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Synthesis and radiolabeling of chelator–RNA aptamer bioconjugates with copper-64 for targeted molecular imaging

William M. Rockey [b], Ling Huang [e], Kyle C. Kloepping [b,c], Nicholas J. Baumhover [b], Paloma H. Giangrande [b,d,*], Michael K. Schultz [a,b,c,*]

[a] Department of Radiology, University of Iowa, 500 Newton Road, ML B180, Iowa City, IA 52242, United States
[b] Department of Radiation Oncology, University of Iowa, Iowa City, IA 52242, United States
[c] Free Radical Radiation Biology Program, University of Iowa, Iowa City, IA 52242, United States
[d] Department of Internal Medicine, University of Iowa, 375 Newton Road, 5202 MERF, Iowa City, IA 52242, United States
[e] Integrated DNA Technologies, Inc., Coralville, IA 52241, United States

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ABSTRACT

Ribonucleic acid (RNA) aptamers with high affinity and specificity for cancer-specific cell-surface antigens are promising reagents for targeted molecular imaging of cancer using positron emission tomography (PET). For this application, aptamers must be conjugated to chelators capable of coordinating PET-radionuclides (e.g., copper-64, ^{64}Cu) to enable radiolabeling for in vivo imaging of tumors. This study investigates the choice of chelator and radiolabeling parameters such as pH and temperature for the development of ^{64}Cu-labeled RNA-based targeted agents for PET imaging. The characterization and optimization of labeling conditions are described for four chelator–aptamer complexes. Three commercially available bifunctional macrocyclic chelators (1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid mono *N*-hydroxysuccinimide [DOTA-NHS]; *S*-2-(4-isothiocyanatobenzyl)-1,4,7-triazacyclononane-1,4,7-triacetic acid [*p*-SCN-Bn-NOTA]; and *p*-SCN-Bn-3,6,9,15-tetraazabicyclo [9.3.1]pentadeca-1(15),-11,13-triene-3,6,9-triacetic acid [*p*-SCN-Bn-PCTA]), as well as the polyamino-macrocyclic diAmSar (3,6,10,13,16,19-hexaazabicyclo[6.6.6] icosane-1,8-diamine) were conjugated to A10-3.2, a RNA aptamer which has been shown to bind specifically to a prostate cancer-specific cell-surface antigen (PSMA). Although a commercial bifunctional version of diAmSar was not available, RNA conjugation with this chelator was achieved in a two-step reaction by the addition of a disuccinimidyl suberate linker. Radiolabeling parameters (e.g., pH, temperature, and time) for each chelator–RNA conjugate were assessed in order to optimize specific activity and RNA stability. Furthermore, the radiolabeled chelator-coupled RNA aptamers were evaluated for binding specificity to their target antigen. In summary, key parameters were established for optimal radiolabeling of RNA aptamers for eventual PET imaging with ^{64}Cu.

1. Introduction

Ribonucleic acid (RNA) aptamers are single-stranded oligonucleotides that bind with high affinity and specificity to targets such as cancer-specific cell-surface antigens.[1–5] This class of biological agents represents an attractive candidate for targeted molecular imaging due to rapid blood clearance, target specificity, and low immunogenicity.[2,5,6] The polyanionic properties of aptamers along with their small size (~15 kDa), favor short circulating plasma half-lives and high permeability in solid tumors, thereby facilitating the delivery of radionuclides for imaging and potentially cancer therapy.[3,4,7] In addition, RNA aptamers are readily chemically synthesized. Chemical synthesis facilitates the inclusion of (1) modified nucleotides (e.g., 2′-fluoro and 2′-*O*-methyl-modified bases), which impart resistance to degradation by in vivo nucleases; and (2) reactive chemical groups (e.g., terminal amines) to allow coupling to chelators for radiolabeling applications.[4,7,8]

Previous work has demonstrated the feasibility of using RNA aptamers for targeted molecular imaging applications using the gamma emitters technetium-99m (^{99m}Tc) and indium-111 (^{111}In) for single photon emission computed tomography (SPECT).[3,4,7] While the results of these studies are encouraging, the radiolabeling of bioconjugates with these gamma-emitting radionuclides generally requires conditions that are problematic for use with RNA aptamers. For example, efficient radiolabeling with ^{111}In and ^{99m}Tc generally requires higher temperatures (>90 °C) and subphysiologic pH conditions (<5 for ^{111}In) at which RNA is chemically unstable. More recently, the spatial resolution and image contrast advantages of positron emission tomography (PET),[9,10] have

* Corresponding authors. Tel.: +1 (319) 384 3242 (P.H.G.), +1 (319) 335 8017 (M.K.S.).
E-mail addresses: paloma-giangrande@uiowa.edu (P.H. Giangrande), michael-schultz@uiowa.edu (M.K. Schultz).

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prompted interest in radiolabeling of aptamers with positron-emitting radionuclides (e.g., copper-64 [^{64}Cu]; fluorine-18, [^{18}F]; gallium-68 [^{68}Ga]; yttrium-86 [^{86}Y]) used in positron emission tomography (PET). While successful radiolabeling of oligonucleotide compounds has been reported with ^{18}F, ^{68}Ga, and ^{86}Y, synthetic strategies and relatively harsh conditions (high temperatures and sub-physiological pH) required for efficient labeling can degrade RNA. For example, radiosynthesis of ^{18}F-labeled compounds requires an on-site cyclotron, as well as specialized radiosynthesis apparatus and shielding for safe and efficient labeling.[4,10,11] While ^{68}Ga has advantages for radiolabeling of small peptides, in our hands reactions carried out under optimum conditions for radiolabeling (>95C, pH 3–4) result in severe degradation to the RNA backbone (data not shown).[12–16] Similarly, radiolabeling of biomolecules with positron emitter yttrium-86 (^{86}Y) requires temperatures in excess of 90 °C for efficient integration of the radiometal in the chelator.[15] While room temperature ^{68}Ga DOTA-peptide labeling has been reported, the optimum pH for these reactions is 3.5, which is corrosive to RNA.[17] In addition, recent results demonstrate that mild temperatures (20–23 °C) can be effective for ^{64}Cu labeling dependent upon the chelator employed.[18] Thus, there is increasing interest in the use of ^{64}Cu for oligonucleotide-based molecular imaging applications. Toward this end, radiolabeling of siRNA with the positron-emitting radionuclide ^{64}Cu was recently described for high-resolution in vivo PET imaging of RNA interference (RNAi).[11,19] The sum of these observations prompted us to examine the use of ^{64}Cu as a candidate for radiolabeling an RNA aptamer for molecular imaging.

^{64}Cu ($t_{1/2}$ = 12.7 h) exhibits both positron emission (β^+: 17.4%, $E_{\beta max}$ = 656 keV) and β-minus decay (β^-: 39%, $E_{\beta max}$ = 573 keV) and can be produced with high specific activity using a medical cyclotron.[20–22] The half-life of ^{64}Cu enables the use of this radionuclide not only with targeting vectors having rapid pharmacokinetics such as low molecular weight (MW) peptides (MW <5 kDa) and aptamers (MW 8–25 kDa), but also with higher MW molecules, such as antibodies (MW 100–150 kDa).[11,23–25] In addition, a half life of 12 h enables centralized production and purification for shipment to other institutions for research and clinical studies. Further, in the current study we show that unlike other positron-emitters (e.g., ^{68}Ga, ^{86}Y),[14,16] optimal labeling with ^{64}Cu can be achieved under the same physical conditions (i.e., pH and temperature) at which RNA is maximally stable (i.e., mild temperatures, aqueous conditions, and neutral pH).[20–22] Based on these properties, ^{64}Cu is an attractive radionuclide for high-resolution, targeted RNA aptamer-based PET imaging agents. While numerous studies describing the use of ^{64}Cu for radiolabeling and molecular imaging of bioactive molecules are available,[20,22,23,26,27] few studies have examined systematically the conditions under which optimum radiolabeling of RNA aptamers can be achieved.

In order to radiolabel a single-stranded RNA oligonucleotide (aptamer) with ^{64}Cu, the aptamer must be modified to include a chelator moiety (Fig. 1). Also, radiolabeling should occur under sufficiently mild conditions to ensure that the aptamer is not degraded during the labeling process. Within this context, four chelator-modified RNA aptamer bioconjugates were prepared to examine effective synthesis strategies and to evaluate the efficiency of radiolabeling with ^{64}Cu. Aqueous synthetic approaches are presented for addition of these chelators to a 5′-amine-modified (12 carbon linker) RNA aptamer (A10-3.2) that binds prostate specific membrane antigen (PSMA) on prostate cancer cells.[2,28]

Three chelators used in these preparations are commercially available in bifunctional forms (i.e., possessing a reactive group to facilitate conjugation): (1) 1,4,7,10-tetraazacyclododecane tetraacetic acid (DOTA) added via the NHS ester; (2) 1,4,7-triazacyclononane triacetic acid (NOTA) added via the *p*-Bn-SCN linker group; and (3) 3,6,9,15-tetraazabicyclo [9.3.1]pentadeca-1(15),11,13-triene-3,6,9-triacetic acid (PCTA), also added via the *p*-Bn-SCN linker group (Fig. 1; **1**,**2**,**3**). The fourth bifunctional chelator, 3,6,10,13,16,19-hexaazabicyclo[6.6.6]icosane-1,8-diamine (diAmSar) (Fig. 1; **4**), was prepared and conjugated to the RNA via a disuccinimidyl suberate linker to allow conjugation to the RNA in a two-step process. Chelator moieties were chosen based on (1) effectiveness of ^{64}Cu complexation; (2) availability of bifunctional versions of the chelator; or (3) the ability to easily adapt the chelator for conjugation if a bifunctional version was not available commercially (as was the case for diAmSar).[26,27,29,30] Additional ^{64}Cu chelators are available and are the subject of further research.[20,21,31–33]

To illustrate a general approach for effective radiolabeling of RNA aptamers with ^{64}Cu, radiolabeling conditions (pH, temperature, and time) were examined for aptamer bioconjugates with the goal of optimizing achievable radiochemical purity and specific activity. The stability of these conjugates in buffer was examined over a 24-h period. The inertness of the radiometal–chelator couplings and the potential for loosely bound ^{64}Cu associated with the RNA backbone were examined by challenge experiments using increasing concentrations of ethylenediaminetetraacetic acid (EDTA) up to 100 mM. Finally, the effects of chelator addition and radiolabeling on the in vitro cell-binding characteristics of the ^{64}Cu-labeled RNA aptamers to prostate cancer cells were studied. In summary, these studies serve to identify optimal chelators and radiolabeling conditions for the future development of effective targeted RNA-based PET molecular imaging agents.

2. Materials and methods

2.1. Chemicals and reagents

All solvents and reagents were used as received unless otherwise noted. RNA aptamers were purchased in desalted, HPLC purified form (Integrated DNA Technologies Inc., Coralville, IA, USA). The RNA molecular targeting sequence for A10-3.2 has been

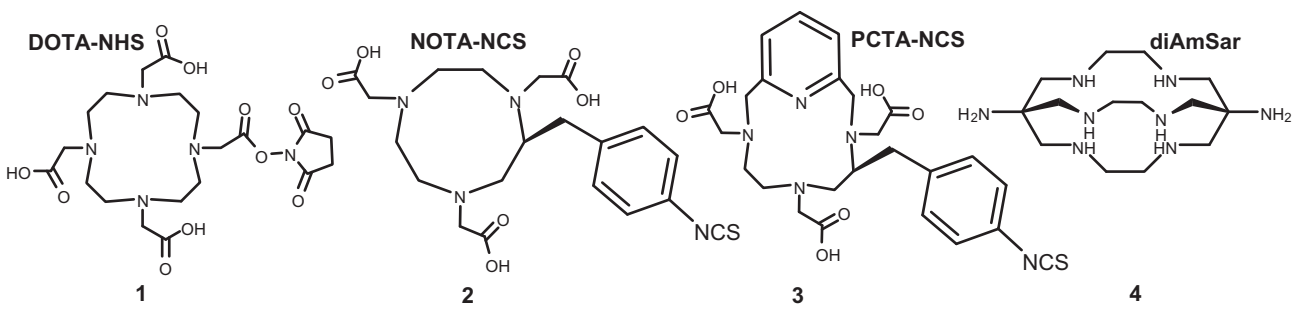

Figure 1. Chelators employed in this study: (1) DOTA-NHS; (2) NOTA-NCS; (3) PCTA-NCS; and (4) diAmSar. The chelators were conjugated to the A10-3.2 PSMA-specific RNA aptamer.

previously described and was modified to include a primary amine group with a 12-carbon linker conjugated to the 5′-end of the RNA aptamer by a phosphoramidite bond.[2,28] The macrocyclic ligand diAmSar, as well as the bifunctional chelators *para*-isothiocyanato-benzyl-NOTA (*p*-SCN-Bn-triNOTA); *p*-SCN-Bn- PCTA; and 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid mono (*N*-hydroxysuccinimide) ester (DOTA-NHS) were acquired from Macrocyclics Inc. (Dallas, TX, USA). Copper-64 was obtained as $^{64}CuCl_2$ solution from the College of Medicine at Washington University in Saint Louis (Missouri, USA).

Unless described otherwise, solutions were prepared using ultrahigh-purity water and chemicals with metal concentrations certified to <20 parts per trillion (Baseline®, Seastar Chemicals, British Columbia, Canada). Alternatively, a 0.22 μm filtered deionized 18 MΩ milliQ Advantage purification system (Millipore, Billerica, MA, USA) was used to obtain highly pure water. Hydrochloric acid and acetic acid used for buffer preparation were also Baseline® grade (Seastar Chemicals). Aptamer bioconjugates were desalted by spin-filtration using 10 kDa molecular weight cutoff (MWCO) Amicon centrifugal filters (Millipore). The pH of all solutions was confirmed with a Horiba Compact pH meter (model B-213; Horiba Scientific Instruments, Ann Arbor, MI, USA), calibrated daily at pH 4.0 and pH 7.0 using standard buffers or colorpHast pH paper (0–14 range; Merck KGaA, Darmstadt, De).

2.2. Synthesis and confirmation of RNA conjugates

Conjugation reactions of the RNA aptamer (A10-3.2) to the various chelators were carried out in plastic 1.5-mL snap-cap vials (USA Scientific, Orlando, FL, USA). Small molecule chemistry was carried out in glass flasks and vessels, which were acid washed in dilute high-purity hydrochloric acid (HCl, Baseline®) to remove trace metal impurities, rinsed in high-purity water, and dried in an oven prior to use. Unless otherwise stated, all conjugation experiments were conducted in triplicate, and the uncertainty associated with results are based on the standard deviation of the three observations. Reaction yields of chelator-modified RNA aptamers were estimated by UV spectrophotometry using a DU800 Spectrophotometer (Beckman Coulter), and the extinction co-efficient for the RNA oligonucleotide supplied by IDT. Reaction conditions to synthesize the different chelator-RNA conjugates are outlined below:

2.2.1. DOTA-RNA (6)

Amide bond formation via the 5′-primary amine was carried out using the bifunctional compound DOTA-NHS (Supplementary Fig. 1A). The most effective strategy for this conjugation step involved the use of a 50-eq excess of the *N*-hydroxysuccinimidal macrocyclic chelator (12.5 μmol). DOTA-NHS was dissolved in a 100 μL solution of 1 M sodium bicarbonate ($NaHCO_3$, Puratronic®), which had been pre-adjusted to pH 8.5–9 with dilute high-purity hydrochloric acid. The solution was cooled to 4 °C in the refrigerator. A 1 mM aliquot of 5′-amine-modified RNA aptamer in 250 μL of Baseline® water stored at −80 °C was allowed to warm to 4 °C in a temperature-controlled reaction block over 2 h prior to the reaction. The bifunctional chelator and RNA solutions were mixed, resulting in an approximate final pH of 8 (estimated by sacrificing approximately 20 μL for pH analysis using colorpHast® pH paper strips (0–14 range, EM Science, Gibbstown, NJ, USA). The reaction mixture was allowed to incubate at 4 °C overnight (~16 h) and was desalted by spin-filter dialysis using a 10 kDa molecular-weight cutoff Amicon centrifugal filter following the manufacturer's recommendations. The resulting RNA template was diluted in 200 μL ultrapure water and purified by peak collection using the semi-preparatory HPLC system described below. Collected fractions were frozen at −80 °C, lyophilized overnight, dissolved in ultrapure water, and stored at −20 °C or −80 °C. For the purposes of this investigation, the chemical yield was assessed by UV spectrophotometry and chemical purity by HPLC. The identity of the conjugate was confirmed by comparison of the theoretical mass to that observed by electrospray ionization mass spectrometry (ESI): the theoretical mass of DOTA-RNA was 13513.51 amu, and the observed M was 13513.4 and 13513.6 amu (2 preparations) (Supplementary Fig. 1A).

2.2.2. NOTA-RNA (10)- and PCTA-RNA (7)

Thiourea bond formation via the 5′-primary amine was carried out using the isothiocyanato-functionalized bifunctional chelators NOTA and PCTA as described previously (Supplementary Fig. 1B).[19,27] Briefly, between 100 and 400 nmol of the 5′-primary amine-modified RNA was dissolved in 250 μL of 1 M $NaHCO_3$ for a final pH of approximately 8 (confirmed by colorpHast pH paper). Fifty equivalents of bifunctional chelator starting material (5–20 μmol) were dissolved in 150 μL of Baseline® water and the solutions were combined. In the case of the NOTA conjugation, a precipitate formed after heating for about 30 min. One milliliter of Baseline® water was added to the reaction mixture, and the precipitate dissolved. In all cases, the reaction mixture was incubated at 37 °C and the reaction progress was monitored by HPLC on an hourly basis. Reactions were complete in about 5 h. In the case of the NOTA reaction, a second aliquot containing 50 equiv of the bifunctional chelator was added to the reaction mixture after approximately 4 h, which appeared to drive the conversion of the remaining amine-modified RNA to the desired product completely. When UV-HPLC analysis indicated that the starting material had been depleted to undetectable or nearly undetectable amounts, the reaction mixture was dialyzed by spin filter as described above and used without further purification. Identity was confirmed by ESI as with other chelator-aptamer conjugates described here. The theoretical mass of NOTA-RNA was 13578.8 amu, and the observed *M* was 13578.1 amu and 13579.1 amu (2 preparations) (Supplementary Fig. 1B). The theoretical mass of PCTA-RNA was 13655.4 amu, and the observed *M* was 13,656.9 amu and 13,656.3 amu (2 preparations) (Supplementary Fig. 1C).

2.2.3. DiAmSar-RNA (9)

DiAmSar starting material was conjugated to the RNA using a two-step synthetic strategy via a disuccinimidyl suberate (bis(2,5-dioxopyrrolidin-1-yl) octanedioate) (DSS) linker (Pierce, Thermo Scientific, Rockford, IL, USA). Reactions were carried out with aqueous reagents either pre-cooled overnight to 4 °C or equilibrated at room temperature (Supplementary Fig. 1D).

2.2.3.1. Step 1. In a typical preparation, 8 mg of the DSS was dissolved in 864 μL DMSO to a concentration of 25 mM. A 220 μL aliquot of this solution (50 equiv relative to RNA) was added to 62.5 nmol of 5′-amine-modified RNA aptamer dissolved in 300 μL of 0.1 M $NaHCO_3$ (Puratronic® $NaHCO_3$ in Baseline™ water), which had been pre-adjusted to pH 8.5–9. The DSS and RNA solutions were mixed, resulting in a final pH of approximately 8 (estimated using colorpHast® pH paper strips, 0–14 range), and the reactants were agitated continuously for 30 min. At the end of 30 min, the reaction mixture was diluted to 4 mL with high-purity water (pre-cooled to 4 °C) and excess DSS was removed by spin-filter dialysis. The dilution and spin-filter dialysis steps were repeated a total of three times resulting in a retentate volume of approximately 150 μL containing the DSS-activated RNA.

2.2.3.2. Step 2. A 50 equiv excess (31.25 μmol) of diAmSar dissolved in 450 μL of 0.1 M $NaHCO_3$, adjusted to pH 8 with high-purity 1 M HCl, was added to the DSS-activated RNA intermediate solution at 4 °C.

Step 2 reactions were conducted overnight at 4 °C or room temperature. The reaction mixture was then desalted by spin-filter dialysis and HPLC purified using a Clarity column (see below). Target retention time was approximately 8.3 min. Collected peaks were desalted by spin filter, lyophilized, dissolved in ultrapure water, and stored at −20 °C or −80 °C. For the purposes of this investigation, the chemical yield was assessed by UV spectrophotometry and chemical purity by HPLC. The identity of the conjugate was confirmed by comparison of the theoretical mass to that observed by electrospray ionization mass spectrometry (ESI). The theoretical mass of diAmSar-RNA was 13,580.45 amu, and the observed mass ***M*** was 13,578.3 amu and 13,580.7 amu (2 preparations) (Supplementary Fig. 1D).

2.2.6. HPLC analysis

Analytical and semi-preparatory high performance liquid chromatography (HPLC) was carried out by reverse phase methods using an Agilent 1200 series HPLC (Agilent Technologies, Santa Clara, CA, USA), equipped with Clarity 5 μm Oligo-RP C18 50 × 4.6 mm columns. Solvent (A) was 100 mM triethylamine acetate, and solvent (B) was acetonitrile. The gradient was 5% B to 30% B over 15 min at a rate of 1 mL min^{-1}. Alternatively, the same solvents and gradient were used with a Zorbax 300SB C-18 HPLC column by Agilent (5 μm and 9.4 × 250 mm). Synthesis and purity of chelator-modified aptamer bioconjugates was further confirmed by electrospray ionization mass spectrometry (ESI), using a Paradigm MS4 HPLC with UV detector (Michrom BioResources, Auburn, CA) and Finnigan TSQ7000 LC/MS system (Thermo Electron, San Jose, CA) operated in negative mode using neutral solutions at Integrated DNA Technologies, Inc. (Coralville, IA, USA). The system is equipped with Xcalibur version 1.2 data system (Thermo Electron) for instrument control and ProMass data processing software (Novacia LLC). Purity of prepared bioconjugates was confirmed by comparison of the theoretical mass of the pure compound and the observed value (reported as *M*, which corresponds to the observed mass). Variation of approximately three amu is expected in the molecular weight range (∼14 kDa) for the ESI technique employed for determinations presented here.

The identity of radiolabeled RNA aptamers was confirmed by comparing radio-HPLC retention times to UV absorption traces of the unlabeled species at wavelengths of 260 nm and 280 nm (Supplementary Figs. 2 and 3). The retention time of unlabeled ('free') ^{64}Cu was determined independently to be approximately 1 min using the above gradient conditions. Radioactivity detection for in-line radio-HPLC was carried out using an in-line liquid scintillation counting (LSC) system (β-RAM, IN/US Systems, Tampa, FL, USA) equipped with a 20 μL internal mixing loop and a 4:1 LSC-cocktail:mobile-phase mixing ratio. Liquid scintillation cocktail used was In-Flow™ 2:1 (IN/US). Radio-HPLC traces were collected using the software program ScintFlow® (IN/US), which integrates direct data collection via Microsoft Excel®.

In addition to using radio-HPLC as described above, the radiochemical purity and stability of ^{64}Cu-labeled chelator-aptamer bioconjugates were also determined by instant thin-layer chromatography (ITLC) using ITLC-SG glass microfiber chromatography paper impregnated with silica gel (Varian, Lake Forest, CA, USA) with a mobile phase of 0.1 M citric acid (Supplementary Figs. 4–6). ITLC strips were cut to 1 cm width × 10 cm height and heated at 110 °C in a conventional laboratory oven for at least 1 h prior to experimental determinations and used within 30 min of removal from the oven.

For the ITLC analyses, two microliters of sample was spotted on the strip origin. Strips were then immersed in mobile phase to just below the spot origin in 50 mL plastic centrifuge tubes, capped, and the mobile phase was allowed to migrate for 3 min, after which the strips were dried with a hairdryer. The ratio of free ^{64}Cu to the radiolabeled chelator-aptamer bioconjugate was determined through exposure of the ITLC strips to a storage phosphor screen (Fujifilm, Minamiashigara, Kanagawa, Japan) and subsequent imaging with a Typhoon™ FLA 7000 phosphorimager (GE Healthcare Bio-sciences AB, Uppsala, Sweden). Data analysis was performed using ImageQuant™ Analysis Toolbox software (TL 7.0, GE Healthcare Bio-sciences AB, Uppsala, Sweden). The percent radiolabeling (radiochemical purity) of chelator-conjugated aptamers was calculated by dividing the integrated signal intensity over the bottom half of the ITLC strips by the total integrated signal intensity over the entire length of the ITLC strips (Supplementary Figs. 4–8).

2.3. Radiochemistry

An examination of the effects of pH, reaction temperature, and reaction time on the formation of ^{64}Cu-chelator-modified bioconjugate complexes was undertaken in sodium acetate/acetic acid buffers. Reactions to examine pH and temperature effects were carried out for 30 min unless otherwise stated. Sodium acetate was obtained as a 3 M high-purity (99.9%, metals grade) solution (Sigma–Aldrich, USA). Acetic acid was ultrahigh-purity (Baseline®). For each bioconjugate, the effect of pH on chelator-modified RNA ^{64}Cu complex formation was first examined by reactions of ^{64}Cu in acetate buffers at pH 4, 5, 6, 7, and 8 (ionic strength ∼0.1 M) (Supplementary Fig. 4). The effect of pH was examined at a mid-range temperature of 50 °C. The reaction was performed in a shaker table with mild agitation.

Following examination of the effect of pH on labeling efficiency for each chelator-modified bioconjugate, the effect of temperature was examined at 5 °C, 20 °C, 40 °C, 60 °C, and 85 °C (Supplementary Fig. 5). The pH chosen for these experiments was in part determined by the experiments described above, which describe the effect of pH on radiolabeling efficiency of the RNA aptamer conjugates. An additional criterion that guided our decision was an a priori expectation that neutral pH is optimum for RNA folding prior to in vitro binding assays or in vivo use. This expectation is based on our previous work in the development of aptamers for other in vivo applications.[2,28] As the results of our investigation of pH for DOTA-, NOTA-, and PCTA-RNA conjugates revealed that radiochemical purity of greater than 96% could be achieved at pH 7 in 30 min at 50 °C, pH 7 was chosen to examine temperature effects for the DOTA-, NOTA-, and PCTA-RNA conjugates. On the other hand, radiolabeling yields for diAmSar-RNA conjugates were low at neutral pH and rose sharply as pH decreased, with a maximum value of 99% radiochemical purity at pH 4. Thus, pH 4 was chosen to examine the effect of temperature for the diAmSar-RNA conjugate. Aliquots employed for the 5 °C experiments were pre-cooled in the refrigerator (∼4 °C) for about 30 min prior to addition of ^{64}Cu. All other samples were held at room temperature, spiked with ^{64}Cu, and then transferred to a temperature-controlled shaker table and incubated with heating and mild agitation.

Measurement of radiolabeling kinetics (i.e., reaction time) was performed by radiolabeling with both 37 MBq and 75 MBq of activity at the optimal pH and temperature conditions as determined using the above methodology (pH 7 for DOTA-, NOTA-, and PCTA-RNA and pH 4 for DiAmSar-RNA and at a temperature of 60 °C for all conjugates based on pH and temperature experiments presented above). The labeling reactions were allowed to proceed at intervals ranging from 5 min to 120 min (Supplementary Fig. 6).

For each test condition (i.e., pH, temperature, or reaction time), between 1.5 and 2 nmol of chelator-modified RNA conjugate were added to 50 μL of acetate buffer (100 mM ionic strength) at the pH and temperature under investigation. The $^{64}CuCl_2$ solution, received in a volume of approximately 20 μL, was diluted in ultrapure water to a volume of 100–200 μL, and an aliquot containing 5–10 MBq (1–2 μL) was added to the solutions containing the

aptamer conjugates. At the conclusion of the incubation period with the chelator-modified RNA, a 2 μL aliquot was withdrawn and immediately spotted at the origin of ITLC strips as described above without further purification (Supplementary Figs. 4–6). For reference, the retention factor of 'free,' unconjugated ^{64}Cu was determined by extracting a diluted sample of the parent ^{64}Cu solution in pH 6 acetate buffer obtained from Washington University for each set of experimental results obtained (e.g., Supplementary Fig. 4B). Using the ITLC system described, free ^{64}Cu migrated with the solvent front, while ^{64}Cu coupled to the chelator-modified RNA aptamer remained stationary at the origin of the spot.

A series of control experiments was conducted to assess loosely bound ^{64}Cu associated with the chelator-modified RNA backbone and to examine the stability of the radiometal-chelator coupling. For these experiments, ^{64}Cu was incubated with chelator-modified RNA at 5 °C, 25 °C, and 60 °C to determine the effect of temperature on the stability of the radiometal–chelator complex (pH 7 for NOTA, DOTA, and PCTA variants; based on pH experiments). These experiments were not conducted for DiAmSar variants because of instability of the conjugate (see Section 3). At the conclusion of the incubation period (30 min), three 10 μL aliquots was removed and transferred to 100 μL solutions of 1 nM, 1 μM, 1 mM ethylenediaminetetraacetic acid (EDTA) and incubated further at room temperature for 30 min and analyzed by ITLC (Supplementary Fig. 7). In separate experiments, ^{64}Cu was incubated at 60 °C (pH 7) with 5′-amine modified RNA aptamer starting material (i.e., no chelator) with and without a secondary incubation with 1 μM EDTA. As a final challenge, NOTA and PCTA conjugates were incubated with ^{64}Cu at 60 °C (pH 7) for 30 min and 10 μL aliquots were transferred to solutions of 100 mM EDTA and allowed to incubate for 30 min at room temperature prior to analysis. Experimental controls were also conducted using ^{64}Cu incubated in pH 7 acetate buffer and ^{64}Cu incubated in pure water (60 °C). At the end of the EDTA incubation period, sample solutions were spotted on ITLC strips as described above. In select cases, a second aliquot was taken for analysis by radioHPLC as a secondary confirmation of the ITLC results (Supplementary Fig. 8).

2.4. In vitro receptor binding assays

One day prior to the binding assay, cells were plated in a 24-well plate at a density of approximately 100,000 cells per well. The PSMA-positive cell line 22Rv1(1.7) and the PSMA-negative cell line PC3 were used. The 22Rv1(1.7) cell line was grown in RPMI 1640 media with 10% FBS and 1% non-essential amino acids, and the PC3 cell line was grown in DMEM/F12 media with 10% FBS. All subsequent procedures were performed on ice to prevent aptamer internalization. For these preliminary binding experiments, aptamers were radiolabeled with ^{64}Cu (5–10 MBq per nmol of RNA) in acetate buffer as described above (60 °C, 45 min, pH 6). Following the radiolabeling reaction, the conjugates were diluted to 4 mL in PBS (with divalent cations Ca^{2+}, Mg^{2+}), incubated at 70 °C for 15 min, and allowed to cool slowly to room temperature (about 20 min) to promote proper RNA folding according to procedures described previously.[2,28] The solution was then spin filtered to a final volume of approximately 150 μL for cell-binding reactions.

Prior to binding, each well was washed twice with 1 ml of ice-cold Dulbecco's phosphate-buffered saline without divalent cations (DPBS −/−) to remove growth media. The concentration of radiolabeled A10-3.2 aptamer was measured with UV–vis absorption spectroscopy, and serial dilutions ranging from 1000 nM to 30 nM were performed. To measure non-specific binding, serial dilutions were also made containing a high fixed concentration of non-radiolabeled A10-3.2 aptamer, generally 5–10 μM. Both sets of dilutions were incubated with the cells in the 24-well plate on ice in a volume of 100 μL. After 1 h, the binding reaction mixture was aspirated off the cells, and the cells were washed twice with 0.5 ml of ice-cold DPBS. Bound RNA was collected by washing with 0.5 ml of 0.5 N NaOH which was added to 3 ml of scintillation fluid, and activity was measured. For each dilution, specific binding was calculated by subtracting the activity of the sample with a high concentration of non-radiolabeled ('cold') aptamer added (i.e., non-specific binding) from the sample without cold aptamer added (i.e., total binding). The data were plotted and fit to a one-site saturation binding model using the non-linear regression algorithm of the software package *Sigma Plot*. Experiments were performed in duplicate.

3. Results and discussion

3.1. Conjugation of chelators to RNA

Several chelator-modified RNA aptamer bioconjugates were prepared to examine effective synthesis strategies and to evaluate the efficiency of radiolabeling these bioconjugates with ^{64}Cu. Three conjugation strategies were used: DOTA via the NHS ester (Supplementary Fig. 1A); NOTA and PCTA via the *p*-SCN-Bn linker group (Supplementary Fig. 1B); and diAmSar via a DSS linker (Supplementary Fig. 1D). Confirmation of the desired product was made by ESI-MS (Supplementary Fig. 1) and purity was assessed by HPLC (Supplementary Fig. 2). The strategies presented use readily available chelator starting materials and are conducted in aqueous solution.

Conjugation of the DOTA chelator through the NHS ester proved highly amenable to the aqueous conditions required for solubilizing the RNA aptamer (Supplementary Fig. 1A; Fig. 2). Three important aspects to this conjugation strategy were revealed through these studies. First, various ratios of DOTA-NHS to RNA were tried in the conjugation reaction, ranging from 1:1 stoichiometry to 100:1. These tests demonstrated that a 50 equiv excess of the bifunctional chelator was required to achieve the goal of >98% desired product without the need for additional purification, beyond spin-filter dialysis to remove excess reagents (data not shown). Second, as previously described, the reaction proceeded most efficiently at a mildly basic pH range (8–9) and could be conducted under aqueous conditions (0.1 M $NaHCO_3$).[11] Third, best results were obtained when the reactions were undertaken at low temperature (4 °C), while investigations at room temperature and 40 °C produced low product yields, indicating (as suggested by others) that the NHS ester was unstable to hydrolysis at higher temperature (data not shown). Under optimal conditions, overall chemical yields exceeding 90% could be achieved for these studies. To achieve high chemical yields, efficient peak collection during HPLC semi-preparative purification was critical.

Despite the successful use of DOTA-NHS, attempted synthesis of a chelator-RNA conjugate using the NOTA-NHS bifunctional chelator obtained from a commercial source was not successful under these conditions (data not shown). It is possible that NOTA-NHS is not stable enough in aqueous solution of pH 8.5–9 for the reaction to occur with the amine-modified RNA. Several attempts were made to perform this reaction in various organic solvents (e.g., acetonitrile, dimethylformamide, dimethyl sulfoxide, dioxane, and *N*-methyl-2-pyrrolidone) and combinations of organic solvents (i.e., organic:organic) without success. Attempts to perform the substitution reaction in mixed solvents (i.e. organic:water) using a small water fraction also proved problematic (data not shown).

Conjugation of the NOTA and PCTA chelators with the *p*-SCN-Bn functionalized groups proved to be highly efficient at pH 8–9 using 50 equiv chelator to 5′-amine-RNA aptamer at 40 °C in 0.1 M $NaHCO_3$ (Supplementary Figs. 1B and 2). As was the case for the

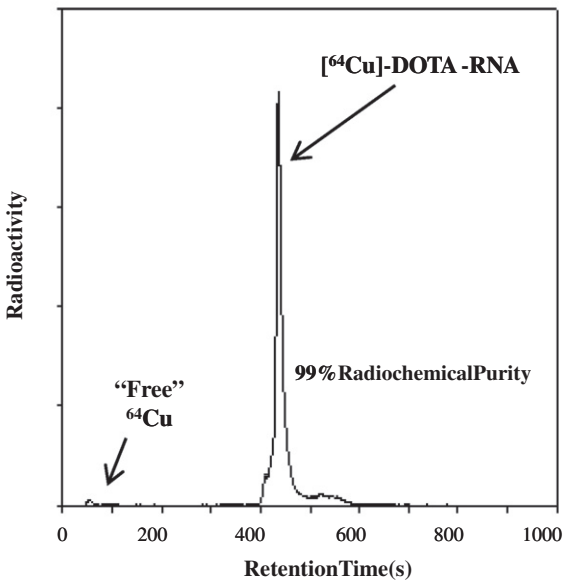

Figure 2. Assessment of radiochemical purity of the DOTA-RNA conjugate following spin-filter dialysis (10 k MWCO). 75 MBq ^{64}Cu was incubated with 1 nmol of DOTA conjugated RNA aptamer (pH 6, 60 °C, 45 min). Following the reaction, the sample was diluted to 4 mL in pure water and spin filtered for 20 min at 3500 RPM. This process was repeated to obtain a final volume of approximately 150 μL and a spin-filter-purified sample of 99% radiochemical purity.

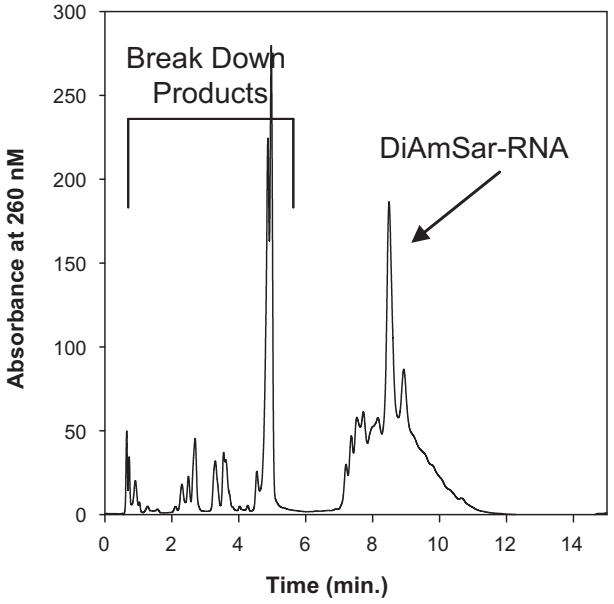

Figure 3. UV absorption chromatogram of diAmSar-RNA conjugate. The diAmSar-RNA compound was prepared according to Section 2 and stored in water at −20 °C for approximately one week prior to radiolabeling with ^{64}Cu. Breakdown products are apparent from the degradation of the diAmSar retention time peak, as well as fragments eluted earlier in the chromatogram. Such degradation is not observed for other chelator-RNA conjugates. Mass spectral analysis (data not shown) of this mixture suggests that the large peak at approximately 5 min is likely to consist of a collection of fragments and is the subject of future studies. The precise mechanism has yet to be revealed.

addition of DOTA, spin-filter dialysis proved to be effective at producing 98% chemical purity without the need for further purification (Supplementary Fig. 2). Importantly, it was observed that the reaction could produce unwanted reaction product impurities if allowed to proceed over 5 h. For these studies, it was found that the best results were obtained when the reaction mixture was monitored by HPLC until the starting material peaks disappeared (usually within about 3 h for reactions on the scale of 100–200 nmol of RNA). Within these reaction parameter constraints, final chemical yields in excess of 80% were obtained for the addition of the PCTA and NOTA chelators with *p*-SCN-Bn functionalized starting materials.

In the case of the addition of the diAmSar macrocyclic to the RNA, the DSS-linker approach proved effective in adding the chelator with the goal of employing simple reactions with a minimal number of steps using readily available starting materials (Supplementary Fig. 1D). The reaction was effective in two steps, but in this case, HPLC purification was required to achieve acceptable chemical purity (data not shown). No effect of reaction temperature (4 °C or room temperature) on yield was observed (data not shown). The final overall chemical yields for addition of the chelator diAmSar to 5′-amine-RNA was quite variable, with an average of 49% (±16%), the variability of which may reflect overall instability of this conjugate. Mass spectral analysis of the resulting purified product produced a single recognizable desired product (Supplementary Fig. 1D). However, UV-HPLC of the purified material revealed an uncharacteristically misshapen product peak (Fig. 3). The same peak shape was seen in both UV and radio-HPLC analyses of the purified product (Supplementary Figs. 1D and 2D; Fig. 3) indicating that the peak shape was related to the presence of the diAmSar chelator. Frequently, wide or multiple peaks indicate the presence of multiple RNA conformations. In many cases, the conformer peaks can be eliminated by diluting the sample to a concentration of <2 μM, heating to 60 °C for several minutes in the presence of divalent cations, and allowing the solution to cool slowly. However, the diAmSar-RNA peak shape could not be altered in this way. Additionally, substantial degradation of the compound was noted by HPLC analysis within days, even when stored at −20 °C (Fig. 3).

3.2. Radiolabeling with copper-64

The aqueous chemistry of ^{64}Cu radiolabeling is seemingly ideal for RNA aptamer applications given the constraints of working with oligonucleotides (aqueous solvents, neutral pH, and moderate to low temperatures). Detailed analyses of the effects of pH, temperature and labeling time on the labeling efficiency of various chelator-RNA conjugates with ^{64}Cu are presented below.

The effect of pH on the radiolabeling efficiency of chelator-modified RNA aptamers was studied in a range of pH 4 to pH 8 (considered to be the relevant range for RNA stability) (Fig. 4; Supplementary Fig. 4). The effect was studied at a specific activity of 5–10 MBq nmol^{-1} in $NaCH_3COO$ metal-free buffer solution using the highest grade purity reagents available to minimize the effect of interfering metal contaminants on radiolabeling effectiveness (see Section 2). The labeling reactions to assess the effect of pH on reaction efficiency were performed at 50 °C, with a reaction time of 30 min chosen based on preliminary experiments. Although a mild downward trend in radiolabeling efficiency was observed from pH 4–8 for the DOTA, NOTA, and PCTA variants examined, radiolabeling efficiency was greater than 96% without the need for further purification (i.e., no spin filtration or HPLC purification was conducted). An abrupt downward change in radiolabeling efficiency was observed at pH 5 for the NOTA-RNA conjugate due to a single unexplainable low result, which is believed to be an analytical artifact.

In contrast, a pronounced downward trend in the radiolabeling efficiency of diAmSar-RNA conjugates was readily identifiable, with the highest efficiency at pH 4 (99%) and trending downward with increasing pH (95% at pH 8) (Fig. 4). Considering that RNA stability is maximal at near-physiologic pH values, the diAmSar con-

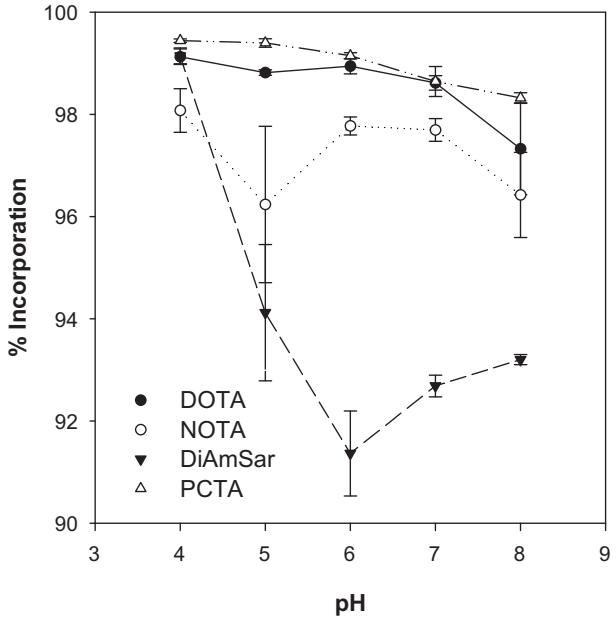

Figure 4. Effect of pH on the % incorporation of ^{64}Cu radiolabeled to RNA aptamer conjugated with chelators DOTA, NOTA, diAmSar, and PCTA. Experiments presented here were conducted at 50 °C for 30 min in 50 μL acetate buffer at 5 MBq nmol^{-1} (see Section 2). Incorporation was virtually quantitative at all pH values for DOTA, NOTA, and PCTA. The highest % incorporation for the diAmSar conjugate (99%) was observed at pH 4, with values trending to 93% at pH 8.

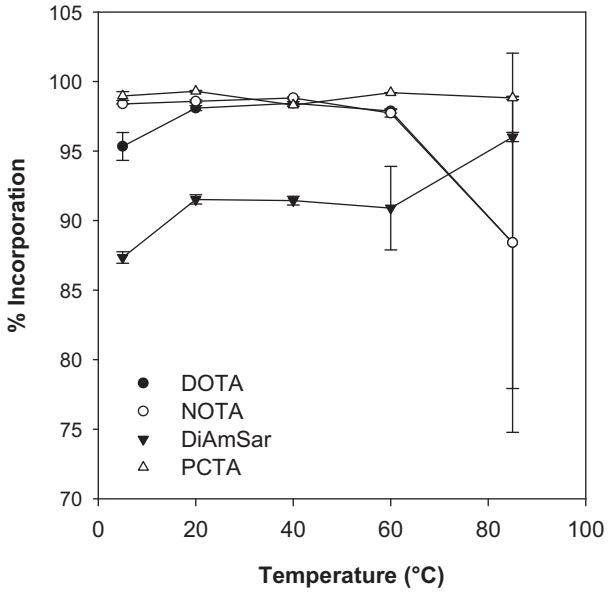

Figure 5. Effect of temperature on the % incorporation of ^{64}Cu radiolabeled to RNA aptamer conjugated with chelators DOTA, NOTA, diAmSar, and PCTA. Experiments presented here were conducted in pH 7 acetate buffer (50 mL) for 30 min at 5 MBq nmol^{-1}. High variability is observed at 85 °C due to apparent degradation of the RNA (see Discussion and Supporting Materials). Temperature has no effect on efficiency (nearly quantitative) from 5 °C to 60 °C for DOTA, NOTA, and PCTA conjugates. Labeling efficiency increases with temperature across this range for diAmSar conjugates.

jugates may be sub-optimal for RNA oligonucleotide applications because the highest radiolabeling efficiency is desired at the most RNA-compatible pH.

Radiolabeling RNA aptamers presents a particular challenge with regard to temperature, because at high temperatures the RNA conformation denatures and may no longer possess the desired activity. Special care must be taken that the aptamer is slowly cooled in relatively dilute concentrations to avoid multimer formation. Before use in their ultimate application (e.g., in vivo imaging), the aptamers are generally refolded by heating to 65 °C for 10 min and cooled to 37 °C over 10 min. It was anticipated that ^{64}Cu labeling would be amenable to a range of temperatures that would allow easy incorporation into the RNA refolding procedure. In concert with previously performed pH examinations (Fig. 4; Supplementary Fig. 4), experiments were carried out from 5 °C to 85 °C at pH 7 for DOTA, NOTA, and PCTA, and pH 4 for diAmSar-RNA conjugates, in $NaCH_3OO$ for 30 min (Fig. 5; Supplementary Fig. 5). There was no significant effect of temperature on radiolabeling efficiency from 5 °C to 60 °C for DOTA, NOTA, and PCTA conjugates under these conditions. While radiolabeling of DOTA, NOTA, and PCTA was nearly quantitative at these temperatures (>95% in 30 min), diAmSar-RNA radiolabeling was not as efficient (~85% at 5 °C to about 93% at 60 °C). For all conjugates, radiolabeling variability and apparent degradation was observed at 85 °C (Fig. 5; Supplementary Fig. 5). As with the pH range experiments previously described (Fig. 4; Supplementary Fig. 4), these results, with regard to radiolabeling efficiency, identify DOTA, NOTA, and PCTA as potentially more suitable chelators for radiolabeling RNA aptamers with ^{64}Cu, versus the chelator diAmSar.

For radiopharmaceutical preparations for clinical applications, it is critical to have bioconjugate compositions that enable rapid radiolabeling at the highest possible specific radioactivity (expressed in MBq per nmol of RNA). A further consideration is a composition that requires the least amount of RNA starting material possible, thus minimizing costs. The amount of RNA starting material used for radiolabeling (1 nmol; 13.5 μg) was determined from previous experiments performed with radiolabeling of peptide bioconjugates (our unpublished data). Relevant reported specific activities for radiolabeling peptide conjugates for clinical applications range from 25 to 100 MBq nmol^{-1}.[12,13,34,35] To evaluate the individual chelator modified RNA derivatives, an assessment was performed of the effect of time on radiolabeling efficiency within the constraints of pH (pH 7 for NOTA, DOTA, and PCTA, and pH 4 for diAmSar conjugates) and temperature (60 °C) at specific activities of 37 MBq nmol^{-1} and 75 MBq nmol^{-1} (Fig. 6; Supplementary Fig. 6). Within these limits of investigation, results indicate that the incorporation of ^{64}Cu is rapid (10 min) and nearly quantitative (>96%) for the NOTA-, PCTA-, and diAmSar-conjugated RNA aptamers at 37 MBq nmol^{-1} (Fig. 6a). Furthermore, there was only a minimal decrease in percent incorporation of ^{64}Cu observed at 75 MBq nmol^{-1} for NOTA and PCTA variants (Fig. 6b).

On the other hand, reaction kinetics were much slower for DOTA-RNA at 37 MBq nmol^{-1} (Fig. 6a). In addition, there was a limit in achievable ^{64}Cu incorporation for DOTA- and diAmSar-RNA conjugates at 75 MBq nmol^{-1} (Fig. 6b). Importantly, these constructs were prepared using identical high-purity solvents, and experiments were carried out using ^{64}Cu from the same production run. It should be noted also that the starting material for these experiments was obtained from the same manufacturer, which specializes in preparation of macrocyclic compounds for radiolabeling applications (Macrocyclics, Inc., Dallas, TX).

A separate set of control experiments was conducted to gauge the stability of the ^{64}Cu-chelator coupling and to assess the possibility of loosely bound ^{64}Cu associated with the RNA backbone. The focus of these experiments was on the NOTA- and PCTA-conjugates based on the radiolabeling results. The experiments comprised incubation of ^{64}Cu-labeled NOTA- and PCTA-modified aptamers with increasing concentrations EDTA (no EDTA, 1 nM, 1 mM, 1 μM) to gauge the stability of the radiometal-chelator coupling. For challenge experiments, NOTA- and PCTA-modified RNA were incubated first with ^{64}Cu at each of three temperatures (5 °C,

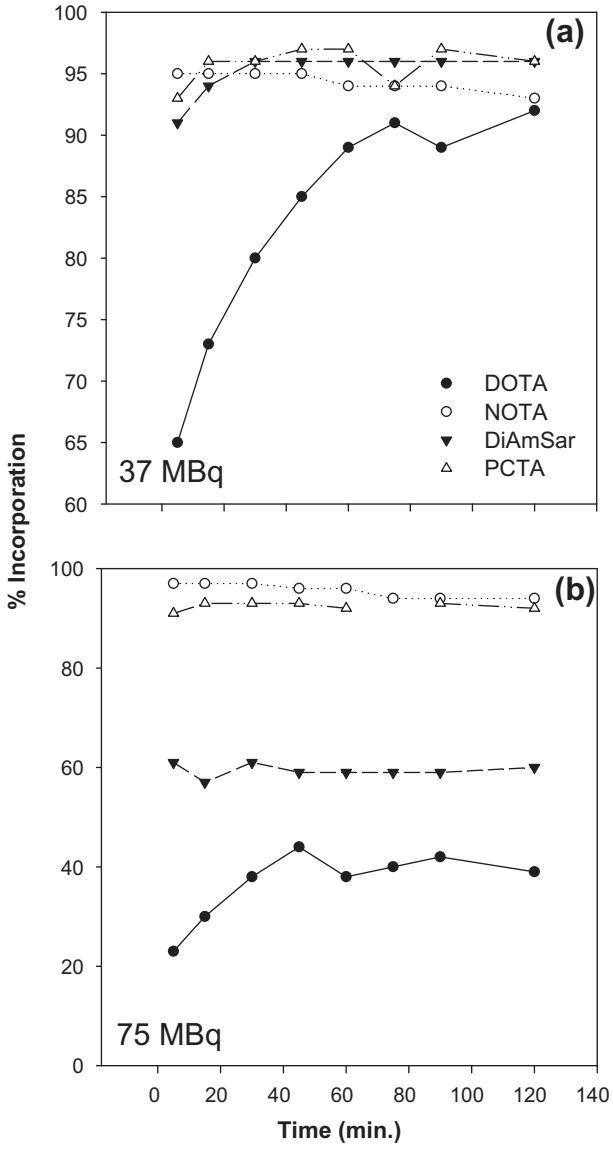

Figure 6. Effect of specific activity on the achievable % incorporation of ^{64}Cu. Experiments were conducted under optimized conditions for 1 nmol each chelator conjugate in 50 mL of acetate buffer solution: (a) Quantitative incorporation of ^{64}Cu is almost instantaneous at 37 MBq nmol^{-1} for NOTA, diAmSar, and PCTA, while DOTA kinetics are slower; (b) At 75 MBq nmol^{-1}, NOTA and PCTA reaction kinetics are rapid and quantitative, while DOTA and diAmSar conjugates were limited in achievable radiochemical purity within the 2 h reaction period. A single data point in the PCTA sequence was lost due to an analytical error at the 75 min time point for the experiment conducted at 75 MBq. Uncertainties are estimated to be <5% at the 1 sigma level).

25 °C, 60 °C) with an incubation time of 30 min for the radiolabeling step at pH 7. Following the radiolabeling step, aliquots of the solution were transferred to separate vials containing EDTA solutions and incubated another 30 min at room temperature. Subsets of experiments were conducted to confirm that the ^{64}Cu coupling was indeed with the chelator moiety and not the result of loose binding to the RNA backbone by incubation of ^{64}Cu with the 5′-amine modified RNA (no chelator) and controls were conducted by incubation of ^{64}Cu in acetate buffer and pure water. A single experiment was conducted in which a DOTA-RNA conjugate was radiolabeled at 60 °C for 30 min at pH 7 in acetate buffer and challenged with 1 μM EDTA solution. Finally, an aggressive challenge was performed of the PCTA- and NOTA-conjugated RNA aptamers in which these conjugates were incubated for 30 min at pH 7 and 60 °C with ^{64}Cu, followed by incubation with 100 mM EDTA for 30 min at room temperature. Assessments were conducted by ITLC by methods described earlier and select radioHPLC measurements were made to validate the ITLC results. Several important points can be made based on these results. First, for NOTA-conjugated RNA, >95% incorporation of ^{64}Cu is observed following radiolabeling and challenge experiments with no EDTA, 1 nM, 1 μM, and 1 mM EDTA incubation conducted at 25 °C and 60 °C (Fig. 7a). A decrease in The ^{64}Cu-NOTA coupling (% incorporation, Fig. 7) is observed for the 30 min radiolabeling at 5 °C, indicating that the ^{64}Cu-NOTA coupling is less stable when the radiolabeling is carried out at the lower temperature. A separate experiment was conducted using a 100 mM concentration of EDTA with radiolabeling conditions of pH 7 and 60 °C. Greater than 95% incorporation was observed also for the NOTA-RNA conjugate in these experiments, which strongly suggest that the ^{64}Cu association is chelator-dependent and not loosely bound to the RNA backbone (Supplementary Fig. 7). Similar results were observed for PCTA variants with an apparent stronger temperature dependence and somewhat less stable coupling when challenged with EDTA (Fig. 7b). Interestingly, a separate experiment in which the DOTA-RNA conjugate was radiolabeled with ^{64}Cu at pH 7 and 60 °C for 30 min and then challenged with 1 μM EDTA corroborates the apparently inferior characteristics of DOTA for copper chelation compared to PCTA and NOTA for this application. In this case, following the 30 min incubation with the EDTA solution, only 37% incorporation of ^{64}Cu was observed (Supplementary Fig. 7). Control experiments with ^{64}Cu incubated with 5′-amine modified RNA starting material at 60 °C for 30 min at pH 7 in the presence of 1 μM EDTA and in the absence of EDTA confirmed no significant association of the ^{64}Cu with the RNA backbone when compared to 'free' ^{64}Cu controls (incubated in acetate or pure water with no RNA) by ITLC and corroborated by radioHPLC results (Supplementary Fig. 8).

Together, these results suggest that the NOTA and PCTA chelators have superior radiolabeling and ^{64}Cu stability characteristics for eventual targeted molecular imaging applications using ^{64}Cu, compared to the DOTA and diAmSar chelators (Figs. 4–6) and the NOTA variant may have advantages in terms of instability to transchelation reactions, which could be important for in vivo stability. Further in vitro and in vivo studies of these conjugates are needed to determine ultimately the best choice for molecular imaging.

3.3. Stability of RNA conjugates

Through the course of these investigations, the stability of the chelator-modified RNA aptamer conjugates was examined by UV-HPLC (Supplementary Fig. 2) and radio-HPLC techniques (Supplementary Fig. 3). Radio-HPLC analysis of raw, undialyzed products did not indicate instability of the RNA aptamer constructs with the exception of the diAmSar-RNA (Supplementary Fig. 2). A small, yet unchanging impurity is observed in UV-HPLC analysis of DOTA-, NOTA-, and PCTA-conjugated RNA (Supplementary Fig. 2). Importantly, no association of this impurity with ^{64}Cu is observed in any of the radio-HPLC analyses (Supplementary Fig. 3). On the other hand, in the case of the diAmSar-RNA conjugates, an impurity was observed by UV-HPLC that increased with time (Fig. 3). The impurity carries a 260 nm to 280 nm UV absorption ratio of approximately 2 that is characteristic of RNA. Mass spectral analysis indicates that the impurity is likely a collection of RNA fragments with molecular weights ranging from 6 kDa to 8.5 kDa (data not shown). The mechanism of the degradation, and the precise identification of the molecular fragments is unclear, but the degradation appears to be unique to the diAmSar-RNA conjugate (perhaps due to the free primary amine and multiple secondary amines of the diAmSar macrocycle). It is also possible that a longer linker is required to mitigate the apparent reaction of the diAmSar

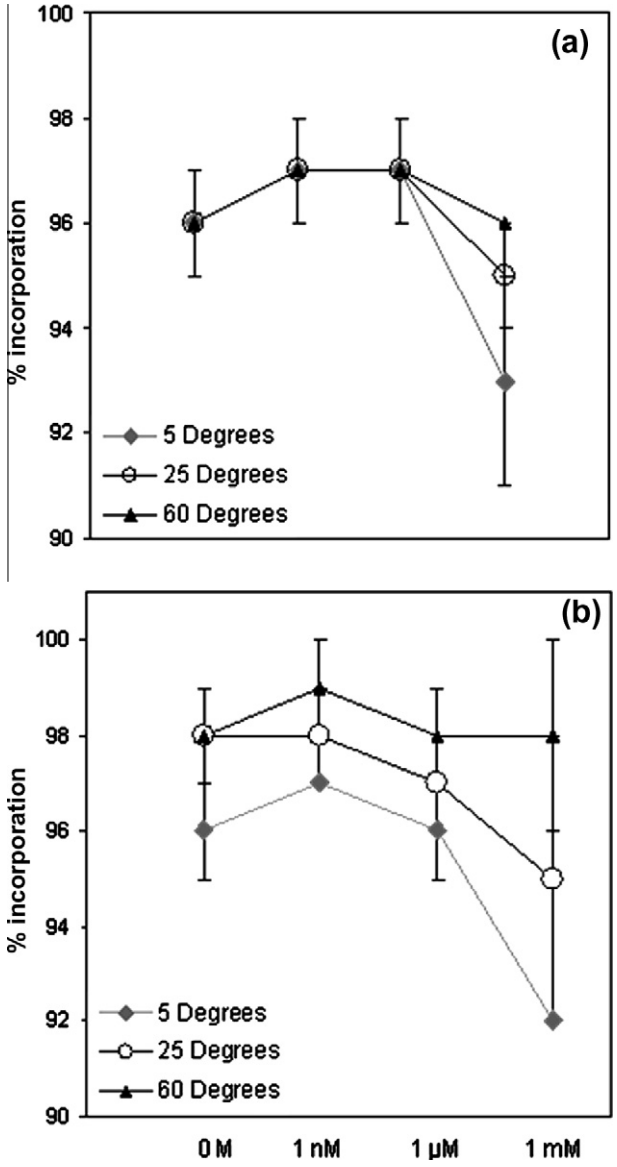

Figure 7. Effect of EDTA on radiochemical % incorporation of [^{64}Cu]-labeled chelator-RNA conjugates. (a) NOTA-RNA; (b) PCTA-RNA. Incubations of chelator-modified RNA with ^{64}Cu were performed at 5 °C, 25 °C, and 60 °C and challenges were performed by incubating aliquots with 0, 1 nM, 1 μM, and 1 mM EDTA for 30 min at room temperature. Assessments were made by spotting ITLC plates and corroborated by radioHPLC of select conjugates ($n = 2$; See Supplementary Figs. 7 and 8).

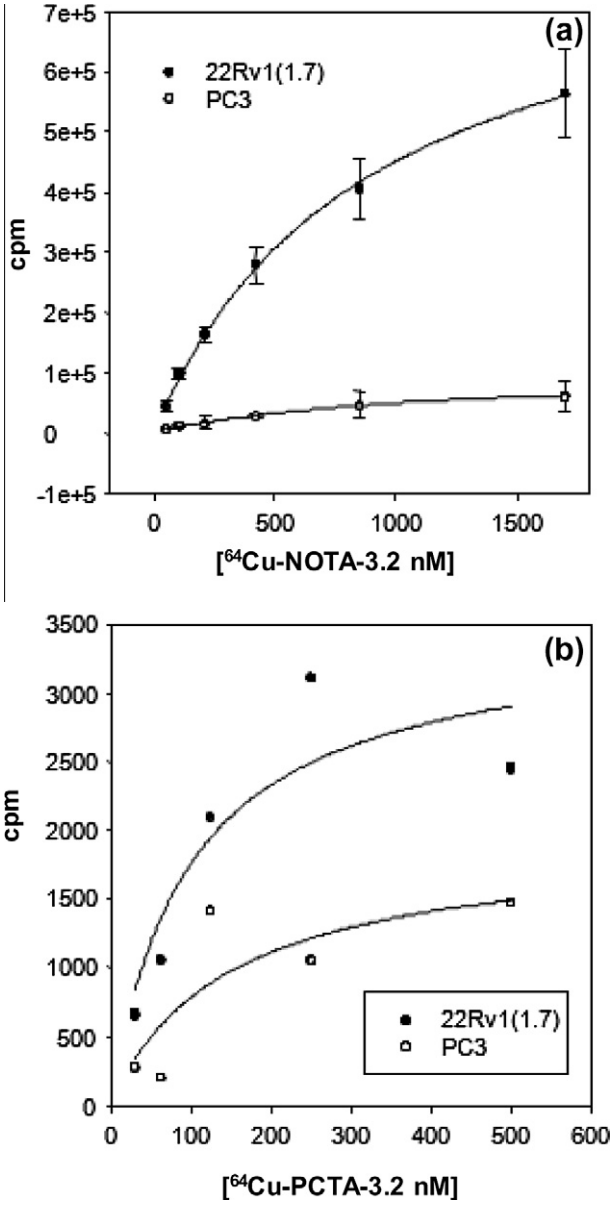

Figure 8. Saturation in vitro binding curves of radiolabeled A10-3.2 aptamer with PSMA-positive (22Rv1[1.7]) and PSMA-negative (PC3) cell lines. Graphs (a) and (b) show the binding of the A10-3.2 aptamer with the appended chelators NOTA and PCTA, radiolabeled with ^{64}Cu. Specificity for PSMA-expressing cells does not appear to be significantly altered by the presence of the chelator.

with the RNA backbone. Nevertheless, this instability indicates a possible disadvantage for using diAmSar in its current form for preparation of ^{64}Cu-labeled RNA aptamers.

3.4. In vitro receptor binding

Cell binding experiments were performed to determine whether the addition of the various chelators to the oligonucleotide A10-3.2 would alter the specificity of the RNA for prostate cancer cells expressing PSMA (Fig. 8). To assess the addition of the chelator, [^{64}Cu]-NOTA-RNA (Fig. 8a) and [^{64}Cu]-PCTA-RNA (Fig. 8b) imaging probes were incubated with either PSMA-negative (PC-3) or PSMA-positive (22Rv1[1.7]) prostate cancer cells.[28] Importantly, specific binding was observed with the RNA probe conjugated to either chelator (Fig. 8a and b). In each case, the binding capacity of PSMA-positive cells for either ^{64}Cu-NOTA-RNA (Fig. 8a) or ^{64}Cu-PCTA-RNA (Fig. 8b), as measured by the asymptote B_{max} in the one-site saturation binding model, is significantly greater for PSMA-expressing 22Rv1(1.7) cells than for PSMA-negative PC-3 cells. Binding affinity of the RNA conjugated to the chelators was found to be similar to that of the unconjugated RNA radiolabeled at the 5′-gamma phosphate position with phosphorous-32 (^{32}P, Supplementary Fig. 9). Qualitatively, these results suggest that the specificity of the modified RNA aptamer is maintained with the addition of both the NOTA and PCTA chelators. On the other hand, based on these results, the NOTA-RNA conjugate appears to have binding affinity advantages in comparison to the PCTA-modified RNA. While the specificity of the PCTA-RNA conjugate is maintained at all concentrations studied, the apparent low affinity indicated by the much reduced signal intensity (Fig. 8b) suggests that the PCTA may degrade the overall binding affinity of the RNA that could be the result of a high concentration

of a conformer with a lower binding affinity. Further experiments are required to explore this possibility.

4. Conclusions

A detailed analysis and characterization is presented for radiolabeling of RNA oligonucleotide aptamers with ^{64}Cu for targeted molecular imaging by PET. Four different chelators (DOTA, NOTA, PCTA, and diAmSar) were successfully conjugated to A10-3.2 PSMA specific RNA aptamer that was functionalized with a 5′primary amine and a 12 carbon alkyl linker. Radiolabeling parameters were established and were found to be favorable (e.g. low temperatures, neutral pH and aqueous conditions) for retaining the stability and target specificity of the RNA aptamer.

Synthesis of DOTA-RNA conjugates via the NHS ester was highly efficient in aqueous bicarbonate at 5 °C, while reactions at higher temperatures resulted in lower yields. This was presumably due to instability of the NHS to hydrolysis at higher temperatures. The conjugation reactions of the chelators *p*-SCN-Bn-PCTA and *p*-SCN-Bn-NOTA to RNA reached completion within 5 h in aqueous bicarbonate at a pH of 8.5–9 and a temperature of 40 °C. Longer reaction times produced addition products necessitating more extensive purification. The –NHS and –NCS functional groups both produced sufficient purity (>98%) to allow purification by simple spin-filter size exclusion. The chelator diAmSar was effectively conjugated via the use of a disuccinimidyl suberate linker in two steps with HPLC purification of the final product required (with lower overall chemical yields). However, fragmentation of the diAmSar-RNA conjugate was observed that indicated a reaction between the chelator and RNA backbone that proceeded at temperatures as low as −20 °C.

Radiolabeling parameters for the four chelator-conjugated RNA imaging probes were as follows. While a slight downward trend in radiolabeling efficiency was observed for DOTA-, NOTA-, and PCTA-RNA conjugates from pH 4 to pH 8, radiochemical purity of greater than 96% could be achieved in 30 min at 50 °C at all pH values examined. These results suggest that pH 7 (optimum for RNA stability) is optimum for ^{64}Cu labeling of RNA aptamers for these chelator-modified conjugates. In contrast, the highest radiolabeling efficiency for diAmSar-RNA conjugate was at pH 4 with a pronounced downward trend in radiolabeling efficiency with increasing pH. Likewise, there was no effect of temperature on radiolabeling efficiency from 5 °C to 60 °C for DOTA-, NOTA-, and PCTA-RNA conjugates, while higher temperatures significantly improved radiolabeling efficiencies for the diAmSar-RNA conjugate. Radiolabeled reaction product variability and apparent degradation of all chelator-RNA conjugates occurred when labeling was performed at 85 °C.

Given the critical importance of specific activity for targeted molecular imaging applications, ^{64}Cu labeling reaction kinetics were examined at 37 MBq nmol^{-1} and 75 MBq nmol^{-1} (1 nmol RNA conjugate, monitored over 120 min). Achievable radiochemical purity (without further purification) was limited for the DOTA- (40%) and diAmSar-RNA (60%) conjugates. On the other hand, for the NOTA- and PCTA-RNA conjugates, greater than 96% radiochemical purity is achievable within 10 min at a pH of 7 and temperature of 60 °C, with no observable change in this result at clinically relevant specific activity (75 MBq nmol^{-1}). These conditions are not only optimal for achieving high radiolabeling yields and radiochemical purity, but also are considered optimum for transition to the final RNA folding step in preparation for in vitro cell binding assays and in vivo examinations of pharmacodynamics. Control experiments confirmed ^{64}Cu coupling is an association with the chelator moieties (not the RNA backbone), and identified a temperature dependence of the chelator-^{64}Cu coupling for NOTA- and PCTA-variants examined. The NOTA-RNA conjugate appeared to have an indentifiable stability to transchelation advantage under the experimental conditions presented here, but the effect was not evident when radiolabeling is carried out at 60 °C in which case >95% incorporation of ^{64}Cu was observed following challenge experiments up to 100 mM EDTA for both PCTA and NOTA conjugates. The ITLC method is useful for assessing trends in radiolabeling efficiency when a large number of experiments are required in a relatively short period of time, but should not be considered quantitative as a small and variable fraction of unlabeled ^{64}Cu signal is retained at the origin, even with the addition of EDTA. Nonetheless, radioHPLC methods can be used to corroborate ITLC results.

Finally, it was shown that the addition of the chelators NOTA and PCTA to the aptamer A10-3.2 does not affect its specificity for its target. Indeed, this has been previously demonstrated in vivo for other chelator-aptamer conjugates labeled with the single-photon emitting radionuclide ^{99m}Tc.[7,36] However, low binding affinity and identifiable variation in cell binding behavior of the PCTA variant was apparent in comparison to the NOTA-RNA conjugate, which may be related to stability of the ^{64}Cu-PCTA coupling or interference of the PCTA chelator with RNA folding. Further experiments are required to fully understand the significance of the effect.

In summary, the NOTA and PCTA constructs appear to be the most promising for further studies of chelator-aptamer derivatives and the results of these optimization studies enable us to progress to examinations of the stability of these chelator-RNA conjugates in small animal models of prostate cancer. Thus, future studies along these lines include evaluations of several other promising chelator variants for use with ^{64}Cu, examinations of alternative bioconjugation techniques to improve synthetic efficiency, and advancing these studies to in vivo models of disease.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.05.010.

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