



Design and synthesis of ^{225}Ac radioimmunopharmaceuticals

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Abstract

The alpha-particle-emitting radionuclides ^{213}Bi , ^{211}At , ^{224}Ra are under investigation for the treatment of leukemias, gliomas, and ankylosing spondylitis, respectively. ^{213}Bi and ^{211}At were attached to monoclonal antibodies and used as targeted immunotherapeutic agents while unconjugated ^{224}Ra chloride selectively seeks bone. ^{225}Ac possesses favorable physical properties for radioimmunotherapy (10 d half-life and 4 net alpha particles), but has a history of unfavorable radiolabeling chemistry and poor metal-chelate stability. We selected functionalized derivatives of DOTA as the most promising to pursue from out of a group of potential ^{225}Ac chelate compounds. A two-step synthetic process employing either MeO-DOTA-NCS or 2B-DOTA-NCS as the chelating moiety was developed to attach ^{225}Ac to monoclonal antibodies. This method was tested using several different IgG systems. The chelation reaction yield in the first step was $93 \pm 8\%$ radiochemically pure ($n = 26$). The second step yielded ^{225}Ac -DOTA-IgG constructs that were $95 \pm 5\%$ radiochemically pure ($n = 27$) and the mean percent immunoreactivity ranged from 25% to 81%, depending on the antibody used. This process has yielded several potential novel targeted ^{225}Ac -labeled immunotherapeutic agents that may now be evaluated in appropriate model systems and ultimately in humans.

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1. Introduction

Alpha-particle-emitters are of considerable interest for radioimmunotherapy applications because of the high linear energy transfer (LET) and short range of alpha particles in tissue (a few cell diameters) (Wilbur, 1991; Humm and Chin, 1993; Zalutsky and Bigner, 1996; Kotz, 1998; McDevitt et al., 1998; Mirzadeh,

1998). A single atom of an alpha-emitting isotope is capable of killing a tumor cell, making it among the most potent cytotoxic agents available (Nikula et al., 1999). The short range of the alpha particle increases its safety profile because nonspecific irradiation of normal tissue around the target cells is greatly reduced or absent. The high LET coupled with a short range makes alpha-particle radioimmunotherapy (RIT) particularly attractive for the treatment of cancers and other diseases.

There are currently three alpha-particle-emitting drugs in clinical investigation. The anti-CD33 drug [^{213}Bi]HuM195 (Jurcic et al., 1999) has been considered for the treatment of leukemias and is currently under investigation in a Phase II trial. This ^{213}Bi isotope is generator produced and the ensuing radioimmunopharmaceutical must be labeled and quality controlled for

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rapid release on site (McDevitt et al., 1999a,b; Nikula et al., 1995) due to its short half-life ($t_{1/2} = 46$ min). The ^{211}At ($t_{1/2} = 7.2$ h) used to label the chimeric anti-tenascin antibody 81C6 is cyclotron produced (Zalutsky et al., 2000); its widespread use is currently limited by the small number of institutions capable of producing this radionuclide on site. ^{224}Ra chloride, has a 3.6 d half-life and decays by 4 net alpha particles. It is currently being examined as a bone-seeking drug for the treatment of ankylosing spondylitis (Kampen et al., 2001; Tiepolt et al., 2001).

^{225}Ac is the parent radionuclide of ^{213}Bi and has been proposed as a potentially useful therapeutic radionuclide (Geerlings et al., 1993; Kaspersen et al., 1995; Lambrecht et al., 1997). It has a 10 d half-life and nets 4 alpha particles per decay. The key to the utilization of ^{225}Ac for in vivo applications is the importance of finding chelant moieties that can stably coordinate this radionuclide. Coordination of the daughter atoms including ^{221}Fr , ^{217}At , and ^{213}Bi , is unlikely with a single chelant and is not proposed as a feasible goal here. Herein we describe the screening of a number of chelant molecules for the ability to coordinate and stably bind ^{225}Ac . Based on this evaluation we selected the DOTA functionality and then developed a synthetic scheme to radiolabel IgGs with ^{225}Ac . We will describe our rationale for using DOTA and develop reliable synthetic methods for the production of sufficient quantities of stable [^{225}Ac]DOTA–IgG constructs. Although it has been suggested that the DOTA chelate would not be suitable for use with ^{225}Ac (Davis et al., 1999; Deal et al., 1999), the data presented here, and the applications of the resulting constructs in animal tumor models (McDevitt et al., 2001) show that such therapeutic agents may have promise as a new class of radiopharmaceutical.

2. Materials and methods

2.1. Radionuclides, reagents, and monoclonal antibodies

The ^{225}Ac was received from Oak Ridge National Laboratory (Oak Ridge, TN) as a nitrate residue. The ^{225}Ac nitrate residue was dissolved in 0.2 M Optima grade HCl (Fisher Scientific, Pittsburgh, PA). Metal-free water (MFW) used for this and all other solutions was obtained from a Purelab Plus system (US Filter Corp., Lowell, MA) and was sterile filtered.

The ^{225}Ac activity was measured with a Squibb CRC-17 radioisotope calibrator (or equivalent model) (E.R. Squibb and Sons, Inc., Princeton, NJ) set at 775 and multiplying the displayed activity value by 5. The parent ^{225}Ac was measured when it was in secular equilibrium with its daughters. Samples were typically measured in a 50 ml conical tube placed at the bottom and center of the

Squibb CRC-17 Radioisotope Calibrator well chamber. The activity value reported using the CRC calibrator was verified by counting a 0.005–0.020 ml (± 0.00015 ml) sample (parent ^{225}Ac in secular equilibrium with daughters) aliquot point source taken from an accurately known volume ($2\text{--}10\text{ ml} \pm 0.02\text{ ml}$) of ^{225}Ac source at a fixed geometry using a HPGe detector with pulse height multi-channel analysis (Canberra Industries, Meriden, CT). The HPGe detector counting efficiency of the ^{213}Bi 440 KeV γ -emission was determined for the same geometry from a plot of counting efficiency $^{-1}$ vs. the γ -energy of standard radionuclide sources. The radioisotope dose calibrator setting of 775 (multiplying the displayed activity value by 5) was selected based upon measurements taken from the pulse height multi-channel analyzed γ -emission spectrum of a ^{213}Bi sample.

The chelating agents diethylenetriaminepentaacetic acid (DTPA) and 1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid (TETA) were obtained from Aldrich Chemical Co. (Milwaukee, WI); 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA), 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrapropionic acid (DOTPA), 1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetrapropionic acid (TETPA), and 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetramethylene-phosphonic acid (DOTMP) were obtained from The Dow Chemical Company (Freeport, TX). The bifunctional chelating agents MeO-DOTA-NCS, (α -(5-isothiocyanato-2-methoxyphenyl)-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid and 2B-DOTA-NCS, 2-(*p*-isothiocyanatobenzyl)-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid were obtained from The Dow Chemical Company and Macrocylics (Dallas, TX), respectively. The structures of these chelating agents are shown in Fig. 1.

Chemicals used in the radiolabeling and purification steps were ACS reagent grade or better. Tetramethylammonium acetate (TMAA), ammonium acetate (NH_4Ac), sodium hydroxide (NaOH), L-ascorbic acid (L-aa), sodium carbonate (Na_2CO_3), sodium hydrogen carbonate (NaHCO_3), Sephadex C-25, ethylenediaminetetraacetic acid (EDTA), diethylenetriaminepentaacetic acid (DTPA), and yttrium chloride (YCl_3) were obtained from Aldrich Chemical Co. Human serum albumin (HSA) (Swiss Red Cross, Bern, Switzerland) and 0.9% NaCl (Abbott Laboratories, North Chicago, IL) were used as received. A 10 DG desalting column (Bio-Rad, Hercules, CA) was used as the stationary phase for the size exclusion chromatographic purification with a 1% HSA mobile phase.

The monoclonal antibodies HuM195 (anti-CD33), mJ591 (anti-PSMA) and huJ591 (anti-PSMA), B4 (anti-CD19), and 3F8 (anti-GD2) were generously provided by Protein Design Laboratories (Sunnyview, CA), Dr. Neil H. Bander (New York Presbyterian Hospital—Weill Medical College of Cornell University, NY), Progenics Pharmaceuticals, Inc. (Tarrytown, NY) and

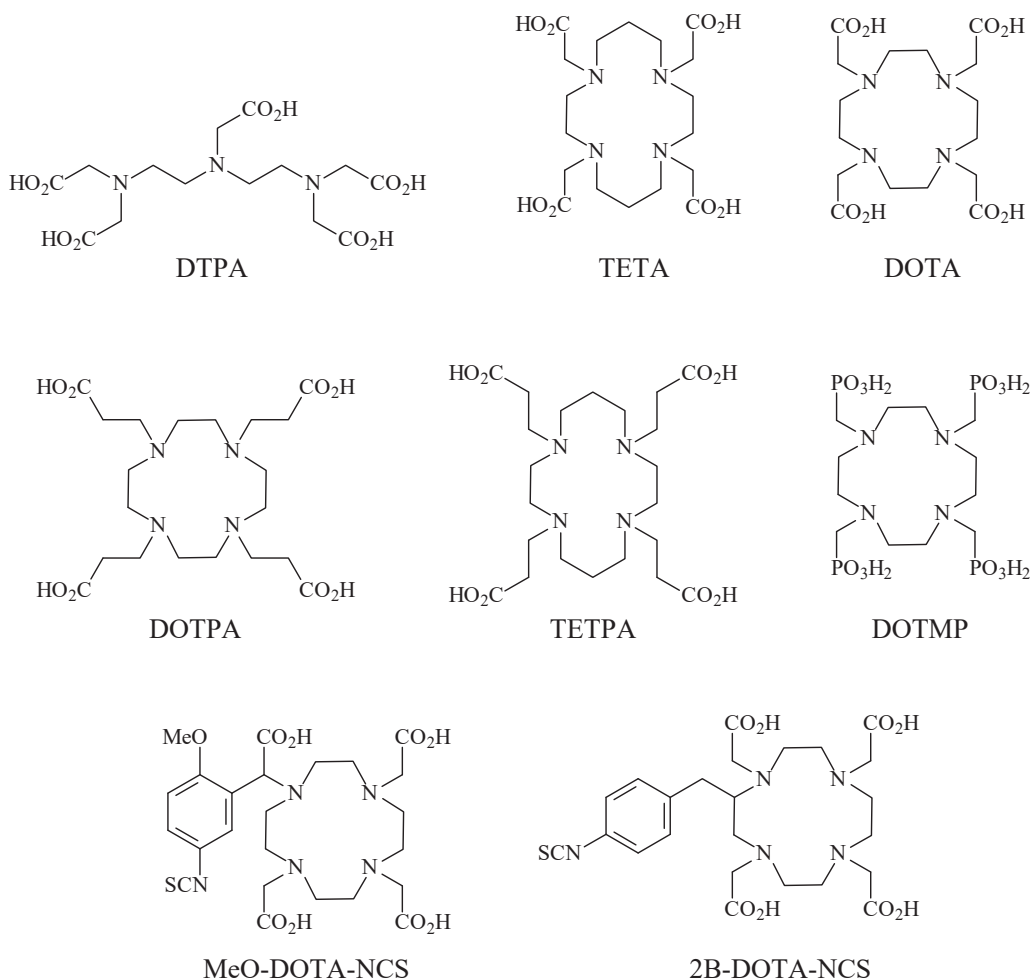


Fig. 1. Structures of the chelating agents used in this study.

Dr. Nai-Kong V. Cheung (Memorial Sloan-Kettering Cancer Center), respectively.

2.2. Reactivity and stability of [^{225}Ac]-chelate species

The reactivity of ^{225}Ac with several chelating agents was determined as follows: Solutions of 0.050 M DTPA, DOTA, TETA, DOTPA, TETPA, and DOTMP were prepared in 0.2 M NH_4Ac . 0.40 ml of each of these chelant solutions was added to a 0.60 ml solution of ^{225}Ac acetate (buffered to pH 6 with 3 M NH_4Ac and MFW (the total reaction volume was 1.0 ml)) and then placed into a 37°C water bath. 0.40 ml of 0.2 M NH_4Ac buffer solution with no chelating agent was added to 0.60 ml solution of ^{225}Ac acetate and served as a control.

The extent of the chelation reaction vs. time was determined in duplicate using instant thin layer chromatography (ITLC) at 0, 30, 60, and 120 min by spotting a small amount (0.001 ml) of each reaction mixture onto a silica gel (SG) impregnated paper strip (Gelman Science Inc., Ann Arbor, MI). The paper strips were

developed using a 9% $\text{NaCl}/10\text{mM}$ NaOH mobile phase. Using this mobile phase, unchelated metal species were characterized by R_f of 0 and the R_f of the metal-chelant complexes was greater than 0. The intact strips were counted after 20 h using a gas ionization detector (Ambis 4000, Ambis Inc., San Diego, CA).

After 120 min of reaction, a 1.5-fold stoichiometric excess of Y(III) to chelant was added as a challenge and to coordinate with any unreacted chelating agent. Following an additional 30 min, each of the yttrium-challenged reaction mixtures was purified by passage over a 1 ml bed of Sephadex C-25 which binds the free ^{225}Ac and the resin was washed with 2.5 ml of 0.9% NaCl . The column was washed a second time with 2.5 ml of 0.9% NaCl to check that all removable activity was recovered. The column and washes were counted immediately and 20 h later to determine the ^{225}Ac activity levels using a Squibb CRC-17 radioisotope Calibrator. The % of activity that washed off the resin was the % ^{225}Ac that was complexed by the chelant moiety.

The stability of the purified ^{225}Ac -chelate species under mock physiological conditions was determined by adding 0.10 ml of each of the first wash solutions from the Sephadex C-25 purification steps to 0.90 ml of 25% human serum and incubating at 37°C. At various time points over a 53 d period, 0.001–0.005 ml aliquots were removed and analyzed in duplicate by ITLC-SG as described above to determine the extent of ^{225}Ac still associated with chelate.

2.3. Synthesis, purification, and quality control of radiolabeled [^{225}Ac]-DOTA-IgG construct

The first step in construct preparation was the ^{225}Ac -DOTA-NCS chelation reaction. The bifunctional isothiocyanato derived MeO-DOTA or the 2B-DOTA can be used interchangeably in this synthetic process as there was no difference in chemistry and these will be referred to only as DOTA-NCS unless specifically noted.

^{225}Ac dissolved in 0.2 M HCl (0.86 ± 0.40 mCi, $n = 31$) (mean $\pm$ SD) was mixed with 10 mg/ml DOTA-NCS in MFW (0.229 ± 0.098 mg, $n = 31$), 0.015–0.020 ml of 150 g/l stock L-aa, and 0.025–0.150 ml of 2 M TMAA. The pH of the resulting reaction mixture was 5.4 ± 0.3 ($n = 31$). The mixture was then heated to $56 \pm 3^\circ\text{C}$ ($n = 30$) for 42 ± 14 min ($n = 31$). At the end of the reaction a 0.001 ml aliquot was spotted onto a 1 ml bed of Sephadex C-25 and the resin washed two times with 3–4 ml of 0.9% NaCl. The column and washes could be counted after 30 min to determine the ^{221}Fr activity or after 6 h to determine the ^{213}Bi activity of each. The % of activity that washed off the resin was the % ^{225}Ac that was chelated by the DOTA-NCS.

The second step in construct preparation was the ^{225}Ac -DOTA-NCS reaction with IgG. There were five different IgGs examined in this study. The ^{225}Ac -DOTA-NCS chelation reaction was mixed with IgG (0.9 ± 0.4 mg, $n = 31$), 0.015–0.020 ml of 150 g/l stock L-aa, and 0.025–0.150 ml of a 1 M carbonate buffer. The pH of the resulting reaction mixture was 8.7 ± 0.3 ($n = 31$). The reaction mixture was then heated to $36 \pm 2^\circ\text{C}$ ($n = 30$) for 52 ± 14 min ($n = 28$). At the end of the reaction period, the mixture was treated with a 0.020 ml addition of 10 mM DTPA to complex any free metals during the size exclusion chromatographic purification.

The reaction mixtures were purified by passage over a 10 DG size exclusion column using 1% HSA as the mobile phase. The column was loaded sequentially with the reaction mixture, a 0.2 ml 1% HSA wash of the reaction vial, and 2.0 ml of 1% HSA which were collected in separate tube (waste). The final product was eluted and collected by the addition of 2.0 ml 1% HSA.

A small amount (0.005 ml) of the final product was used to determine the radiochemical purity of the final product using ITLC-SG. The paper strips were developed using two different mobile phases. Mobile phase I was 10 mM EDTA and II was 9% NaCl/10 mM NaOH. The R_f of the radiolabeled antibody was 0 and both the free metal species and metal chelates were characterized by R_f of 1.0 in mobile phase I. In mobile phase II, the radiolabeled antibody and free metal species were characterized by R_f of 0 and the R_f of the metal chelates was 1.0. The strips were counted intact using the Ambis 4000 gas ionization detection system.

Immunoreactivity was determined using a cell-based assay with excess antigen as described previously (Nikula et al., 1999). The ^{225}Ac activity in the cell pellets and media aspirates were counted after 20 h from separation of the components using a Beckman LS 6000IC beta scintillation counter (Beckman Instruments, Inc., Fullerton, CA).

3. Results and discussion

3.1. Reactivity of the various chelants with ^{225}Ac

Fig. 2 shows at equilibrium the percentage of ^{225}Ac that was associated with the C-25 column and the percentage of ^{225}Ac that was complexed by the chelant and washed off the column from each of the six reaction mixtures and the blank ^{225}Ac acetate. The C-25 column bound 100% of the ^{225}Ac that was present in the positive control blank reaction and in the DTPA, TETA, DOTPA, and TETPA reaction mixtures indicating the inability of these chelates to strongly bind the ^{225}Ac after 2 h at 37°C and challenge with a 1.5-fold stoichiometric excess of yttrium ion. In contrast, the DOTA chelant was able to bind 100% of the ^{225}Ac and the DOTMP chelant was able to bind 78% of the ^{225}Ac under similar conditions. A qualitative examination of the rate of reaction of DOTA with ^{225}Ac showed little or no complexation at time 0 and almost complete complexation after 30 min as determined by the ITLC-SG developed in 9% NaCl/10 mM NaOH. The other chelant systems were not considered further as they were inherently unstable to challenge by the cationic exchange binding sites on the C-25 resin.

3.2. The stability of the ^{225}Ac chelates in vitro

The stability of the purified ^{225}Ac -DOTA and ^{225}Ac -DOTMP chelates in 25% serum at 37°C were determined vs. ^{225}Ac acetate control. The data are shown in Fig. 3. The ^{225}Ac -DOTMP complex dissociated rapidly in 25% serum while the ^{225}Ac -DOTA dissociated slowly with only 10% lost from the chelate after one ^{225}Ac $t_{1/2}$ of 10 d. It was clear that DOTA was a more stable

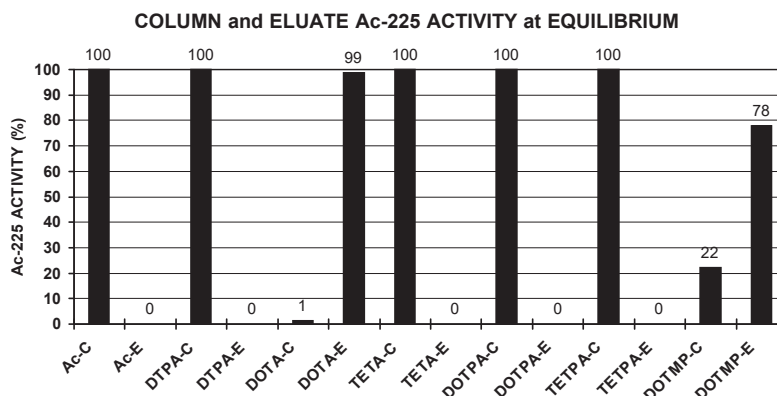


Fig. 2. The percentage ^{225}Ac activity at equilibrium bound to the respective chelate and eluted from the resin (E) and the percentage free or released ^{225}Ac activity bound by the Sephadex C-25 cation exchange resin (C).

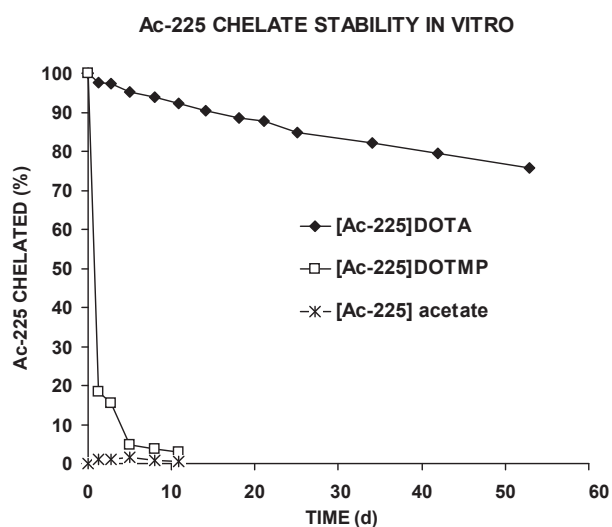


Fig. 3. The stability in vitro of ^{225}Ac -DOTA, ^{225}Ac -DOTMP, and ^{225}Ac -acetate in 25% human serum at 37°C .

chelating agent than DOTMP in vitro under these conditions, designed to mimic those expected in vivo. DOTA was selected as the chelating moiety to pursue in the development of a radioimmunotherapeutic pharmaceutical agent.

3.3. The two-step synthetic process to prepare ^{225}Ac -DOTA-IgG constructs

Given the favorable reactivity and stability of ^{225}Ac -DOTA complexes relative to DTPA and other chelating agents we focused our attention on DOTA-functionalized drugs. Initial attempts to label a DOTA-IgG construct with ^{225}Ac by conventional techniques (McDevitt et al., 1999b) resulted in such low yields of product as to make further development unfeasible. Test labeling with ^{111}In chloride using the same DOTA-IgG construct with the same reagents resulted in an 85%

yield of ^{111}In -DOTA-IgG after 20 min reaction (unpublished results) demonstrating the integrity of the construct, reagents, and methods. Higher temperatures were investigated as a method to increase the efficacy of stable ^{225}Ac complexation. However, such conditions are unsuitable for antibody stability and retention of function. Therefore, a two-step procedure was used. The first step of the synthetic scheme allowed a bifunctional DOTA derivative to chelate the ^{225}Ac at 50 – 60°C for a short period of time (30–60 min). The second step of the process was the reaction of the ^{225}Ac -DOTA-NCS with the IgG.

Thirty-one different labelings employing this two-step construct syntheses with ^{225}Ac were performed (see Table 1). The % radiochemical (RC) yield, specific activity (in units of Ci/g), % immunoreactivity (IR), and % radiochemical (RC) purity for the five different monoclonal antibodies tested as well as the usage of the MeO-DOTA or the 2B-DOTA are reported (mean $\pm$ SD values).

The percent radiochemical yields were low, $9.8 \pm 4.5\%$ ($n = 31$); however, this was a result of attaching approximately 5.4 DOTA per IgG. This was based upon the mean number of moles of IgG ($6 \text{ E-}9 \text{ mol}$), the mean number of moles of DOTA chelants ($330 \text{ E-}9 \text{ mol}$) and the mean number of moles of ^{225}Ac ions ($0.066 \text{ E-}9 \text{ mol}$) in these experiments. The ^{225}Ac -DOTA chelation efficiency of the first step was $93 \pm 8\%$ ($n = 26$); therefore, only 1 in about 5000 DOTA molecules was coordinating an ^{225}Ac ion. This translates into a final drug with about 1 in 775 IgG molecules that were ^{225}Ac radiolabeled. Efforts are underway in our laboratory to improve the overall radiochemical yield of this process. However, because of the extraordinary potency of ^{225}Ac , this methodology has been sufficient to yield enough drug, of a reasonable specific activity ($0.11 \pm 0.07 \text{ Ci/g}$, $n = 28$) in each labeling for extensive preclinical therapeutic studies in vitro and in vivo involving dozens of mice per experiment, toxicology studies in 5 kg

Table 1
[²²⁵Ac]–DOTA–IgG labeling results

Antibody	HuM195	B4	huJ591	mJ591	3F8	All data
RC yield (%)	10.6±3.6 <i>n</i> = 8	9.8±5.0 <i>n</i> = 8	8.2±1.1 <i>n</i> = 3	9.0±5.8 <i>n</i> = 6	10.6±5.4 <i>n</i> = 6	9.8±4.5 <i>n</i> = 31
Sp. act. (Ci/g)	0.11±0.06 <i>n</i> = 7	0.12±0.06 <i>n</i> = 7	0.08±0.02 <i>n</i> = 3	0.10±0.08 <i>n</i> = 6	0.10±0.11 <i>n</i> = 5	0.11±0.07 <i>n</i> = 28
IR (%)	81±4 <i>n</i> = 3	49±17 <i>n</i> = 6	43±33 <i>n</i> = 3	25±9 <i>n</i> = 6	58±11 <i>n</i> = 3	47±24 <i>n</i> = 21
RC purity (%)	96±5 <i>n</i> = 6	94±4 <i>n</i> = 6	96±2 <i>n</i> = 3	91±7 <i>n</i> = 6	98±2 <i>n</i> = 6	95±5 <i>n</i> = 27
MeO–DOTA	<i>n</i> = 2	<i>n</i> = 5	<i>n</i> = 0	<i>n</i> = 5	<i>n</i> = 0	<i>n</i> = 12
2B–DOTA	<i>n</i> = 6	<i>n</i> = 3	<i>n</i> = 3	<i>n</i> = 1	<i>n</i> = 6	<i>n</i> = 19

primates, and potentially for human clinical trials (estimated doses of 0.05–0.5 mCi). Therefore, other than for economic considerations, increasing yields may not be essential. The values tabulated underestimate the radiochemical labeling efficiency since only a fraction (approximately 80%) of the IgG mass used in the reaction was recovered in order to obtain a >90% pure product.

The heating to 50–60°C for 30–60 min allows for the ²²⁵Ac–DOTA reaction to go to completion rapidly and presumably yield the most stable species. Previous attempts to synthesize the constructs at lower temperatures yielded less stable complexes that were more prone to dissociate in serum stability studies (data not shown). A complete analysis of stability in vitro of an [²²⁵Ac]DOTA–IgG construct vs. similarly prepared [¹⁷⁷Lu]DOTA–IgG construct demonstrates the robust nature of these drugs (McDevitt et al., 2001).

The constructs thus prepared were 95±5% radiochemically pure (*n* = 27) and the mean percent immunoreactivity ranged from 25% to 81%, depending on the antibody being examined. The low percentage of immunoreactivity values more directly reflect the initial immunoreactivity of each IgG rather than a loss of activity resulting from the labeling steps as determined by checking the immunoreactivity of corresponding ¹¹¹In, ⁹⁰Y, and ²¹³Bi-labeled IgGs (McDevitt et al., 1999b, 2000; Nikula et al., 1999). The two-step synthetic process described herein allows the higher heating step to precede the combination with IgG thus sparing the protein harsh denaturing conditions. In addition, the potentially radiolytic alpha-particle emitting ²²⁵Ac was dilute enough to prevent damage to the IgG construct during labeling, although the addition of an aliquot of 150 g/l L-aa served as a radioprotectant

species for the reactions occurring in the first and second steps.

The reported radiochemical yields are lower than the actual ²²⁵Ac–DOTA–IgG labeling efficiencies since these were optimized to result in >90% radiochemically pure products for preclinical studies. The reported yields underestimate the coupling reaction efficiency as a consequence of the purification methodology used to clean up the reaction mixture. We are in the process of increasing reaction yields and optimizing the purification methods.

In summary, we provide here methods to readily coordinate ²²⁵Ac to a bifunctional DOTA and subsequently attach this moiety to an IgG or other suitable carrier protein. Compounds prepared in this fashion are quite stable and have been demonstrated to be potent in killing tumor cells in disseminated lymphoma and solid prostate carcinomas in vivo (McDevitt et al., 2001). Of course, this technology may be applied to other targeting agents such as antibody fragments, peptides, and other small molecules to yield a variety of potent radioimmunotherapeutic agents.

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