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Rapid and Efficient Production of Radiolabeled Antibody Conjugates Using Vacuum Diafiltration Guided by Mathematical Modeling

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Abstract

Increasing interest in the use of radiolabeled antibodies for cancer imaging and therapy drives the need for more efficient production of the antibody conjugates. Here, we illustrate a method for rapid and efficient production of radiolabeled antibody conjugates using vacuum diafiltration guided by mathematical modeling. We apply this technique to the production of 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA)-conjugated antibodies at the milligram and gram production scale and achieve radiolabeling efficiencies >95% using In-111. Using vacuum diafiltration, antibody-chelate conjugation and purification can be accomplished within the same vessel, and the entire process can be completed in <24 h. Vacuum diafiltration also offers safer and gentler processing conditions by eliminating the need to keep the retentate vessel under positive pressure through applied gas pressure or shear-inducing restriction points in the retentate flow path. Experimental data and mathematical model calculations suggest there exists a weak binding affinity ($\sim 10^4 \text{ M}^{-1}$) between the charged chelate molecules (e.g., DOTA) and the antibodies that slows the removal of excess chelate during purification. By analyzing the radiolabeling efficiency as a function of the number of diavolumes, we demonstrate the importance of balancing the removal of free chelate with the introduction of metal contaminants from the diafiltration buffer and also illustrate how to optimize radiolabeling of antibody conjugates under a variety of operating conditions. This methodology is applicable to the production of antibody conjugates in general.

INTRODUCTION

Radiolabeled antibodies have been used for therapy and imaging of cancer for over two decades (1). Radioimmunotherapy has been particularly effective in the treatment of hematologic malignancies (lymphomas), evidenced by the two FDA-approved radiolabeled anti-CD20 antibodies, Zevalin and Bexxar (2). The use of antibodies to achieve targeted delivery of radiation provides benefits not achievable by monoclonal antibodies or external beam radiation alone. Metal chelators, such as DOTA, can be covalently attached to antibodies and subsequently used to bind radioisotopes (3, 4). However, antibody-conjugated chelators can be hampered by slow radiolabeling kinetics and poor radiolabeling efficiencies (5). While functionalization of the chelate, as in the conjugation to lysines on a

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Supporting Information Available: Tabular presentation of the experimentally determined radiolabeling efficiencies and additional contour plots showing the impact of different operating parameters on the predicted radiolabeling efficiency as a function of the number of diavolumes. This information is available free of charge via the Internet at <http://pubs.acs.org>.

protein, has been shown to slow the metal loading rate and lower the overall thermodynamic stability of the metal complex (4, 6, 7), other factors such as metal contamination or unconjugated free chelate also contribute significantly to the low radiolabeling efficiencies.

Several techniques have been proposed to address the issues of metal contamination and removal of unconjugated chelate. Besides minimizing contact with metal containing materials, buffers can be processed with chelating resins such as Chelex 100 to reduce the metal burden (8–12). Care must be taken when using chelating resins, such as iminodiacetate (IDA), whose metal binding affinity may be orders of magnitude lower than chelators such as DOTA or diethylenetriaminepentaacetic acid (DTPA). If the resin is allowed to equilibrate with a solution containing the chelate (e.g., DOTA-antibody conjugate), then the metal can be thermodynamically driven to bind to the DOTA instead of the chelating resin depending on the relative concentrations. Pretreatment of the buffers using a column of the chelating resin can avoid such complications, and previous reports have demonstrated >99% removal of trace metal contaminants by column operation of the Chelex 100 resin (12).

Dialysis is a commonly used method for purification because of its ease of scalability and gentle conditions. Each dialysis-based buffer exchange or purification step is time-intensive and can require multiple days depending on the number of buffer changes required. Furthermore, dialysis can require a large amount of buffer volume that can also increase the risk of introducing metal contaminants. Other membrane-based purification strategies, such as ultrafiltration, can offer faster processing times with reduced buffer volumes. Application of ultrafiltration requires convecting the fluid toward the membrane, and the membrane can be designed to retain larger molecules, such as antibodies, while allowing low molecular weight impurities to penetrate through the membrane. If repeated cycles of ultrafiltration are used to remove impurity-containing fluid by replacing the fluid removed with impurity-free fluid, the process is called diafiltration. Rapid changes in antibody concentration resulting from the cycles of ultrafiltration and buffer replacement can negatively impact antibody stability. This problem is avoided by using constant-volume diafiltration, where the impurity-free buffer is added to the retentate at the same rate as the fluid is removed. Previous studies have demonstrated the feasibility of constant-volume diafiltration for the preparation of radiolabeled antibody conjugates (13, 14).

Here, we describe the use of a constant-volume vacuum-driven diafiltration process for the rapid buffer exchange and purification of conjugated antibodies in preparation for radiolabeling. A mathematical model of the diafiltration and radiolabeling steps is used to predict optimum operating conditions and elucidate possible mechanisms to explain experimental observations. We demonstrate the utility of this technique through production of DOTA-conjugated monoclonal antibodies at the milligram and gram production scale. Observed radiolabeling efficiencies with In-111 exceeded 95%, and model calculations are used to specifically illustrate how metal contamination and excess chelate can both contribute to low radiolabeling efficiencies. Using vacuum diafiltration, the entire conjugation and radiolabeling procedure can be accomplished within 24 h. Besides expediting the conjugation and purification process, the use of vacuum diafiltration avoids risks associated with membrane-based processing strategies that employ positive pressure gradients as the driving force. When the retentate vessel is under positive pressure, the potential for catastrophic leakage and loss of material is greater since fluid in the retentate will be driven out under pressure through any leak in the system; such a problem is avoided under vacuum diafiltration since the retentate vessel remains near atmospheric pressure. In certain embodiments of ultrafiltration or diafiltration processes the positive pressure gradient is created by applying a restriction in the retentate flow path. This restriction creates a region of increased shear stress that can exacerbate protein degradation. Vacuum diafiltration

provides more gentle processing conditions, and the stability of the applied vacuum enables strict control of the pressure differential across the membrane for the duration of the diafiltration process. The prevalence of small vacuum pumps or house vacuum systems make vacuum diafiltration widely applicable and easily scalable from the lab to clinical production, and we have used this technique for the conjugation of a variety of molecules (e.g., biotin and fluorochromes) with antibodies, thereby making it generally applicable to bioconjugations.

MATERIALS AND METHODS

Proteins

Rituximab (Rituxan), a chimeric monoclonal IgG1 against the CD20 antigen, was purchased from Genentech (South San Francisco, CA). Albuminar-25, a 25% w/w human serum albumin preparation with 0.02 M sodium N-acetyl-DL-tryptophan and 0.02 M sodium caprylate, was purchased from Aventis Behring (King of Prussia, PA).

Radioisotope

High-purity indium-111 (indium chloride) was obtained in 0.05 M HCl from Trace LifeSciences (Denton, TX).

Buffers

Dulbecco's phosphate-buffered saline without calcium and magnesium was purchased from Mediatech (21-031-CV, Herndon, VA). Sodium bicarbonate buffer was prepared using sodium bicarbonate (7412, Mallinckrodt Baker, Phillipsburg, NJ) and plasma grade water (W9, Fisher Chemical, Fairlawn, NJ). Ammonium acetate buffer was prepared using ammonium acetate (372331, Aldrich, St. Louis, MO) and plasma grade water (W9, Fisher Chemical). Sodium acetate buffer was prepared using sodium acetate (3462-01, Mallinckrodt Baker, Phillipsburg, NJ) and plasma grade water (W9, Fisher Chemical).

Chelex 100 trace metal removal

Biotechnology Grade Chelex 100 chelating resin (Bio-Rad, 100–200 mesh, 143–2832) was used in column mode for trace metal removal from the ammonium acetate buffer. The Chelex 100 resin (100-g) was initially washed with ~400 mL of 0.1N NaOH and allowed to stand for 18 hours for depyrogenation. Following depyrogenation, the liquid was removed by vacuum filtration and the resin was rinsed with 500 mL of plasma grade water. Subsequently, the resin was washed with ~400 mL 0.1 N HCl followed by ~750 mL of plasma grade water. The resin was then washed twice with 0.25 M ammonium acetate buffer (pH 7) and poured into a 5-cm diameter XK50 column (GE Healthcare, Chalfont St. Giles, United Kingdom) connected to an AKTA FPLC (GE Healthcare). In the column mode, the resin was further rinsed with ~600 mL of 0.25 M ammonium acetate buffer (pH 7) such that the measured pH and conductivity of the effluent from the column matched that of the inlet ammonium acetate buffer. The final bed volume of the Chelex 100 resin in the ammonium form was ~150 mL. Ammonium acetate buffer (0.25 M, pH 7) was pumped through the equilibrated Chelex 100 column at a flow rate of 5 mL/min and the effluent was collected directly from the outlet of the column into a sterile 2-L Stedim Flexboy Bag (Stedim Biosystems, Aubagne, France).

Pilot-scale vacuum diafiltration

A schematic of the pilot-scale vacuum diafiltration setup is shown in Figure 1A. Vacuum diafiltration was performed at room temperature using a MidGee Hoop (GE Healthcare) cross flow hollow fiber filter with a 30 kDa MWCO polysulfone membrane, two 1-mm

inner diameter fibers, and a membrane area of 73 cm². The retentate solution (containing the protein to be diafiltered) was circulated through the hollow fiber membrane at a flow rate of 25–50 mL/min using a peristaltic pump. A vacuum pump was used to apply vacuum to the filtrate side of the membrane to achieve a net transmembrane pressure (TMP) of approximately 8 psi. Under these operating conditions, the flux was 20–25 L m⁻² h⁻¹ (LMH) during diafiltration of the antibody solutions. A weighing balance was used on both the filtrate and retentate vessels to monitor changes in mass (i.e., volume). The diafiltration buffer was pumped into the retentate vessel by a peristaltic pump at a flow rate equal to the flow rate of the filtrate to achieve constant-volume diafiltration.

Lab-scale vacuum diafiltration

A schematic of the lab-scale vacuum diafiltration setup is shown in Figure 1B. By using the inlet port, normally utilized for attachment of a gas pressure line, to introduce additional diafiltration buffer under a closed-system operation using HPLC tubing and fittings, constant-volume diafiltration could be achieved. This apparatus requires no additional equipment to achieve constant-volume diafiltration, and it can be operated under vacuum generated from a house vacuum line or a small vacuum pump. If desired, multiple diafiltration devices could be run in parallel using a common vacuum line, offering flexibility that would facilitate screening multiple variations of an antibody conjugate during the development stage.

Lab-scale diafiltration experiments were performed at room temperature using an Amicon Stirred Cell Model 8010 (10-mL sample volume, 4.1 cm² effective membrane area) with a Biomax ultrafiltration membrane (PBQK, Millipore, Billerica, MA) with a 50 kDa MWCO. The stirring rate was adjusted such that the vortex created was approximately one-third the depth of the liquid in the stirred cell. Vacuum was applied to the filtrate side of the membrane using a vacuum pump to achieve a net TMP of approximately 8.5 psi. Under these operating conditions, the flux was typically 25–35 LMH during diafiltration of the antibody solutions. The diafiltration buffer reservoir was connected to the stirred cell using 1/16" i.d. HPLC tubing connected by HPLC fittings so that the outlet for the diafiltration buffer tubing dripped onto the wall of the stirred cell as shown in Figure 1B (to minimize bubble formation and to prevent contact with any other parts of the stirred cell device). Because the diafiltration setup was operated as a closed system, the diafiltration buffer was pulled into the stirred cell at the same rate as the filtrate flowed out of the stirred cell.

Removal of acetyltryptophanate (AT) in the presence of human serum albumin (HSA) or Rituxan by vacuum diafiltration

The removal of AT in the presence of both HSA and Rituxan by vacuum diafiltration was studied using the pilot-scale diafiltration setup with a MidGee Hoop cross flow hollow fiber filter and a retentate flow rate of 25 mL/min. The HSA was used at a concentration of 7 mg/mL, diluted in PBS from Albuminar-25 (which contains 0.02 M AT). Rituxan was used at a concentration of 8.3 mg/mL along with N-acetyl-L-tryptophan at a concentration of 4×10⁻⁴ M⁻¹. The HSA was initially at a concentration of 7 mg/mL in PBS, and it was diafiltered with approximately 6 diavolumes of 0.1 M bicarbonate buffer (pH 8.3). The Rituxan was initially at a concentration of 8.3 mg/mL in PBS, and it was first diafiltered with 5 diavolumes of 0.1 M bicarbonate buffer (pH 8.3). Subsequently, the N-acetyl-L-tryptophan was added to the Rituxan solution and the mixture was diafiltered with 7 diavolumes of 0.25 M sodium acetate buffer (pH 7.2). Samples (~100 µL) were removed from the retentate vessel during the diafiltration. The concentration of HSA/Rituxan and AT in each sample was determined by integrating the A280 peak area of the trace from a Superdex 200 column (30 cm × 10 mm i.d.; GE Healthcare) using an AKTA purifier HPLC (GE Healthcare).

Removal of DOTA or DTPA in the presence of Rituxan by vacuum diafiltration

The removal of DOTA or DTPA in the presence of Rituxan by vacuum diafiltration was studied using both the pilot-scale and lab-scale diafiltration setup. For the pilot-scale diafiltration, the Rituxan was initially at a concentration of 8.3 mg/mL in PBS, and it was buffer-exchanged by diafiltration with 5 diavolumes of 0.1 M bicarbonate buffer (pH 8.3). Subsequently, 1 mM DOTA (Parish Chemical Company, Orem, UT) was added to the Rituxan solution and the mixture was diafiltered with 7 diavolumes of 0.25 M sodium acetate buffer (pH 7.2). For the lab-scale diafiltration, the Rituxan was initially at a concentration of 7 mg/mL in PBS, and it was buffer-exchanged into 0.1 M bicarbonate buffer (pH 8.3). After addition of either 1 mM DOTA or 1 mM DTPA, the mixture was diafiltered with 7 diavolumes of 0.25 M ammonium acetate buffer (pH 7). Samples (~50–100 μ L) were removed from the retentate vessel during the diafiltration. Each experiment was also repeated in the absence of Rituxan.

The concentration of DOTA or DTPA in each sample was determined by a colorimetric assay using arsenazo III. Arsenazo III exhibits a bathochromic shift from 548 nm to 660 nm upon binding to free gadolinium. To the DOTA- or DTPA-containing samples in microcentrifuge tubes were added 20 nmol of gadolinium chloride in 0.1 M ammonium acetate buffer (pH 6). The reaction mixture was allowed to incubate for 15 min at 75°C to enable complex formation between the gadolinium and chelate. Using the elevated temperature also helps to minimize interference in the assay from the potential binding between IgG and arsenazo III since IgG molecules show rapid denaturation and aggregation at these temperatures (15). Subsequently, the reaction mixture was combined with an equal volume of 1 mM arsenazo III, and the absorbance at 655 nm was measured with a Bio-Rad 680 microplate reader (Bio-Rad, Hercules, CA) to determine the amount of free gadolinium remaining. The relative concentration of DOTA or DTPA was calculated from the change in the level of gadolinium-arsenazo III complexes formed (as indicated by absorbance at 655 nm) since the chelated gadolinium cannot interact with arsenazo III.

Pilot-scale production of radiolabeled antibodies using vacuum diafiltration

Buffer exchanges and purification required in the preparation of DOTA-conjugated Rituxan were performed using the pilot-scale diafiltration setup with a MidGee Hoop cross flow hollow fiber filter and a retentate flow rate of 50 mL/min. The Rituxan was conjugated with the NHS-activated form of the macrocyclic chelator, DOTA, which reacts with exposed amine groups on the antibody. Rituxan was processed at a concentration of 7 mg/mL in a volume of 50 mL. It was initially exchanged into 0.1 M bicarbonate buffer (pH 8.3) with 1 mM DTPA (metal scavenger) for conjugation. This was achieved with 5 diavolumes and was performed in ~2 hours, after which a 19.5-molar excess of DOTA-NHS (Macrocyclics, Dallas, TX) was added and allowed to react with the Rituxan for 2 hours at room temperature. Dissolving the DOTA-NHS in water instead of the pH 8.3 buffer can help to reduce unwanted NHS hydrolysis prior to addition to the reaction vessel due to the low pH of the DOTA-NHS solution. Subsequently, the unreacted DOTA-NHS was removed and the conjugate exchanged into 0.25 M sodium acetate buffer (pH 7.2) with 10 diavolumes, and this diafiltration was performed in ~4 hours. Diafiltration with an additional 5 diavolumes of 0.25 M sodium acetate buffer was performed 5 days later. After each diavolume during the post-conjugation diafiltration, a 100 μ L sample was removed and subjected to radiolabeling with In-111. Additionally, each sample was subjected to HPLC analysis on a Superdex 200 column (30 cm $\times$ 10 mm i.d.; GE Healthcare) to analyze antibody concentration and aggregate formation by following absorbance at 280 nm.

Lab-scale production of radiolabeled antibodies using vacuum diafiltration

For preparation of DOTA-conjugated antibodies with the lab-scale vacuum diafiltration setup, diafiltration was performed using an Amicon stirred cell and a Biomax ultrafiltration membrane with a 50 kDa MWCO. The Rituxan was processed at a concentration of 7 mg/mL in a volume of 5 mL. The initial exchange into the 0.1 M bicarbonate buffer (pH 8.3) with 1 mM DTPA was achieved with 5 diavolumes and was performed in ~2 hours, after which a 15-molar excess of DOTA-NHS (Macrocyclics, Dallas, TX) dissolved in water was added and allowed to react with the antibody inside the stirred cell for 1 hour at room temperature. Subsequently, the unreacted chelate was removed and the conjugate exchanged into 0.25 M ammonium acetate buffer (pH 7, processed with Chelex 100 as described previously) with 18 diavolumes (still using the same stirred cell device) in ~8 hours. During the post-conjugation diafiltration, samples (~75 μ L each) were removed at specified diavolumes and subjected to radiolabeling with In-111. Each sample was also subjected to HPLC analysis on a Superdex 200 column (30 cm $\times$ 10 mm i.d.; GE Healthcare) to analyze antibody concentration and aggregate formation by following absorbance at 280 nm.

Radiolabeling efficiency

Radiolabeling reactions were performed at a ratio of 5 mCi/mg antibody conjugate in acetate buffer (pH 5.5), and the reaction was allowed to proceed for 45 minutes at 43°C. Analysis of radiolabeling efficiency was measured by instant thin layer chromatography (ITLC). Two equal aliquots of the reaction mixture were withdrawn, and 10 mM DTPA solution (pH 6) was added to one aliquot to give a final concentration of 1 mM DTPA. This was incubated for 10 minutes at room temperature. Samples from the radiolabeling reaction with and without the DTPA chase were then analyzed on 2.25" $\times$ 0.5" Tec-Control ITLC strips (Cat. # 151-020, Biodex Medical Systems, Shirley, NY) using 0.9% saline as the mobile phase. Each strip was cut into 0.5-cm segments, and each segment was counted in a Cobra Auto-gamma counter. The ratio of radioactivity at the origin (In-111 bound to the DOTA-antibody conjugate or free, hydrolyzed In-111) to the radioactivity near the solvent front (DOTA- or DTPA-chelated radioisotope) was calculated. An estimate of the amount of free radioisotope remaining was obtained from the difference between the fraction of the radioactivity measured at the origin (bottom half) for ITLC strips run with or without the DTPA chase.

Mathematical model calculations

Model calculations were performed using MATLAB 7 (The MathWorks, Inc., Natick, MA). The differential equations governing the clearance of solutes during diafiltration were solved numerically using the forward Euler method with a step size equal to 1/1000 the total number of diavolumes analyzed. Chelate-metal complex formation is assumed to reach equilibrium (both during diafiltration and during the radiolabeling reaction) to simplify calculation of metal loading and overall radiolabeling efficiency.

RESULTS

Theoretical development

Impurity removal by vacuum diafiltration—Removal of low molecular weight impurities during constant-volume diafiltration can be calculated from a mass balance on the impurity in the retentate. If the impurity exhibits a reversible binding affinity interaction with the retained molecule, such as an antibody, then impurity removal will proceed more slowly as a result of the binding interaction. The impact of protein binding during diafiltration has been previously analyzed using D-tryptophan and bovine serum albumin as a model system (16). The change in the concentration of impurity, C_p , during constant-volume diafiltration is given by the equation

$$V \frac{dC_i}{dt} = -f_i C_i S_i J_v A \quad [1]$$

where V is the constant retentate volume, f_i is the unbound fraction of impurity i , S_i is the sieving coefficient of impurity i , J_v is the filtrate flux through the membrane, and A is the membrane area. The binding interaction between the impurity and a retained protein can be expressed in terms of an equilibrium binding constant, K_a , which is defined as

$$K_a = \frac{[C_{i,b}]}{[C_{i,f}][C_{p,f}]} \quad [2]$$

where $C_{i,b}$ is the bound concentration of impurity i , $C_{i,f}$ is the free concentration of impurity i , and $C_{p,f}$ is the free concentration of the protein which interacts with the impurity. This expression can be used to derive an expression for f_i the unbound fraction of impurity i , given by the following equation

$$f_i = \frac{-nC_p + C_i - \frac{1}{K_a} + \sqrt{\left(nC_p - C_i + \frac{1}{K_a}\right)^2 + \frac{4C_i}{K_a}}}{2C_i} \quad [3]$$

where n is the number of binding sites per protein for impurity i . For an impurity that has no protein binding ($f_i = 1$), the concentration of impurity remaining after a given number of diavolumes, N_D , is equal to

$$C_i = C_{i,0} e^{-N_D S_i} \quad [4]$$

where $C_{i,0}$ is the initial impurity concentration and the concentration of remaining impurity decays exponentially with the number of diavolumes.

Chelate-metal complex formation—Chelation of metal species by compounds such as DOTA can be approximated as an association reaction between the metal species, M , and the chelate, C , to form a metal-chelate complex, $M-C$. This equilibrium binding can be described by

$$K_f = \frac{[M-C]}{[M][C]} \quad [5]$$

where K_f is the stability constant of the metal-chelate complex. This equation can be used to estimate the equilibrium metal loading into the chelate molecules given the stability constant and the initial concentrations of M and C (17). Metal loading is calculated for both free chelate (not protein-bound), C_f , and chelate molecules covalently coupled to the antibody molecule, $C-Ab$. For example, separate equilibrium relationships can be written for the free and conjugated chelate,

$$K_{f1} = \frac{[M-C_f]}{[M][C_f]} \quad [6]$$

$$K_{f2} = \frac{[M-C-Ab]}{[M][C-Ab]} \quad [7]$$

where K_{f1} is the free chelate-metal stability constant and K_{f2} is the antibody-chelate-metal stability constant.

For the model calculations, any non-radioactive metal present in the buffer during diafiltration or the radiolabeling reaction is also allowed to form complexes with either free chelate or antibody-chelate according to the equilibrium relationships described by equations [6] and [7]. Chelate (remaining after diafiltration) or chelate-antibody conjugate that binds metal during the diafiltration stage is no longer available for binding to additional metal species (radioactive or non-radioactive) during the radiolabeling reaction. Since the thermodynamic stability for the DOTA-metal or DTPA-metal complexes is generally characterized by high $\log K_f$ values, a $\log K_f$ value of 23 was used for all calculations regardless of the metal species (18). This is justified for these calculations since the equilibrium is essentially shifted completely in favor of the complex formation for such large $\log K_f$ values. More complicated model calculations could incorporate the relative formation rate constants for the DOTA or DTPA binding to the metal species, but such equations were not incorporated into the model used here. The model calculations used here also restrict nonspecific antibody binding (equation [3]) to the metal-free chelate, meaning any chelate that has formed a metal complex is no longer available for antibody binding.

Since the fraction of the radiolabeled metal, M^* , that is bound to free chelate or chelate-antibody conjugates is equal to the fraction of total metal bound by each (under the assumption of equivalent chelation of all metal species), the radiolabeling efficiency is calculated as

$$RE = \frac{[M - C - Ab]}{[M]_{tot}} = \frac{[M^* - C - Ab]}{[M^*]_{tot}} \quad [8]$$

where M_{tot} is the total concentration of all metal species present, M^*-C-Ab is the concentration of radioisotope-labeled chelate-antibody conjugates, and M^*_{tot} is the total concentration of radioisotope added to the radiolabeling reaction.

Solute removal by vacuum diafiltration

It has been previously established that tryptophan and its derivative acetyltryptophan reversibly bind human serum albumin with a stoichiometry of approximately 1:1, and this interaction can be characterized by affinity constants of 10^4 – 10^5 M^{-1} for L-tryptophan and 10^3 – 10^4 M^{-1} for D-tryptophan (19). Furthermore, Klotz et al. estimated the binding affinity between N-acetyl-DL-tryptophan (acetyltryptophanate) and bovine serum albumin to be $\sim 4 \times 10^3$ M^{-1} (20). Using equation [1], the concentration of acetyltryptophanate (AT) can be predicted during diafiltration in the presence of human serum albumin. The predicted removal of AT was compared to experimentally determined values obtained by sampling the retentate during vacuum diafiltration. The results shown in Figure 2A reveal that the experimental data closely match the predicted removal of a solute with a sieving coefficient of 1 and an affinity constant for human serum albumin of approximately 8×10^3 M^{-1} , within the same order of magnitude as those binding constants reported previously for the interaction between tryptophan and albumin. No such binding was observed between N-acetyl-L-tryptophan and the monoclonal antibody, Rituxan, however (Figure 2A).

We extended this analysis to the interaction between the metal chelators, DOTA or DTPA, and the monoclonal antibody, Rituxan. Because each Rituxan molecule contains >100 negatively or positively charged amino acids, we hypothesized that it would potentially act as an ion exchanger with a weak affinity for charged molecules such as DOTA or DTPA, which contain multiple amine and carboxylate groups. Least squares regression was first used to estimate the sieving coefficient of the chelate in the absence of Rituxan from the data in Figure 2, yielding an average value of $S = 0.95$. Although the Biomax (polyethersulfone) and polysulfone membranes do exhibit a negative surface charge density at the pH values used in these studies, the negatively charged DOTA or DTPA molecules

still exhibit a sieving coefficient of near unity, as indicated by the observed clearance of each chelator in the absence of Rituxan (Figure 2). Shao et al. determined that small charged impurities can be strongly rejected by charged membranes (including Biomax) at low flux or low ionic strength (21). Because the buffers used in our experiments had relatively high ionic strengths, these charge interactions were minimal. Subsequently, the affinity constant characterizing the binding between DOTA or DTPA and Rituxan was estimated using the data for their clearance in the presence of Rituxan (Figure 2). Assuming two binding sites per antibody ($n = 2$) based on the symmetrical IgG1 structure containing two identical heavy chains and two identical light chains, the magnitude of the affinity constant, $K_d \sim 1 \times 10^4 - 3 \times 10^4 \text{ M}^{-1}$, is similar to the weak binding observed between AT and HSA. Since the magnitude of this affinity constant will vary with the exact antibody or buffer used during diafiltration, the slightly lower affinity constant observed at the lab-scale relative to the pilot-scale may be the result of using ammonium acetate instead of sodium acetate buffer. The ammonium ions, more than sodium ions, may compete with the amines on the antibody for binding with the chelate carboxylates. Such a hypothesis is supported by the observation that ammonium ions have a greater affinity toward cation exchangers than sodium ions (22). Future studies must be done to more precisely examine the impact that different processing conditions can have on the interaction between the chelate and antibody molecules. Comparison can also be made between the affinity constants measured using antibodies with or without prior chelate modification, as the presence of covalently conjugated chelate may impact the binding affinity toward nonspecifically bound chelate.

Production of radiolabeled antibodies using vacuum diafiltration

During the production of radiolabeled antibodies, DOTA is first conjugated to the antibody at a molar excess of DOTA-NHS:antibody. The unreacted DOTA-NHS must be removed since it will directly compete with the conjugated DOTA-Ab for binding to the radioactive isotope during the radiolabeling reaction. The chelate-antibody nonspecific affinity constant estimated from the data in Figure 2 can be combined with equations [1]–[3] to estimate the number of diavolumes needed to reduce the free chelate concentration below a desired threshold. Furthermore, the use of equations [6]–[8] can allow estimation of the predicted radiolabeling efficiency which depends on the amount of free chelate remaining as well as the total metal contaminants introduced during diafiltration. Such information allows the rational design of DOTA-Ab production to yield high radiolabeling efficiencies. To demonstrate such a procedure, a DOTA-Rituxan conjugate was synthesized and purified using both pilot-scale and lab-scale vacuum diafiltration and subsequently radiolabeled with In-111. The results of radiolabeling efficiency versus number of diavolumes are shown graphically in Figure 3A and provided in tabular format in the Supporting Information. Each sample was also subjected to gel filtration chromatography on a Superdex 200 column to analyze relative antibody concentration as well as the formation of any aggregates. At all diavolumes tested, there was negligible fluctuation in the measured antibody concentration (Figure 3B) and the aggregate formation remained <1.5%.

Theoretical considerations for maximizing radiolabeling efficiencies

Because variability in radiolabeling efficiencies has often plagued the production of radiolabeled antibodies, we sought to elucidate key parameters controlling the radiolabeling efficiency using a mathematical model of the vacuum diafiltration and radiolabeling processes. Additional parameters for the model calculations are given in Table 1. The solid lines in Figures 4A and 4B show the good fit that can be achieved between the predicted and experimentally determined radiolabeling efficiencies under the conditions used at both the pilot and lab scales, respectively. The dashed lines show the radiolabeling efficiency predicted if the chelate molecules exhibited no antibody binding. In the absence of antibody binding, significant radiolabeling efficiency should begin to appear as early as 4 diavolumes.

Instead, measurable radiolabeling began to appear after >6 diavolumes had been processed, indicating hindered removal of the free chelate. This hindered removal can be accounted for by a weak binding affinity ($\sim 10^4 \text{ M}^{-1}$) between the free chelate and the antibody molecules (Figure 2). The rapid sigmoidal rise in radiolabeling efficiency between diavolumes 6 and 10 would be expected if the free chelate (DOTA or DTPA) was able to bind the metal more readily than the DOTA-antibody conjugate. Previous studies have demonstrated that the stability constant for metals binding to monofunctionalized DOTA molecules (such as those containing amide bond linkages to the lysines on proteins) are approximately two orders of magnitude lower than the stability constant for metals binding to free DOTA (7, 23). Furthermore, there is a decreased metal loading rate when DOTA is conjugated to protein (6). Consistent with these observations, the model predictions closely matched the experimental data observed here if the metal preferentially loaded into the free chelate relative to the DOTA-Ab conjugate. This was implemented in the model by setting the actual metal loading as a fraction of the maximum equilibrium loading value (equations [6] and [7]) depending on the relative concentrations of the two species. The preferential binding by free chelate was realized by multiplying the free chelate concentration by 100 (higher effective concentration) to enhance the relative fraction of metal bound by free chelate. Such a preferential binding by free chelate enhances the sigmoidal shape of the curve and inhibits radiolabeling at low diavolumes when there is still sufficient free chelate (DOTA or DTPA) to compete with the DOTA-Ab for metal binding. However, after approximately 6 DV, the free chelate is predicted to be at a low enough concentration that it no longer can bind to all of the metal present in the radiolabeling reaction and the radiolabeling efficiency rapidly increases with additional diavolumes. When the radiolabeling efficiency begins to plateau after ~ 10 DV, there is a negligible amount remaining of free chelate available for radioisotope binding (it has either been removed in the filtrate or already occupied with non-radioactive metal), so all of the subsequent radioactive or non-radioactive metal in the radiolabeling reaction will bind to the DOTA-Ab conjugate. Once there is no longer any additional free chelate to be removed, then additional diavolumes will potentially add metal contaminants (depending on the metal content of the diafiltration buffer) to the DOTA-Ab conjugate until a point is reached when enough of the DOTA-Ab conjugate has been occupied by metal contaminants that it no longer can bind all of the radioactive isotope during radiolabeling, thus decreasing the radiolabeling efficiency. However, the model predicts that addition of metal can enhance radiolabeling efficiencies if there is substantial free chelate remaining in the radiolabeling reaction since the free chelate would otherwise be able to bind the majority of both the radioactive and non-radioactive metal present. Only after this extra free chelate is saturated with bound metal will the DOTA-Ab be able to bind a significant portion of the radioactive metal, meaning higher radiolabeling efficiencies can be achieved by addition of non-radioactive metal even when free chelate remains at low diavolumes.

To test this hypothesis experimentally, the same batch of In-111 that was used for the pilot-scale radiolabeling reactions shown in Figure 3 was again used to radiolabel the samples from DV 8–10 of the pilot-scale diafiltration, although the In-111 had decayed for an additional 5 days before these labelings were performed. The DOTA-Ab conjugate was also subjected to an additional 5 diavolumes by pilot-scale diafiltration and samples were removed at each diavolume for radiolabeling with this same batch of decayed In-111. The samples from DV 8–10 and the new samples from DV 11–15 were radiolabeled under the same reaction conditions that were used 5 days previously, and the radiolabeled samples were subjected to ITLC. The ITLC analysis was performed with and without a DTPA chase to differentiate between radioisotope that had been bound by free chelate remaining in the DOTA-Ab preparation versus free radioisotope that was still available to bind to the DTPA chase. Under the ITLC conditions without the DTPA chase, both free radioisotope and radioisotope bound to DOTA-Ab will remain at the origin, while radioisotope bound by any

residual free chelate (DOTA or DTPA not removed during diafiltration) will migrate with the solvent. However, if an additional DTPA chase is included during ITLC, then any free radioisotope that would have remained at the origin will bind to DTPA and migrate with the solvent. The difference between the radioactive concentrations at the origin can thus be used to indicate the presence of free radioisotope remaining after the radiolabeling reaction. An overview of the experimental procedure is depicted in Figure 5A, while the experimental data and model calculations are summarized in Figures 5B and 5C, respectively. Figure 5B depicts the results of the ITLC measurements with and without a DTPA chase. The radiolabeling efficiencies are provided in tabular format in Supporting Information. Free radioisotope began to appear after 12–13 DV, and the model calculations offer a possible explanation for these observations, as shown in Figure 5C. As hypothesized, the use of the decayed In-111 (which contains a higher relative amount of non-radioactive metal contaminants and thus a lower radioisotope purity) enhances the observed radiolabeling efficiency at low diavolumes because the additional non-radioactive metal saturates the remaining free chelate and thereby allows the remaining radioactive metal to be bound by the DOTA-Ab conjugate. However, at higher diavolumes, the level of free chelate has been reduced to low levels and instead of helping the radiolabeling efficiency, the additional non-radioactive metal from the diafiltration buffer (in this case, sodium acetate buffer that was not pre-treated with the Chelex 100 resin) and the decayed In-111 preparation now saturates the available DOTA-Ab binding sites and prevents the conjugate from being able to bind all of the radioactive metal. This is indicated by the detection of free radioactive isotope by ITLC performed with and without the DTPA chase. On the other hand, no free radioactive isotope was detected by ITLC for samples even out to 18 diavolumes when using the Chelex 100-treated ammonium acetate buffer which should have reduced metal contamination (Supporting Information). Previous studies have shown that the complexes formed between antibody-conjugated DOTA and metals are extremely stable, with total release of bound In-111 of ~3% after 240 hours in the presence of 1 mM DTPA (23). Such observations suggest that most of the bound metal is stably coordinated by the DOTA conjugates and the contribution of metal binding to other sites on the antibody is likely to be minimal. Furthermore, this stability of the metal within the DOTA complexes enables the use of a DTPA chase to probe for the presence of free remaining radiometal after the labeling reaction is completed. The data shown in Figure 5 therefore corroborate the predictions made with the model calculations and also offer important insights into potential mechanisms for the variability in radiolabeling efficiencies.

As described earlier, the model calculations assume equilibrium binding between the chelate and metal, although the residence time during the constant diafiltration process may limit the accuracy of such an assumption. The assumption of equilibrium therefore represents a worst-case scenario for metal contamination from the diafiltration buffers. Notwithstanding, there is likely an optimum number of diavolumes that will achieve the highest radiolabeling efficiency, representing a balance between removal of free material and introduction of non-radioactive metal with the diafiltration buffer or the radioisotope preparation. The plots in Figure 6 illustrate the impact of different operating parameters (antibody concentration during diafiltration, radioisotope purity, diafiltration buffer metal concentration, and the radiolabeling reaction ratio) on the predicted radiolabeling efficiency for the pilot-scale diafiltration parameters. Similar analyses were performed for the lab-scale diafiltration parameters as well as for antibody conjugates containing a higher conjugation efficiency with 5 DOTA per antibody (Supporting Information). While these plots apply to the specific operating conditions used in this study, the observed trends are generally applicable for the production of radiolabeled antibodies using diafiltration.

DISCUSSION

In this report, we describe the use of constant-volume vacuum diafiltration for the production of radiolabeled antibodies. The method is a modification of standard diafiltration techniques whereby the transmembrane pressure is created by applying a vacuum to the filtrate side of the membrane as opposed to using positive pressure on the retentate side of the membrane. Vacuum diafiltration can be quickly and easily implemented with minimal equipment requirements since a house vacuum line is sufficient to generate the transmembrane pressure required for filtration. Furthermore, the use of vacuum as the driving force minimizes the risk for leakage or loss of expensive antibody materials since the membrane interface is the only region of significant pressure differential that makes contact with the antibody, thereby meaning recoverable loss into the filtrate is the most likely failure. In contrast, applying a positive pressure to the retentate (antibody) side of the membrane creates the potential for catastrophic leakage out of the retentate vessel if any of the seals were to fail under the applied pressure differential. Such safety precautions become especially important at the clinical production scale. Vacuum diafiltration is easily scalable, as we have demonstrated here on both the milligram and gram production scale, and overall processing times and buffer volumes required can be drastically reduced relative to other purification strategies such as dialysis. While we illustrate the use of the method in the production of radiolabeled antibodies, vacuum diafiltration offers an easily accessible strategy for preparing any antibody conjugate. Besides using vacuum diafiltration to achieve high radiolabeling efficiencies (>90%) for several different antibodies in addition to Rituxan, we also have used it for labeling antibodies with fluorochromes and biotin.

Through the use of mathematical modeling combined with experimental data, we also elucidate several factors that can contribute to variable radiolabeling efficiencies. Since excess free chelate can directly compete with chelate-antibody conjugates for radiolabeling, it must be stringently removed prior to radiolabeling to ensure maximum yield. The experimental data presented here suggest a weak binding affinity between DOTA or DTPA and antibodies, further complicating the removal of excess chelate. Notwithstanding, free chelate can be reduced to acceptably low levels through extensive purification, as illustrated here by the high radiolabeling efficiencies achieved at diavolumes greater than approximately 10. An interesting result of following the radiolabeling efficiency as a function of the number of diavolumes was the observation that there can be an optimum number of diavolumes for maximizing radiolabeling efficiency, beyond which point the yield will decrease if metal contaminants from the buffers outweigh the benefit derived from removing additional free chelate. Controlling the level of metal contaminants therefore emerged as one of the most important factors influencing the consistency of the radiolabeling efficiency.

The influence of metal contamination becomes most significant with respect to the purity of the radioisotope used for radiolabeling. Metal contamination during the initial conjugation and diafiltration steps can be minimized by including metal scavengers (e.g., DTPA) in the buffers used for washing or diafiltration (although one must then also ensure that sufficient diavolumes are processed during diafiltration to reduce the free DTPA concentration to levels that will not interfere with radiolabeling). Additionally, treatment of the diafiltration buffers with chelating resins, such as Chelex 100, can significantly reduce the potential burden of metal contamination (8–12). In our experience, high radiolabeling efficiencies (>95%) are achieved using Chelex 100-treated ammonium acetate buffer even out to 18 diavolumes. Removal of trace metal contaminants in the diafiltration buffer by chelating resins helps to minimize variability that could be introduced by fluctuations in buffer metal content, thereby widening the range of diavolumes that can achieve high radiolabeling efficiencies and yielding a more robust process (Figure 6 and Supporting Information).

Unfortunately, neither of these methods is feasible for separation of the radioactive metal from non-radioactive metal in the radioisotope preparation. Evaluation of the certificate of analysis provided by several vendors of radioisotope preparations reveals that even though the radionuclide purity may be >99.9%, there can be sufficient metal contaminants to impact radiolabeling efficiencies. For example, the specifications for In-111 (Trace LifeSciences) require the metal contaminants (including Cd, Cu, Fe, Ni, Pb, and Zn) to be at a level below 0.1 $\mu\text{g/mCi}$. Given the specific activity of In-111 (4.7×10^7 Ci/mol), then the In-111 can represent <1 mol% of the total metal contaminants and meet specifications. A typical radiolabeling reaction might be performed at a concentration of 5 mCi/mg antibody conjugate, meaning there is ~100-fold molar excess of Ab-conjugated DOTA molecules relative to the moles of In-111 in 5 mCi. The resulting radiolabeling efficiency represents the fraction of the total In-111 atoms that will be bound by the Ab-conjugated DOTA molecules. If the In-111 represents 0.5% of the total metal atoms present in the radioisotope preparation, for example, then a 200-fold excess of available Ab-conjugated DOTA molecules would be required for all of the In-111 to be bound (assuming, for simplicity, that all of the metal atoms can bind to DOTA with equal efficiency). If the radiolabeling ratio results in only a 100-fold excess of Ab-conjugated DOTA molecules present (as described above), then the maximum theoretical radiolabeling efficiency would be 50%. These calculations illustrate the ease with which variations in radioisotope purity can alter the radiolabeling efficiencies under typical reaction conditions, even when the radioisotope is within the manufacturer's specification limits for metal contaminants.

To facilitate more consistent radiolabeling efficiencies, the metal contaminants in radioisotope preparations must be stringently controlled. The specifications for current preparations make radiolabeling reactions inherently subject to variability. Regardless of any changes made in the production of radioisotopes, several steps can be taken toward achieving more consistent radiolabeling efficiencies. First, metal contaminants must be reduced in the steps prior to the radiolabeling reaction. By minimizing the amount of metal contaminants in the buffers through the use of metal scavengers or chelating resins, it is possible to more accurately predict the ratio of available antibody-conjugated DOTA molecules to the total metal in the radiolabeling reaction. Otherwise, metal contamination prior to the radiolabeling reaction can lead to a significant fraction of the antibody-conjugated DOTA molecules already being occupied by metals, making them unavailable for radiolabeling. Second, it is important to analyze the certificate of analysis provided with the radioisotope preparation and estimate its purity (moles radioisotope relative to total moles of metal present). This purity can then be used to predict the radiolabeling reaction ratio (mCi/mg antibody conjugate) needed to ensure that there is sufficient capacity to bind all of the radioisotope during the radiolabeling reaction. Third, care must be taken to account for the decay of the radioisotope preparation, meaning the purity of the radioisotope will decrease with time as the radioisotope decays. As illustrated in Figure 5, this change in purity can either increase or decrease the radiolabeling efficiency depending on the presence of additional free chelate (which can be estimated from the number of diavolumes processed) and the ratio of radioisotope to antibody-conjugated DOTA molecules (mCi/mg antibody conjugate) used during the radiolabeling reaction.

In summary, vacuum diafiltration offers a convenient and scalable method that can be easily implemented for the production of radiolabeled antibody conjugates. Mathematical models enable a rational approach for optimizing operating conditions for the production of radiolabeled antibodies, including controlling metal contamination and free chelate removal. Several suggestions to improve overall radiolabeling efficiencies were offered according to model predictions and experimental observations. The study presented here facilitates production of radiolabeled antibodies with increased consistency and higher efficiency,

offering benefits for patient safety, therapeutic efficacy, and cost minimization in the clinical application of radioimmunotherapeutics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Schematic for vacuum diafiltration at the pilot-scale (**A**) and lab-scale (**B**).

Figure 2.

Effect of protein binding on the removal of acetyltryptophanate, DOTA, or DTPA by vacuum diafiltration. **(A)** Experimentally determined removal of acetyltryptophan by pilot-scale vacuum diafiltration in the presence of 7 mg/mL HSA (circles) or 8.3 mg/mL Rituxan (diamonds). **(B)** Experimentally determined removal of DOTA by pilot-scale vacuum diafiltration in the presence of 8.3 mg/mL Rituxan (circles) or no protein (diamonds). **(C)** Experimentally determined removal of DOTA by lab-scale vacuum diafiltration in the presence of 7 mg/mL Rituxan (circles) or no protein (diamonds). **(D)** Experimentally determined removal of DTPA by lab-scale vacuum diafiltration in the presence of 7 mg/mL Rituxan (circles) or no protein (diamonds). Predicted clearances are shown for protein binding (solid lines) and no protein binding (dashed lines).

Figure 3. Production of radiolabeled Rituxan using vacuum diafiltration. **(A)** Experimentally determined radiolabeling efficiency as a function of the number of diavolumes by pilot-scale and lab-scale vacuum diafiltration. **(B)** Relative Rituxan concentration determined by HPLC (Superdex 200, 280 nm) as a function of the number of diavolumes during vacuum diafiltration.

Figure 4.

Comparison of predicted and experimentally determined radiolabeling efficiency as a function of the number of diavolumes. **(A)** Model calculations are shown for the pilot-scale diafiltration when a 19.5-molar excess of DOTA-NHS is reacted with Rituxan and the excess free chelate exhibits either a weak affinity for Rituxan of $3 \times 10^4 \text{ M}^{-1}$ (solid line) or no affinity for Rituxan (dashed line). The pilot-scale diafiltration data from Figure 3A are shown for comparison (circles). **(B)** Model calculations are shown for the lab-scale diafiltration when a 15-molar excess of DOTA-NHS is reacted with Rituxan and the excess free chelate exhibits either a weak affinity for Rituxan of $1 \times 10^4 \text{ M}^{-1}$ (solid line) or no

affinity for Rituxan (dashed line). The lab-scale diafiltration data from Figure 3A are shown for comparison (circles). Additional model parameters are given in Table 1.

A **B** **C** **Figure 5.**

Effect of metal contamination on radiolabeling efficiency. **(A)** Flow chart depicting the experimental procedure. **(B)** Radiolabeling efficiency as a function of the number of diavolumes on day 0 (fresh radioisotope) and on day 5 (decayed radioisotope). ITLC was performed with or without a DTPA chase to detect the presence of free metal remaining after the radiolabeling reaction. The day 0 data points with DTPA (circles) are the same data as shown in Figure 3; additionally, the ITLC samples without DTPA (squares) are shown for comparison, demonstrating the absence of any free metal. The day 5 data points represent samples taken from DV 8–10 from the initial diafiltration and samples taken from an additional 5 diavolumes of the same DOTA-antibody conjugate preparation (DV 11–15). Data are shown for ITLC samples with DTPA (diamonds) or without DTPA (triangles), demonstrating the appearance of free metal beginning at DV 12–13. **(C)** The predicted radiolabeling efficiency as a function of the number of diavolumes was calculated for a

radioisotope purity of 0.025 (day 0, solid line) and 0.008 (day 5, dashed line). Experimental data of the ITLC samples with DTPA from day 0 (circles) and day 5 (diamonds) are shown for comparison.

Figure 6.

Contour plots showing the impact of different operating parameters on the predicted radiolabeling efficiency as a function of the number of diavolumes for pilot-scale diafiltration. Model calculations are used to estimate the predicted radiolabeling efficiency for a range of (A) antibody concentrations, (B) radioisotope purities, (C) metal concentrations in the diafiltration buffer, and (D) radiolabeling reaction ratios given as the amount of radioactivity per mass of antibody. Fixed parameter values were chosen to match those of the pilot-scale diafiltration experiment as given in Table 1. Lighter color represents higher radiolabeling efficiencies, and labels indicate the radiolabeling efficiency of a given contour line.

Table 1

Additional parameters used for model calculations to predict chelate clearance and radiolabeling efficiency.

Parameter Description	Value	Determination
Impurity sieving coefficient (DOTA, DTPA)	0.95	Least squares regression of experimental data in Figure 2
Relative chelating ability (free vs. conjugated chelate)	100	Estimated from experimental data, supported by literature (7, 23)
K_{fl} , free chelate-metal association constant (M^{-1})	1×10^{23}	Literature (17, 18)
K_{f2} , antibody-chelate-metal association constant (M^{-1})	1×10^{21}	Calculated from K_{fl} and the relative chelating ability of free vs. conjugated chelate
K_{ns} , chelate-antibody nonspecific binding association constant (M^{-1})	3×10^4 = pilot 1×10^4 = lab	Least squares regression of experimental data in Figure 2
n , number of nonspecific binding sites per antibody	2	Estimated from experimental data and the symmetry of IgG molecules
Number of conjugated DOTA per antibody	2	Literature (4)
Buffer metal concentration (M)	4×10^{-6} = pilot 2×10^{-7} = lab	Estimated from max specifications from certificate of analysis (sodium acetate, pilot-scale) and an estimated 90–95% reduction from max specifications by Chelex 100 treatment (ammonium acetate, lab-scale)
Radioisotope purity at calibration time and after 5-day decay	0.025 = day 0 0.008 = day 5	Estimated from certificate of analysis; decayed value calculated using In-111 half-life of 67 h