesized by the same method used for preparation of *C*-NE3TA-NCS [20] (Scheme 1). Removal of *t*-butyl groups in **1** [15] by the treatment of **1** with 4M HCl/1,4-dioxane provided compound **2** which was further reacted with thiophosgene to provide *N*-NE3TA-NCS (**3**) in the isothiocyanate form. *C*-NE3TA-Bn containing the nitro group **4** [16] was subjected to hydrogenation to afford compound **5** containing the amino group which was converted to the NCS group in compound **6** using thiophosgene.

The NE3TA-based bifunctional chelates in the NCS form were conjugated to transferrin (Tf), and concentration of the corresponding transferrin conjugates was quantified by spectrometric method [20,21]. The known macrocyclic chelating agent *C*-DOTA was conjugated to Tf to produce *C*-DOTA-Tf for comparative serum stability studies. The UV-VIS spectrophotometric assay using Cu(II)-arsenazoIII (AAIII) was performed for the determination of the number of NE3TA ligands linked to Tf (L/P ratio). [21] The ligand to protein (L/P) ratio for *N*-NE3TA-Tf (**7**), *C*-NE3TA-Tf (**8**), *C*-NE3TA-Bn-Tf (**9**), and *C*-DOTA-Tf (**10**) was found to be 4.5, 3.1, 6.6, and 2.9, respectively.

Radiolabeling and specific activity of NE3TA-Tf conjugates with ^{64}Cu

The NE3TA-Tf conjugates were evaluated for radiolabeling reaction kinetics with ^{64}Cu at room temperature (Table 1 and Supporting Information). Each conjugate (in 0.25 M NH_4OAc , pH 5.5) was radiolabeled with ^{64}Cu for 30 min at room temperature, and the radiolabeling efficiency (%) was determined using TLC. At the designated time points (1, 10, and 30 min), a sample of the reaction mixture was withdrawn and quenched with DTPA solution (10 mM). Copper-64-radiolabeled conjugates ($R_f = 0.50$) migrated less than unbound ^{64}Cu ($R_f = 0.75$). The *N*-NE3TA-Tf conjugate (**7**) instantly bound to ^{64}Cu , and almost complete radiolabeling of the conjugate was achieved in 1 min after the reaction. *C*-NE3TA-Tf (**8**) and *C*-NE3TA-Bn-Tf (**9**) were relatively slower in binding ^{64}Cu as compared to *N*-NE3TA-Tf (**7**), and the respective radiolabeling efficiency of 99%, 95% and 80% at the 10 min time point was observed with *N*-NE3TA-Tf (**7**), *C*-NE3TA-Tf (**8**) and *C*-NE3TA-Bn-Tf (**9**). *N*-NE3TA-Tf (**7**), *C*-NE3TA-Tf (**8**), and *C*-NE3TA-Bn-Tf (**9**) were labeled with ^{64}Cu for 30 min at specific activities of 2.98, 2.60, and 2.93 (mCi/mg), respectively.

A control experiment was conducted to check if ^{64}Cu was bound to holotransferrin (Tf). A solution of naïve holo-Tf (20 μg) in 0.25 M NH_4OAc buffer (pH 5.5) was mixed with ^{64}Cu (60 μCi), and the resulting mixture was subjected for radiolabeling under the same reaction condition used for testing NE3TA-Tf conjugates for 30 min at room temperature. At the designated time points (1, 10, and 30 min), a sample of the mixture was withdrawn and quenched with DTPA solution (10 mM) and analysed using both radio-TLC and SE-HPLC. The data (Supporting information) clearly indicate that ^{64}Cu was completely complexed with DTPA, and there was almost no bound ^{64}Cu -transferrin present in the reaction mixture as quenched with DTPA.

The more efficient conjugates *N*-NE3TA-Tf (**7**) and *C*-NE3TA-Tf (**8**) were further evaluated for radiolabeling with ^{64}Cu in higher specific activity. A dilute solution of *C*-NE3TA-Tf or *N*-NE3TA-Tf (1 μg) was tested for radiolabeling with ^{64}Cu (30 μCi) at room temperature for 1 h (Supporting Information). *N*-NE3TA-Tf (**7**) and *C*-NE3TA-Tf (**8**) rapidly bound to ^{64}Cu with respective specific activities of 29.2 mCi/mg (2.22 Ci/ μmol) and 28.3 mCi/mg (2.15 Ci/ μmol). As expected from the radiolabeling efficiency data (98.9% and 88.2% at 1 min for *N*-NE3TA-Tf and *C*-NE3TA-Tf, respectively), *N*-NE3TA-Tf (**7**) is slightly more efficient than *C*-NE3TA-Tf (**8**). This result suggests that the position of a functional group in the NE3TA backbone is an important factor affecting radiolabeling efficiency.

Serum stability of ^{64}Cu -radiolabeled NE3TA-Tf conjugates and ^{64}Cu -C-DOTA-Tf

N-NE3TA-Tf (**7**) and *C*-NE3TA-Tf (**8**) conjugates that were efficient in binding ^{64}Cu were further screened for complex stability in human serum and compared with *C*-DOTA-Tf (Figure 2). *In vitro* serum stability was performed to determine if the conjugates radiolabeled with ^{64}Cu remained stable in human serum. Stability was assessed by measuring the transfer of ^{64}Cu from the complex to human serum using HPLC. Copper- ^{64}Cu -radioabeled conjugates were readily prepared from the reactions of the conjugates with ^{64}Cu at room temperature and purified for serum stability studies (pH 7 and 37 $^{\circ}\text{C}$). The ^{64}Cu -radiolabeled complexes of *N*-NE3TA-Tf (**7**) were stable in human serum for 2 days, while a considerable amount of ^{64}Cu (~5%) was released from ^{64}Cu -*C*-NE3TA-Tf (**8**) (Figure 2 and Supporting Information). *N*-NE3TA-Tf (**7**) was shown to have complex stability comparable to *C*-DOTA-Tf. The radiolabeling kinetics and serum stability data indicate that among three NE3TA analogues tested, *N*-NE3TA is the most effective chelating agent of ^{64}Cu .

In vitro cellular uptake assay

N-NE3TA-Tf (**7**) and *C*-NE3TA-Tf (**8**) were evaluated for binding affinity to TfR using PC-3 cancer cells with elevated expression of TfR. The bifunctional chelate *N*-NE3TA in the nitro form [10] and *N*-NE3TA-CA (cholic acid) [15] containing a cholic acid moiety targeting a bile acid receptor were used as negative controls. Aliquots of 3×10^6 cells of PC-3 cells were incubated with ^{64}Cu -radiolabeled Tf conjugate (10 μCi) at 37 $^{\circ}\text{C}$, and the radioactivity bound to the cells was measured at designated time points. As shown in Figure 3, binding of ^{64}Cu -*N*-NE3TA-Tf and ^{64}Cu -*C*-NE3TA-Tf to the tumor cells was rapid and increased up to 30 min of incubation. ^{64}Cu -*N*-NE3TA-Tf had higher uptake in PC3 cells than ^{64}Cu -*C*-NE3TA-Tf. Both *N*-NE3TA-CA and *N*-NE3TA used as the negative controls displayed very low binding to PC3 cells.

Biodistribution study

Biodistribution studies were conducted to investigate the tumor and normal tissue uptake of ^{64}Cu -*N*-NE3TA-Tf in SCID mice bearing PC-3 xenografts. As presented in Figure 4, accumulation of ^{64}Cu -*N*-NE3TA-Tf in peripheral tissues like lung, liver, spleen and kidney was relatively high. The radioactivity in lung, liver, and kidney was shown to gradually decrease over the time points (11.22% ID/g, 11.54% ID/g, and 6.72% ID/g at 24 h position injection, respectively). Uptake of the tracer in muscle, bone, and pancreas remained low at all time points (24 h post injection, 3.03%, 2.90%, 4.00% ID/g, respectively). The tumor uptake increased in a sequential manner (Figure 4a), where the accumulation was 1.4 ± 0.4 %ID/g, 3.8 ± 0.2 %ID/g and 10.0 ± 1.5 %ID/g for 1, 4 and 24 h post-injection, respectively. As shown in Figure 4(b), the blood concentration decreased in a corresponding manner. All tumor and blood concentrations measured at 1, 4 and 24 h were observed to be statistically different with $p < 0.05$. With the continuing trend of decreasing blood and increasing tumor uptake values (Figure 4c), perhaps a longer evaluation time point, such as 48 h, might be beneficial to determine the maximum tumor: blood ratio.

Conclusion

Bifunctional NE3TA-based chelating agents were synthesized and conjugated to transferrin (Tf) as a tumor targeting vector. The corresponding NE3TA-Tf conjugates were evaluated for the effect of the functional unit in the chelates on complexation with ^{64}Cu . Among the three NE3TA-Tf conjugates tested, *N*-NE3TA-Tf conjugate was found to be the most effective in binding ^{64}Cu . Radiolabeling of *N*-NE3TA-Tf with ^{64}Cu was achieved with a high specific activity and extremely fast kinetics at room temperature. The lead ^{64}Cu -*N*-NE3TA-Tf conjugate displayed rapid blood clearance and increased tumor uptake in prostate tumor-bearing mice over 24 h. The *in vitro* and *in vivo* data suggest that the bifunctional chelate *N*-NE3TA can be further evaluated for efficient ^{64}Cu radiolabeling of tumor targeting antibodies or peptides for potential use in PET imaging.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgement

This work was *partly* supported by the National Institutes of Health (R01 CA136695). The authors would like to thank the small animal imaging facility at Washington University School of Medicine for technical assistance in conducting biodistribution studies. We would also like to acknowledge the Isotope Production Group at Washington University for production of ^{64}Cu . The authors acknowledge Jesse J. Parry and Stephanie Krieger for providing technical assistance with cell lines and xenografted mice, as well as Donald Connor for the preparation of graphic artwork. We also thank the Department of Veterans Affairs, for providing resources and use of facilities at the Harry S. Truman Memorial Veterans' Hospital in Columbia, MO.

Abbreviations

PET	positron emission tomography
FDG	2-Fluoro-2-deoxy-glucose

Tf	Transferrin
DOTA	1,4,7,10-tetraazacyclododecane- <i>N,N',N'',N'''</i> -tetraacetic acid
TETA	1,4,8,11-tetraazacyclotetradecane- <i>N,N',N'',N'''</i> -tetraacetic acid
CB-TE2A	1,4,8,11-tetraazabicyclo[6.6.2]hexadecane-4,11-diacetic acid
NE3TA	7-[2-[carboxymethyl]amino]ethyl]-1,4,7-triazacyclononane-1,4-diacetic acid
N-NE3TA	7-[2-[(carboxymethyl)amino]-2-(4-nitrophenyl)methyl]-1,4,7-triazacyclononane-1,4-diacetic acid
C-NE3TA	7-[2-[carboxymethyl]amino]-3-(4-nitrophenyl)propyl]-1,4,7-triazacyclononane-1,4-diacetic acid
C-NE3TA-Bn	7-[2-[(carboxymethyl)amino]-2-(phenylmethyl)]-3-(4-nitrophenyl)propyl]-1,4,7-triazacyclononane-1,4-diacetic acid
DTPA	diethylenetriamine pentaacetic acid
SCID	severe combined immunodeficient
AAIII	2,2-(1,8-dihydroxy-3,6-disulfonaphthylene-2,7-bisazo) bis-benzenearsonic acid
HEPES	N-(2-hydroethyl)-piperazine- <i>N'</i> -ethanesulfonic acid
FBS	fetal bovine serum
BSA	bovine serum albumin

References

- Gambhir SS. Nat. Rev. Cancer. 2002; 2:683–693. [PubMed: 12209157]
- Rohren EM, Turkington TG, Coleman RE. Radiology. 2004; 231:305–332. [PubMed: 15044750]
- Cherry SR, Clin J. Pharmacol. 2001; 41:482–491.
- Shokeen M, Anderson CJ. Acc. Chem. Res. 2009; 42:832–841. [PubMed: 19530674]
- Wadas TJ, Wong EH, Weisman GR, Anderson CJ. Chem. Rev. 2010; 110:2858–2902. [PubMed: 20415480]
- Smith SV, Inorg J. Biochem. 2004; 98:1874–1901.
- Bass LA, Wang M, Welch MJ, Anderson CJ. Bioconjugate Chem. 2000; 11:527–532.
- Sun X, Wuest M, Weisman GR, Wong EH, Reed DP, Boswell CA, Motekaitis R, Martell AE, Welch MJ, Anderson CJ, Med J. Chem. 2002; 45:469–477.
- Boswell CA, Sun X, Niu W, Weisman GR, Wong EH, Rheingold AL, Anderson CJ. J. Med. Chem. 2004; 47:1465–1474. [PubMed: 14998334]
- De Silva RA, Jain S, Lears KA, Chong H-S, Kang CS, Sun X, Rogers BE. Nucl. Med. Biol. 2012; 39:1099–1104. [PubMed: 22743158]
- Chong H-S, Mhaske S, Lin M, Bhuniya S, Song HA, Brechbiel MW, Sun X. Bioorg. Med. Chem. Lett. 2007; 17:6107–6110. [PubMed: 17911020]
- Inoue T, Cavanaugh PG, Steck PA, Brünner N, Nicolson GA. J. Cell. Physiol. 1993; 156:212–217. [PubMed: 8314858]
- Qian GM, Li H, Sun H, Ho K. Pharmacol. Rev. 2002; 54:561–587. [PubMed: 12429868]
- Thorstensen K, Romslo I. Biochem. J. 1990; 271:1–9. [PubMed: 2222403]

15. Chappell LL, Ma D, Milenic DE, Garmestani K, Venditto V, Beitzel MP, Brechbiel MW. Nucl. Med. Biol. 2003; 30:581–595. [PubMed: 12900284]
16. Woods M, Kovacs Z, Kiraly R, Brucher E, Zhang S, Sherry AD. Inorg. Chem. 2004; 43:2845–2851. [PubMed: 15106971]
17. Cole WC, DeNardo SJ, Meares CF, McCall MJ, DeNardo GL, Epstein AL, O'Brien HA, Moi MK. Nucl. Med. Biol. 1986; 13:363–368.
18. Chong H-S, Song HA, Ma X, Milenic DE, Brady ED, Lim S, Lee H, Baidoo K, Cheng D, Brechbiel MW. Bioconjugate Chem. 2008; 19:1439–1447.
19. Chong H-S, Chen Y, Kang CS, Sun X, Wu N. Bioorg. Med. Chem. Lett. 2015; 25:1082–1085. [PubMed: 25661712]
20. Chong H-S, Sun X, Dong P, Kang CS. Eur. J. Org. Chem. 2011; 33:6641–6648.
21. Song HA, Kang CS, Baidoo KE, Milenic DE, Chen Y, Dai A, Brechbiel MW, Chong H-S. Bioconjugate Chem. 2011; 22:1128–1135.

Highlights

- Synthesis of three chelating agents based on NE3TA (7-[2-[carboxymethyl]amino]ethyl)-1,4,7-triazacyclononane-1,4-diacetic acid) for potential use in Cu-64-PET imaging.
- Rapid Cu-64 radiolabeling of NE3TA-transferrin (Tf) conjugates at room temperature.
- Excellent complex stability of ^{64}Cu -NE3TA-Tf conjugates in human serum.
- Rapid blood clearance and increasing tumor uptake of ^{64}Cu -N-NE3TA-Tf in tumor-bearing mice.

Figure 1.
Structure of NE3TA and bifunctional NE3TA ligands for targeted PET imaging application

Figure 2.

(a) HPLC chromatograms of ^{64}Cu -radiolabeled transferrin conjugates in human serum at 0 h. (b) HPLC chromatogram of ^{64}Cu -radiolabeled complexes in human serum at 48 h.

Figure 3. Binding affinity of ^{64}Cu -N-NE3TA-Tf (7) and ^{64}Cu -C-NE3TA-Tf (8) to PC3 cancer cells. N-NE3TA-CA (cholic acid) conjugate and N-NE3TA used as negative controls.

Figure 4. Biodistribution of ^{64}Cu -radiolabeled *N*-NE3TA-Tf (7) in SCID mice with PC-3 tumors. (a) Tumor and (b) blood uptake of ^{64}Cu -*N*-NE3TA-Tf at 1, 4, and 24 h post-injection in female SCID mice with PC-3 xenografts; (c) Tumor:blood uptake ratio in a XY plot.

Scheme 1.
Synthesis of bifunctional NE3TA ligands

Table 1

Evaluation of NE3TA-transferrin (Tf) conjugates for radiolabeling efficiency (%) with ^{64}Cu (RT, 0.25M NH_4OAc , pH 5.5, TLC).

Time	N-NE3TA-Tf (7)	C-NE3TA-Tf (8)	C-NE3TA-Bn-Tf (9)
1 min	98.9 $\pm$ 0.1	88.2 $\pm$ 1.7	67.4 $\pm$ 1.9
10 min	99.3 $\pm$ 0.2	94.5 $\pm$ 1.1	79.6 $\pm$ 1.5
30 min	99.3 $\pm$ 0.4	97.6 $\pm$ 0.4	86.7 $\pm$ 1.8