



# Preparation of $^{212}\text{Pb}$ -labeled monoclonal antibody using a novel $^{224}\text{Ra}$ -based generator solution <sup>☆</sup>



Sara Westrøm <sup>a,b,c</sup>, Roman Generalov <sup>d</sup>, Tina B. Bønsdorff <sup>a</sup>, Roy H. Larsen <sup>a,e,\*</sup>

<sup>a</sup> Oncoinvent AS, Oslo, Norway

<sup>b</sup> Department of Tumor Biology, Institute for Cancer Research, The Norwegian Radium Hospital, Oslo University Hospital, Oslo, Norway

<sup>c</sup> Institute of Clinical Medicine, University of Oslo, Oslo, Norway

<sup>d</sup> Nordic Nanovector ASA, Oslo, Norway

<sup>e</sup> Sciencons AS, Oslo, Norway

## ARTICLE INFO

### Article history:

Received 22 February 2017

Received in revised form 6 April 2017

Accepted 15 April 2017

### Keywords:

Radioimmunoconjugate

Lead-212

$^{212}\text{Pb}$

TCMC-trastuzumab

Radium-224

Targeted alpha therapy

## ABSTRACT

**Introduction:** Alpha-emitting radionuclides have gained considerable attention as payloads for cancer targeting molecules due to their high cytotoxicity. One attractive radionuclide for this purpose is  $^{212}\text{Pb}$ , which by itself is a  $\beta$ -emitter, but acts as an *in vivo* generator for its short-lived  $\alpha$ -emitting daughters. The standard method of preparing  $^{212}\text{Pb}$ -labeled antibodies requires handling and evaporation of strong acids containing high radioactivity levels by the end user. An operationally easier and more rapid process could be useful since the 10.6 h half-life of  $^{212}\text{Pb}$  puts time constraints on the preparation protocol. In this study, an *in situ* procedure for antibody labeling with  $^{212}\text{Pb}$ , using a solution of the generator nuclide  $^{224}\text{Ra}$ , is proposed as an alternative protocol for preparing  $^{212}\text{Pb}$ -radioimmunoconjugates.

**Methods:** Radium-224, the generator radionuclide of  $^{212}\text{Pb}$ , was extracted from its parent nuclide,  $^{228}\text{Th}$ . Lead-212-labeling of the TCMC-chelator conjugated monoclonal antibody trastuzumab was carried out in a solution containing  $^{224}\text{Ra}$  in equilibrium with progeny. Subsequently, the efficiency of separating the  $^{212}\text{Pb}$ -radioimmunoconjugate from  $^{224}\text{Ra}$  and other unconjugated daughter nuclides in the solution using either centrifugal separation or a PD-10 desalting size exclusion column was evaluated and compared.

**Results:** Radiolabeling with  $^{212}\text{Pb}$  in  $^{224}\text{Ra}$ -solutions was more than 90% efficient after only 30 min reaction time at TCMC-trastuzumab concentrations from 0.15 mg/mL and higher. Separation of  $^{212}\text{Pb}$ -labeled trastuzumab from  $^{224}\text{Ra}$  using a PD-10 column was clearly superior to centrifugal separation. This method allowed recovery of approximately 75% of the  $^{212}\text{Pb}$ -antibody-conjugate in the eluate, and the remaining amount of  $^{224}\text{Ra}$  was only  $0.9 \pm 0.8\%$  ( $n = 7$ ).

**Conclusions:** The current work demonstrates a novel method of producing  $^{212}\text{Pb}$ -based radioimmunoconjugates from a  $^{224}\text{Ra}$ -solution, which may be simpler and less time-consuming for the end user compared with the method established for use in clinical trials of  $^{212}\text{Pb}$ -TCMC-trastuzumab.

© 2017 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

## 1. Introduction

The benefit of using  $\alpha$ -emitting radionuclides in cancer therapy is well established [1]. Their high linear energy transfer and short range in tissue give them advantages over  $\beta$ - and  $\gamma$ -emitting radionuclides in terms of more effective cancer cell inactivation and less damage to surrounding normal tissue when used in targeted therapy. The awareness of  $\alpha$ -emitting radionuclides for cancer therapy has increased after the FDA approval of  $^{223}\text{Ra}$ -dichloride, Xofigo®, as the first in class

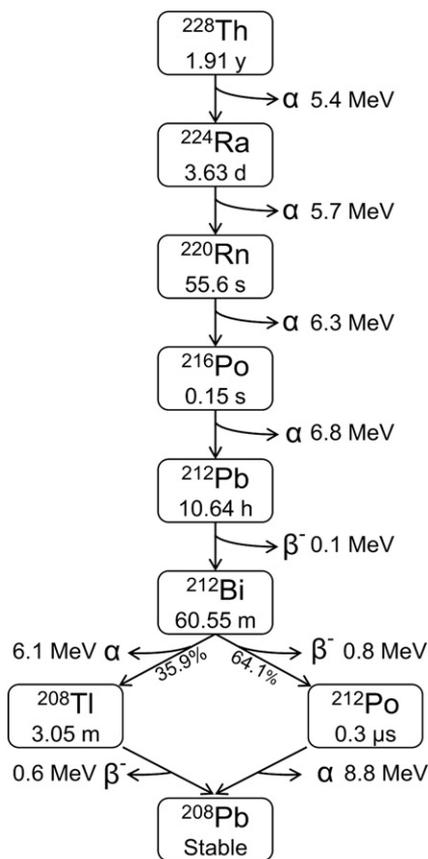
$\alpha$ -emitting radiopharmaceutical. Xofigo® is approved for treatment of patients with metastatic castration-resistant prostate cancer disseminated to the bones. In contrast to Xofigo®, where the resemblance of the  $^{223}\text{Ra}$ -isotope to calcium naturally guides the radionuclide to the bones, the majority of proposed  $\alpha$ -therapies require the use of a targeting molecule. Most widespread is the use of a protein, such as a monoclonal antibody or a peptide, as the targeting moiety.

Even if the potential of  $\alpha$ -emitters in targeted cancer therapy is well-known, very few  $\alpha$ -emitting radionuclides are suitable for radiotherapeutic purposes. Many have shortcomings related to inconvenient half-lives (either too short or too long), lack of viable chemistry to link them to targeting molecules, difficulties in production and/or availability concerns [2]. One radionuclide which has been suggested and examined for applications in targeted  $\alpha$ -therapy is  $^{212}\text{Pb}$ . It is part of the  $^{228}\text{Th}$  decay chain (Fig. 1) and decays via  $\beta$ -emission to the therapeutically

<sup>☆</sup> This is a free access article and can also be viewed on the journal's Web site ([www.nucmedbio.com](http://www.nucmedbio.com)). Complimentary access to this article is available until the next issue publishes online.

\* Corresponding author at: Oncoinvent AS, Kjelsåsveien 168 B, 0884, Oslo, Norway. Tel.: +47 920 16 487.

E-mail address: [royhlars1@yahoo.no](mailto:royhlars1@yahoo.no) (R.H. Larsen).



**Fig. 1.** The decay chain of  $^{228}\text{Th}$ , including details on each nuclide's half-life, main mode of decay and mean energies.

potent  $\alpha$ -emitting daughter  $^{212}\text{Bi}$ . To avoid challenges with the short half-life of  $^{212}\text{Bi}$  (60.6 min), a strategy has been to use  $^{212}\text{Pb}$  as an *in vivo* generator for  $^{212}\text{Bi}$ . In addition to the convenient longer half-life of  $^{212}\text{Pb}$ , another advantage with the  $^{212}\text{Pb}$ - $^{212}\text{Bi}$  *in vivo* generator is that it delivers about ten times the dose per administered activity unit compared to  $^{212}\text{Bi}$  alone [3], thus greatly reducing the amount of activity required. A  $^{212}\text{Pb}$ -based radioimmunoconjugate is currently in clinical testing against peritoneal cancer [4–6], after the preclinical efficacy [7–10] and safety [11,12] of the product have been thoroughly demonstrated.

The process for generating  $^{212}\text{Pb}$  and the protocols for radiolabeling have evolved in line with the growing interest of  $^{212}\text{Pb}$  as a therapeutic radionuclide. The first reported  $^{212}\text{Pb}$  generator was based on a  $^{228}\text{Th}$ -source [13]. The first generator had problems with radiolytic damage to the resin which caused decreased yields with time and was also associated with serious radiation safety issues. Generator systems based on emanation of  $^{220}\text{Rn}$  [14,15] improved radiation safety, but concerns with radiolytic damage were not completely overcome. A generator based on  $^{224}\text{Ra}$  [16] has been designed to address challenges arising from use of  $^{228}\text{Th}$ -based generators. This principle has become the most common way of generating  $^{212}\text{Pb}$ . In the  $^{224}\text{Ra}$ -based generator,  $^{224}\text{Ra}$  is first separated from  $^{228}\text{Th}$  using an anion exchange resin, followed by absorption of  $^{224}\text{Ra}$  onto a macroporous, organic cation-exchange resin column. Subsequently,  $^{212}\text{Pb}$  can be eluted from the column using high acid concentrations.

In terms of  $^{212}\text{Pb}$ -radiolabeling, the main improvement in protocol came with the emergence of the efficient lead-chelator TCMC (2-(4-isothiocyanatobenzyl)-1,4,7,10-tetraaza-1,4,7,10-tetra-(2-carbamoyl methyl)-cyclododecane) [17]. Many chelators have been assessed [18], including the commonly used p-SCN-Bn-DOTA (2-(4-isothiocyanatobenzyl)-1,4,7,10-tetraazacyclododecane tetraacetic acid), but TCMC has become the chelation

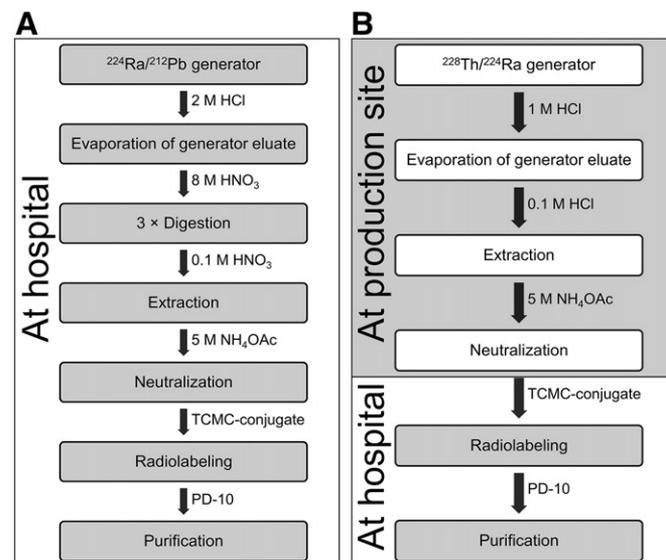
chemistry of choice as it creates a more stable complex at lower pH and provides more efficient conjugation than its DOTA analog [17].

The 10.6 h half-life of  $^{212}\text{Pb}$  imposes time constraints to its practical application and therefore rapid, efficient and reliable production and purification procedures are required. The previously mentioned  $^{212}\text{Pb}$ -radioimmunoconjugate undergoing clinical evaluation is based on the monoclonal antibody trastuzumab conjugated to TCMC. Baidoo and colleagues [19] have provided a detailed review of the protocol used for  $^{212}\text{Pb}$  elution and radiolabeling of TCMC-conjugates that were generated for use in this trial. To summarize the protocol; A  $^{212}\text{Pb}$ -generator consisting of  $^{224}\text{Ra}$  adsorbed on a column is shipped to the radiopharmacy at the hospital where  $^{212}\text{Pb}$  is separated from  $^{224}\text{Ra}$  when it is eluted in acid from the generator. The  $^{212}\text{Pb}$ -eluate is then evaporated and digested three times with acid. The series of acid digestions are performed by dissolving  $^{212}\text{Pb}$  in concentrated nitric acid (8 M) before evaporating the solution. The next step is reconstitution of  $^{212}\text{Pb}$  in dilute nitric acid (0.1 M) before neutralization and radiolabeling. In Fig. 2A, a flow chart of the process is depicted. The authors reported that the preparation of an injectable dose required approximately 3.5 h, and after that some time will pass when quality control of the product is performed. Drawbacks with the protocol described above such as loss of  $^{212}\text{Pb}$  due to time-consuming steps involving work and evaporation of strong acid solutions motivated us to investigate an alternative method of labeling monoclonal antibodies with  $^{212}\text{Pb}$  which may be easier to perform in a hospital environment. Herein we have evaluated an *in situ* procedure of  $^{212}\text{Pb}$ -labeling of TCMC-conjugated antibody in a solution of  $^{224}\text{Ra}$  in equilibrium with daughter nuclides, which functions as a liquid  $^{212}\text{Pb}$  generator, and subsequent removal of  $^{224}\text{Ra}$  as an alternative strategy for preparing  $^{212}\text{Pb}$ -based radioimmunoconjugates.

## 2. Methods

### 2.1. Radioactivity measurements

Radioactive samples were measured in the window 70–80 keV on a Cobra II Autogamma counter (Packard Instruments, Downer Grove, IL, USA) or from 60 to 110 keV and 520–640 keV on a Hidex Automatic Gamma Counter (Hidex, Turku, Finland). The energy ranges below



**Fig. 2.** A flow chart comparing  $^{212}\text{Pb}$  production and labeling of TCMC-conjugates between the method established for use in clinical trials of  $^{212}\text{Pb}$ -TCMC-trastuzumab presented by Baidoo and colleagues (A) and the  $^{224}\text{Ra}$  solution protocol suggested in this paper (B). The details of the method described by Baidoo et al. are based on the information given in reference [19].

110 keV are assumed to mainly count X-rays and  $\gamma$ -radiation from  $^{212}\text{Pb}$  with very little contribution from other radionuclides in the series. Since  $^{224}\text{Ra}$  decay results in modest  $\gamma$ -emission in an energy region with more abundant  $\gamma$  from  $^{212}\text{Pb}$ , the  $^{224}\text{Ra}$  activity was determined indirectly from the counts in the 70–80 keV or 60–110 keV window. This was carried out by re-measuring the samples after minimum 3 days, when the initial  $^{212}\text{Pb}$  present in the sample had decayed and equilibrium between  $^{224}\text{Ra}$  and newly produced  $^{212}\text{Pb}$  had been established. The 520–640 keV window was used to determine  $^{212}\text{Bi}$  indirectly from the highly abundant  $^{208}\text{Tl}$   $\gamma$ . To quantify the amount of  $^{212}\text{Bi}$  correctly, samples were measured after approximately 20 min, when transient equilibrium between  $^{208}\text{Tl}$  and the  $^{212}\text{Bi}$  parent had been established. In Table 1 all  $\gamma$ -rays in the  $^{224}\text{Ra}$ -series with higher abundance than 1% are listed together with an overview of which nuclides in the series have X- and/or  $\gamma$ -rays that fall within the 60–110 and 520–640 keV windows respectively. A radioisotope calibrator (CRC-25R, Capintec Inc., Ramsey, NJ, USA) was used to measure amounts of radioactivity higher than 50 kBq.

## 2.2. The $^{224}\text{Ra}$ -generator

Radium-224 was extracted from a generator based on  $^{228}\text{Th}$  (Eckert & Ziegler, Braunschweig, Germany) immobilized on a DIPEX® (Eichrom Technologies LLC, Lisle, IL, USA) actinide resin. The details of the  $^{224}\text{Ra}$ -generator setup have been described elsewhere [20]. In brief,  $^{228}\text{Th}$  in 0.1 M  $\text{HNO}_3$  was mixed with a portion of actinide resin and after a few hours a column was prepared by first applying a small amount of inactive actinide-resin before the portion containing  $^{228}\text{Th}$  was loaded onto the column. The inactive resin was laid in the bottom to serve as a catcher layer in case of some release of  $^{228}\text{Th}$  during operation of the generator. Radium-224 could be eluted regularly from the generator column with 1 M HCl. This crude eluate was loaded onto a second actinide resin cartridge for further purification. The second cartridge was washed with 1 M HCl to produce an eluate containing  $^{224}\text{Ra}$ . This solution was evaporated to dryness and the residue was dissolved in 0.2 mL or more of 0.1 M HCl. All work described in this paragraph was performed in a glove-box with negative pressure. At its maximum capacity, the  $^{224}\text{Ra}$ -generator column contained approximately 2 MBq of  $^{228}\text{Th}$ . The  $^{224}\text{Ra}$ -generator column was stored inside the glove-box in a 2.5 cm thick lead pot which reduced the dose rate at 30 cm distance from the source to approximately 1.3  $\mu\text{Sv/h}$ . The handling of the generator and the produced eluates was also performed behind similar shielding using lead-bricks.

**Table 1**

Overview of which nuclides in the  $^{224}\text{Ra}$ -series having X- and/or  $\gamma$ -lines in the 60–110 keV and/or 520–640 keV energy range. Gamma lines which are outside these energy ranges are shown in the “Other” column. The abundance of the lines is given in parentheses and all radiation with 1% or higher abundance is shown.

Nuclide	60–110 keV	520–640 keV	Other
$^{224}\text{Ra}$			241.0 keV (4.1%)
$^{220}\text{Rn}$			
$^{216}\text{Po}$			
$^{212}\text{Pb}$	74.8 keV (10.3%) 77.1 keV (17.1%) 86.8 keV (2.1%) 87.4 keV (4.0%) 87.4 keV (1.5%)		238.6 keV (43.6%) 300.1 keV (3.3%)
$^{212}\text{Bi}$			727.3 keV (4.3%)*
$^{212}\text{Po}$			
$^{208}\text{Tl}$	75.0 keV (1.2%)†	510.8 keV (8.1%)† 583.2 keV (30.5%)†	277.4 keV (2.4%)† 860.6 keV (4.5%)† 2614.5 keV (35.8%)†

\* Branching corrected for 64.1%.

† Branching corrected for 35.9%.

## 2.3. Radiolabeling of antibodies

The humanized anti-HER2 IgG1 monoclonal antibody trastuzumab (Herceptin, Roche, Basel, Switzerland) conjugated to a chelator, TCMC (Macrocylics Inc., Dallas, TX, USA), was used for radiolabeling with  $^{212}\text{Pb}$ .

Before conjugation to TCMC, the original buffer of trastuzumab was exchanged with carbonate buffer (0.1 M  $\text{NaHCO}_3$  and 5 mM  $\text{Na}_2\text{CO}_3$  in metal free water of pH. Eur grade). To achieve this, a solution of trastuzumab was washed four times with carbonate buffer using a centrifugal concentrator (Vivaspin 15R, 30 or 50 kDa MWCO, Sartorius Stedim Biotech, Göttingen, Germany). During each wash trastuzumab was concentrated by a factor of ten. The concentration of antibody in the new buffer was determined by UV-spectrophotometry (Hitachi U-1900, Hitachi High-Technologies Corporation, Tokyo, Japan) using the standard absorbance value of 1.4 for immunoglobulins at 280 nm for 0.1% solutions. A solution of TCMC dissolved in 5 mM HCl was added to trastuzumab in carbonate buffer in a five- to tenfold molar excess of chelator to antibody. The mixture reacted for 2 h at room temperature with gentle agitation. To remove unconjugated chelator from TCMC-trastuzumab and reduce pH, the carbonate buffer was exchanged with 0.9% NaCl, using a centrifuge filtering cartridge (Vivaspin 15R, 30 or 50 kDa MWCO). The sample was diluted 1:10 with 0.9% NaCl and the TCMC-antibody conjugate was concentrated tenfold by centrifugation. This procedure was repeated a total of three times. The described protocol for TCMC-conjugation has previously shown to yield a chelator to antibody ratio of 2–5 [10,17]. The TCMC-trastuzumab conjugate was stored at 4 °C until radiolabeling.

A solution of  $^{224}\text{Ra}$  in equilibrium with progeny in 0.1 M HCl and 0.5 M  $\text{NH}_4\text{OAc}$  was used for radiolabeling. The pH of the solution was verified to be approximately 5–6 using pH paper (Merck Millipore general pH indicator paper, Merck KGaA, Darmstadt, Germany). TCMC-trastuzumab and the  $^{224}\text{Ra}$ -solution were mixed and incubated usually for minimum 30 min on a ThermoMixerR (Eppendorf AG, Hamburg, Germany) at 37 °C and 750 rpm. This solution, consisting of  $^{212}\text{Pb}$ -labeled trastuzumab and free  $^{224}\text{Ra}$  and daughters is referred to as the “reaction mixture”. Different concentrations of TCMC-trastuzumab conjugate (in the range from 0.1 to 6 mg/mL) were radiolabeled using the described method. Typically, the reaction mixture volume was between 30 and 130  $\mu\text{L}$ . In one experiment, the influence of incubation time on yields of  $^{212}\text{Pb}$  and  $^{212}\text{Bi}$  labeling of TCMC-trastuzumab (4 mg/mL) was evaluated by taking out samples from the reaction vial after 5, 15 and 30 min. The radiochemical purity (RCP) of the samples was analyzed by instant thin layer chromatography (ITLC). Experiments to examine possible radiolysis of the antibody during labeling was also performed. The reaction mixture was stored overnight to obtain elevated self-dose to the radiolabeled product followed by analysis with high-performance liquid chromatography (HPLC) or measurement of the immunoreactive fraction.

## 2.4. Instant thin layer chromatography assay procedure

The RCP of the  $^{212}\text{Pb}$  labeled antibody in the reaction mixture was evaluated using ITLC strips (model # 150–772, Biodex Medical Systems Inc., Shirley, NY, USA). An aliquot of reaction mixture was mixed with a twofold excess (by volume) of formulation buffer consisting of 7.5% human serum albumin, 5 mM EDTA in Dulbecco's PBS, and adjusted to pH 7 with NaOH. The reaction mixture with added formulation buffer was whirl-mixed for 4–5 s and left for another 5–10 min to allow chelation of unbound radioisotopes with EDTA. An ITLC strip was spotted with 1–5  $\mu\text{L}$  of sample at the origin line and placed in a small beaker with about 0.5 mL of 0.9% NaCl for development. After the solvent front had moved to the designated solvent front line, the strip was cut in half at the cut line and each half was placed in a glass tube for counting. In this system  $^{212}\text{Pb}$ -TCMC-trastuzumab does not migrate

from the bottom half (B) whereas  $^{212}\text{Pb}$  (and other free radionuclides) complexed with EDTA migrates to the upper half (U). The percent radionuclide bound to the antibody was determined as:

$$\%RCP = \frac{CPM(B)}{CPM(B) + CPM(U)} \times 100$$

where CPM denotes the counts per minute.

### 2.5. Measurement of immunoreactive fraction

The immunoreactive fraction of  $^{212}\text{Pb}$ -TCMC-trastuzumab was determined in a one-point, live-cell binding assay, performed according to a previously published procedure [21]. Briefly, samples of  $16\text{--}20 \times 10^6$  HER2 expressing human osteosarcoma cells, OHS [21,22], were prepared and incubated at room temperature with  $^{212}\text{Pb}$ -TCMC-trastuzumab or blocked with an excess of trastuzumab prior to addition of  $^{212}\text{Pb}$ -TCMC-trastuzumab. The percent bound activity after washing was determined for each sample, and the immunoreactive fraction of  $^{212}\text{Pb}$ -TCMC-trastuzumab was estimated to be the total bound minus the unspecific bound in the blocked samples.

### 2.6. High-performance liquid chromatography

HPLC was performed in a 1260 Infinity VL System (Agilent Technologies, Santa Clara, CA, USA) using a size exclusion TSKgel G3000SWxl column (Tosoh Bioscience, Griesheim, Germany, product number 08541) with UV (220 and 280 nm) and radiometric (Radiomatic 150TR Flow Scintillator Analyzer, Perkin Elmer, Waltham, MA, USA) detection. The mobile phase was 50 mM sodium phosphate (pH 7.0) containing 250 mM NaCl with a flow rate of 0.8 ml/min.

### 2.7. Purification of radiolabeled antibodies

Two different methods of purification were evaluated in this study: purification using a centrifugal concentrator and purification with a desalting column. In the first case the reaction mixture was loaded in a concentrator spin tube (Vivaspin 4, 50 kDa MWCO, Sartorius Stedim Biotech, Göttingen, Germany) and diluted with 0.9% NaCl until the total volume was 4 mL. The content was concentrated tenfold by centrifugation. The concentrate (C) was collected and measured immediately ( $t = 0$ ). The yield (Y) of the purification process was estimated as the percentage of antibody-bound  $^{212}\text{Pb}$ -activity in the concentrate:

$$\%Y(^{212}\text{Pb}\text{--TCMC}\text{--trastuzumab}) = \frac{CPM(C)_{t=0}}{CPM(T)_{t=0} \times RCP_{\text{reaction mixture}}} \times 100$$

The total activity (T) loaded onto the spin concentrator tube was determined from a sealed reference sample prepared from an aliquot of the reaction mixture taken prior to purification. A minimum of 3 days after the first measurement ( $t = \text{eq}$ ), when equilibrium between  $^{224}\text{Ra}$  and  $^{212}\text{Pb}$  was reached, all samples were measured again and the percentage of  $^{224}\text{Ra}$  remaining in the concentrate was calculated:

$$\%^{224}\text{Ra} = \frac{CPM(C)_{t=\text{eq}}}{CPM(T)_{t=\text{eq}}} \times 100$$

The second purification method utilized a Sephadex G-25 PD-10 column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) for separation of the radiolabeled TCMC-trastuzumab from unbound radionuclides. The column was equilibrated with approximately 25 mL of Dulbecco's PBS supplemented with 0.5% bovine serum albumin as elution buffer. Both the crude reaction mixture and reaction mixture added a tenfold excess (by volume) of formulation buffer was purified with PD-10. In the latter case the reaction mixture was reacted with formulation buffer

for at least 10 min to allow chelation of unbound radioisotopes with EDTA before application. Quenching of the reaction mixture with EDTA prior to application on the PD-10 column was performed to investigate whether this could lead to more efficient separation of the radiolabeled antibody from unconjugated nuclides. The sample was loaded on top of the column and allowed to enter the column bed completely before elution buffer was added. The elution process was continued until seven fractions of 1 mL each was collected in Eppendorf tubes, before the activity in all fractions were measured immediately. The radiolabeled antibody was typically eluted in fractions 3–5 (F3–F5), and the yield of the process was estimated to be the percentage of antibody-bound  $^{212}\text{Pb}$ -activity in these fractions:

$$\%Y(^{212}\text{Pb}\text{--TCMC}\text{--trastuzumab}) = \frac{CPM(F3 + F4 + F5)_{t=0}}{CPM(T)_{t=0} \times RCP_{\text{reaction mixture}}}$$

The total activity (T) loaded onto the PD-10 column was determined from a sealed reference sample prepared from an aliquot of the reaction mixture taken prior to purification. RCP of the product in fraction 4 was determined by the previously described ITLC procedure. After at least 3 days, the samples were re-measured and the percentage of  $^{224}\text{Ra}$  remaining in fraction 3–5 was calculated:

$$\%^{224}\text{Ra} = \frac{CPM(F3 + F4 + F5)_{t=\text{eq}}}{CPM(T)_{t=\text{eq}}}$$

To assess co-elution of radionuclides with the protein fractions an experiment was performed where the radiolabeling and PD-10 purification were performed as described above, but unconjugated trastuzumab was used instead of TCMC-conjugated trastuzumab. The presence of  $^{212}\text{Pb}$  and  $^{212}\text{Bi}$  in the seven collected fractions was determined by measurement in the 60–110 and 520–640 keV windows. By measuring the samples 5 min, 20 min, 1 h, 1 day and 5 days after the PD-10 purification was finalized, the decay rate could also be assessed. This was necessary to ascertain if the  $\gamma$ -activity in the 520–640 keV window was reflecting the presence of  $^{212}\text{Bi}$  in addition to  $^{208}\text{Tl}$ . From the day 5 measurements, when all samples had reached equilibrium, the amount of  $^{224}\text{Ra}$  was determined.

### 2.8. Retention of the $\alpha$ -emitting $^{212}\text{Pb}$ daughter $^{212}\text{Bi}$ by the TCMC-chelator

In a  $^{224}\text{Ra}$ -solution in equilibrium the ratio of  $^{212}\text{Bi}$  activity to  $^{212}\text{Pb}$  activity is approximately equal to one. A sealed  $^{224}\text{Ra}$  sample in equilibrium with progeny was used as a reference to determine an efficiency factor (Bq/CPM) for the 60–110 and 520–640 keV windows. After PD-10 purification of reaction mixture quenched with EDTA, fraction 4 was measured 10 min, 20 min, 60 min and 22 h after end of purification. The  $^{212}\text{Bi}$  to  $^{212}\text{Pb}$  ratio at the different time points was estimated using the efficiency factors. An online universal decay calculator (<http://www.wise-uranium.org/rcc.html>) was used to determine the theoretical  $^{212}\text{Bi}$  to  $^{212}\text{Pb}$  ratios as a function of time based on different initial  $^{212}\text{Bi}$  to  $^{212}\text{Pb}$  ratios, ranging from a sample of pure  $^{212}\text{Pb}$  without any  $^{212}\text{Bi}$  present to a sample where the activity ratio is equal to one. Under the assumption that all activity in fraction 4 straight after purification was bound to the antibody-conjugate, an estimate of the portion of  $^{212}\text{Bi}$  retained in the TCMC-chelator could be deduced by comparing the experimentally determined ratios with plots of ingrowth for different theoretical  $^{212}\text{Bi}$  to  $^{212}\text{Pb}$  ratios.

## 3. Results and discussion

### 3.1. Radiolabeling of TCMC-trastuzumab

Radiolabeling of TCMC-trastuzumab with  $^{212}\text{Pb}$  in a solution of  $^{224}\text{Ra}$  in equilibrium with daughter nuclides was successful. The procedure yielded a product with RCP above 90% already at 0.15 mg/mL of

antibody conjugate, and above 95% from 1 mg/mL and at higher concentrations (Table 2). In three of the labeling experiments, the immunoreactive fraction of the product was determined. It ranged from 57% to 66%, which is in line with previously published results on the immunoreactivity of  $^{212}\text{Pb}$ -TCMC-trastuzumab [10,23] labeled with the method described by Baidoo et al. [19].

Because the labeling was performed in a solution of  $^{224}\text{Ra}$  in equilibrium with daughters,  $^{212}\text{Bi}$  will be present during the incubation period. The RCP was therefore also measured in the  $^{208}\text{Tl}$  window after transient equilibrium was reached to account for  $^{212}\text{Bi}$ . This resulted in values ranging from 56% to 86%, indicating that  $^{212}\text{Bi}$  also conjugates directly to TCMC under these labeling conditions. It was found that  $^{212}\text{Pb}$  was complexed almost quantitatively already after 5 min reaction time at a TCMC-trastuzumab concentration of 4 mg/mL (Table 3). At the same time, more than 70% of the  $^{212}\text{Bi}$  was complexed, and it increased up to 86% after 30 min. Altogether, these results demonstrate that TCMC-trastuzumab is labeled with  $^{212}\text{Bi}$  in addition to  $^{212}\text{Pb}$ , albeit the reaction kinetics with  $^{212}\text{Bi}$  is slower.

The successful  $^{212}\text{Pb}$ -labeling over a range of antibody concentrations demonstrates that a variety of specific activities of the radioimmunoconjugate can be achieved. Since this study mainly was intended to show proof of concept, relatively low activity levels were used compared to what is expected in a clinical setting. The radiolabeling was therefore performed in quite small volumes, typically from 30 to 130  $\mu\text{L}$ , to simulate relevant clinical activity concentrations. Due to the low volumes, it was possible to achieve relatively high specific activities of the end product, despite the low activity used. The highest specific activity of  $^{212}\text{Pb}$ -TCMC-trastuzumab achieved in this study was approximately 30 MBq/mg which is comparable to what was used in a recent clinical study with  $^{212}\text{Pb}$ -TCMC-trastuzumab [4]. Upon complete decay of  $^{224}\text{Ra}$ , stable  $^{208}\text{Pb}$  is formed, which can compete with  $^{212}\text{Pb}$  on complexation with the TCMC chelator. With the activity levels used here, there was no indication that the presence of  $^{208}\text{Pb}$  influenced the yield of the radiolabeling due to the relatively high specific activity it was possible to achieve. However, the situation might differ when higher  $^{224}\text{Ra}$  activity is used and the following estimation was made: Assume 1 mg antibody labeled in a solution of 100 MBq  $^{224}\text{Ra}$ . This corresponds to  $4 \times 10^{15}$  molecules, where we can assume 2–5 TCMC chelators per antibody [10,17], giving  $8\text{--}20 \times 10^{15}$  possible binding sites for lead. Complete decay of 100 MBq  $^{224}\text{Ra}$  will form approximately  $4.5 \times 10^{13}$   $^{208}\text{Pb}$  atoms. These numbers indicate that the presence of  $^{208}\text{Pb}$  should not influence the yield of radiolabeling to a significant extent, not even for a product with high specific activity.

### 3.2. Separation of $^{212}\text{Pb}$ -TCMC-trastuzumab from $^{224}\text{Ra}$

The desired end-product of the process described in this study is a solution of pure  $^{212}\text{Pb}$ -labeled TCMC-antibody. To achieve the desired end-product, the solution with  $^{212}\text{Pb}$ -labeled antibody conjugate was purified to remove free  $^{224}\text{Ra}$  and other unconjugated daughter nuclides. Two different purification methods were evaluated; purification by centrifugal concentration and purification with a desalting column.

**Table 2**

Radiochemical yield after labeling of TCMC-trastuzumab conjugate with  $^{212}\text{Pb}$  in a solution containing  $^{224}\text{Ra}$  in equilibrium with daughter nuclides. The radiochemical purity (RCP) for  $^{212}\text{Pb}$  was determined using instant thin layer chromatography strips.

TCMC-trastuzumab concentration (mg/mL)	RCP (%)	n
0.00	$3 \pm 1$	5
0.10	$54 \pm 37$	3
0.15	$93 \pm 5$	3
0.20	$93 \pm 1$	2
0.25	$93 \pm 2$	6
1.00	$95 \pm 2$	6
4.00	$96 \pm 2$	8
6.00	$97 \pm n/a$	1

**Table 3**

Radiochemical yield of  $^{212}\text{Pb}$  and  $^{212}\text{Bi}$  after different incubation times of TCMC-trastuzumab conjugate (4 mg/mL) in a solution of  $^{224}\text{Ra}$  in equilibrium with daughter nuclides. The radiochemical purity (RCP) for  $^{212}\text{Pb}$  and  $^{212}\text{Bi}$ , respectively, was determined using instant thin layer chromatography strips.

Incubation time (min)	RCP (%)	
	$^{212}\text{Pb}$	$^{212}\text{Bi}$
5	$95.3 \pm 0.0$	$71.4 \pm 1.1$
15	$96.2 \pm 0.3$	$81.5 \pm 0.9$
30	$96.4 \pm 0.1$	$86.1 \pm 0.3$

Both methods are based on a size-dependent separation of the antibody-conjugate from low molecular weight compounds such as free ions, unbound chelator molecules and salts.

Use of the centrifugal concentrator for separation of  $^{212}\text{Pb}$ -TCMC-trastuzumab from cationic  $^{224}\text{Ra}$  and other unconjugated daughter nuclides yielded  $70.5 \pm 9.6\%$  ( $n = 6$ ) of the antibody-bound  $^{212}\text{Pb}$  activity in the concentrate. The loss of approximately one third of  $^{212}\text{Pb}$ -labeled trastuzumab due to the procedure is significant, but still in line with reported yields ( $73 \pm 3\%$ ) of  $^{212}\text{Pb}$ -TCMC-trastuzumab after PD-10 column purification [19]. The amount of  $^{224}\text{Ra}$  remaining in the concentrate was  $25.9 \pm 13.1\%$  ( $n = 6$ ), i.e., the separation of  $^{224}\text{Ra}$  from the radioimmunoconjugate was only 75% complete. The ratio of  $^{212}\text{Pb}$ -TCMC-trastuzumab to  $^{224}\text{Ra}$  improved from 1:1 to only about 3:1, which is not a satisfactory result for biomedical use of  $^{212}\text{Pb}$ -labeled radioimmunoconjugates. We observed in addition a slight trend towards higher percentage of  $^{224}\text{Ra}$  remaining in the concentrate after purification when higher amounts of antibody-conjugate were applied. This observation might indicate a saturation or clogging of the membrane with protein which decreases the efficiency of filtration of ions through the membrane.

Separation of  $^{212}\text{Pb}$ -TCMC-trastuzumab from  $^{224}\text{Ra}$  and other unconjugated daughter nuclides was more successful when a PD-10 gel filtration column was used. The use of gel filtration columns like PD-10 is common for purification of radiolabeled antibodies [19,24,25] and allows rapid removal of low molecular weight substances, such as unconjugated radionuclides, from the antibody containing solutions. The data for separation of  $^{212}\text{Pb}$ -labeled trastuzumab from free  $^{224}\text{Ra}$  is presented in Table 4. The recovery of  $^{212}\text{Pb}$ -trastuzumab was very favorable, with a yield of approximately 80% in fraction 3–5, independent of quenching the reaction mixture with EDTA. From Table 4 it is also evident that the majority (about 70%) of the protein conjugate was eluted from 3 to 4 mL (fraction 4). This is consistent with Baidoo et al. reporting 73% yield in the collected PD-10 eluate from 2.5–4.2 mL (1.7 mL) [19]. Removal of  $^{224}\text{Ra}$  from the solution containing  $^{212}\text{Pb}$ -TCMC-trastuzumab was quite effective with typically less than 4%  $^{224}\text{Ra}$  remaining in fraction 3–5, with a trend towards more efficient separation when EDTA was used to quench the reaction mixture. It was seen in some of the experiments that  $^{224}\text{Ra}$  started to elute after 4.5 mL, and to minimize the amount of  $^{224}\text{Ra}$  it was decided to exclude fraction 5 from the analysis. The breakthrough of  $^{224}\text{Ra}$  could then be reduced to  $0.9 \pm 0.8\%$  and  $2.7 \pm 3.6\%$  with and without EDTA, but came at the expense of a modest reduction in yield of  $^{212}\text{Pb}$ -TCMC-trastuzumab of approximately 5%, to  $76.7 \pm 11.7\%$  and  $76.1 \pm 5.9\%$  respectively. ITLC analyses of fraction 4 gave increased RCP of  $^{212}\text{Pb}$ -trastuzumab compared to prior to purification, with an average of  $98 \pm 1\%$  ( $n = 8$ ). The results altogether indicate that the use of PD-10 purified  $^{212}\text{Pb}$ -trastuzumab prepared from  $^{224}\text{Ra}/^{212}\text{Pb}$  mixtures is feasible.

To examine whether any of the radionuclides in the  $^{224}\text{Ra}$ -solution bind non-specifically to trastuzumab and thereby co-elute with the protein fractions, an experiment was performed where the radiolabeling protocol and PD-10 purification were performed as usual, except that TCMC-trastuzumab was replaced with trastuzumab. The presence of  $^{212}\text{Bi}$ ,  $^{212}\text{Pb}$  and  $^{224}\text{Ra}$  was assessed by measuring the seven collected fractions at different time points after the PD-10 purification was

**Table 4**  
Purification of  $^{212}\text{Pb}$ -labeled TCMC-trastuzumab from  $^{224}\text{Ra}$  and other unconjugated daughter nuclides using Sephadex G-25 PD 10 gel filtration desalting single-use columns.

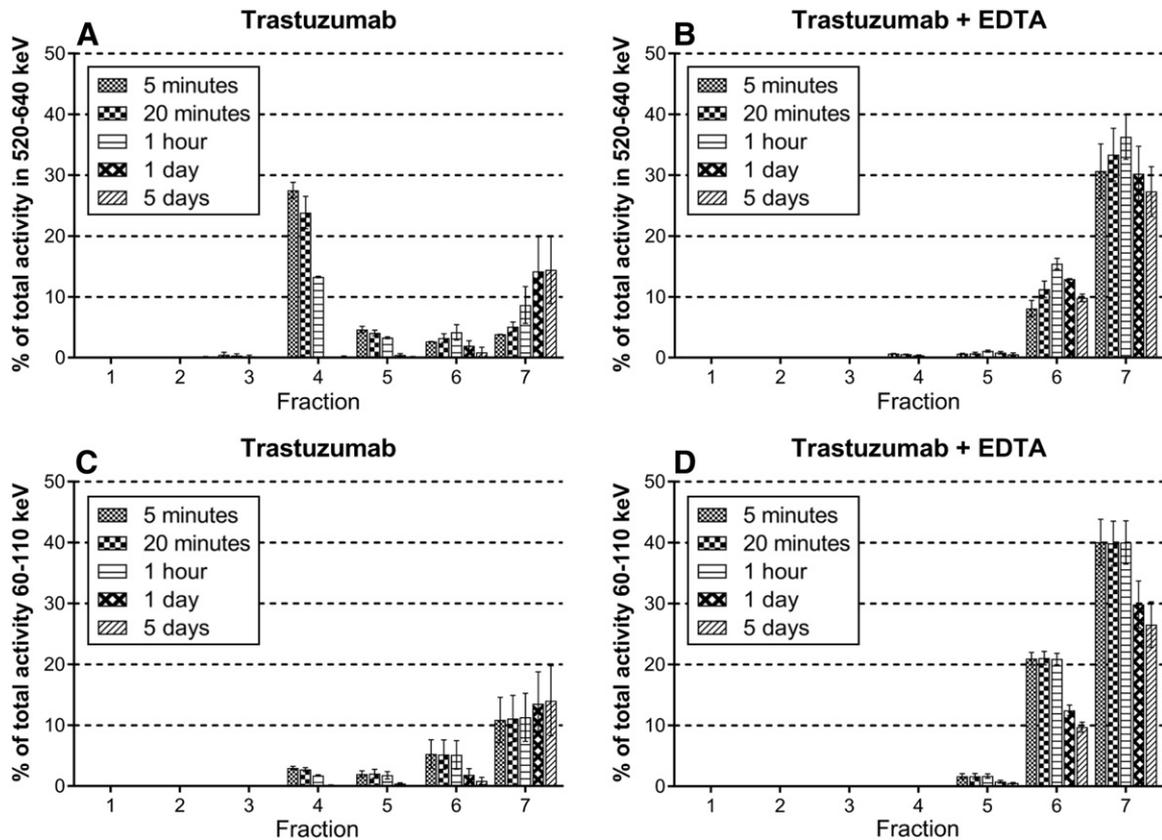
		No EDTA (n = 6)	EDTA (n = 7)
Yield of $^{212}\text{Pb}$ -labeled TCMC antibody	Fraction 3	4.8 ± 3.8%	7.2 ± 5.1%
	Fraction 4	71.3 ± 5.2%	69.5 ± 9.1%
	Fraction 5	5.6 ± 5.0%	4.6 ± 2.0%
	Total	81.7 ± 7.2%	81.3 ± 11.1%
	Amount of $^{224}\text{Ra}$ remaining in purified fractions	Fraction 3	0.6 ± 1.2%
	Fraction 4	2.1 ± 2.6%	0.8 ± 0.7%
	Fraction 5	0.5 ± 0.6%	1.3 ± 0.7%
	Total	3.2 ± 4.2%	2.2 ± 1.1%

finalized. The results are presented in Fig. 3 as a percentage of total activities in the 60–110 and 520–640 keV windows respectively. Fig. 3A shows a significant amount (28%) of  $^{212}\text{Bi}$  co-eluting with the antibody in fractions 4 and 5 when no EDTA was present. It is seen from the decay rate that the activity measured in this window (520–640 keV) clearly originates from ingrowth of  $^{208}\text{Tl}$  from  $^{212}\text{Bi}$  as it decays with the half-life of the mother  $^{212}\text{Bi}$ . When EDTA was used to quench the reaction mixture (Fig. 3B), the co-elution of  $^{212}\text{Bi}$  was reduced to 1.3% in the same fractions. Co-elution of  $^{212}\text{Pb}$  was insignificant in fraction 3 and 4 of the PD-10 eluate when EDTA was present (Fig. 3D), and below 2% in fraction 5. Fig. 3C shows that without EDTA present, approximately 5% of the total  $^{212}\text{Pb}$  activity co-eluted with the antibody in fractions 3–5. As can be seen from the measurements on day 5, co-elution of  $^{224}\text{Ra}$  was negligible (less than 0.7%) in both cases. Altogether, the results clearly show that using EDTA to quench the reaction mixture prior to purification on the PD-10 column maximizes the product purity when a  $^{224}\text{Ra}$ -solution is used for preparing  $^{212}\text{Pb}$ -based radio-immunoconjugates. This measure removes non-specific bound  $^{212}\text{Bi}$

and  $^{212}\text{Pb}$  from the antibody fractions, and at the same time gives less remaining  $^{224}\text{Ra}$  in the end product.

### 3.3. Retention of $^{212}\text{Bi}$ by the TCMC-chelator upon $^{212}\text{Pb}$ decay

It is of interest to have knowledge about the fate of  $^{212}\text{Bi}$  formed when  $^{212}\text{Pb}$  chelated to TCMC decays. To avoid radiotoxicity caused by free  $^{212}\text{Bi}$  it is desirable that a substantial fraction of  $^{212}\text{Bi}$  is retained by the TCMC-chelator upon decay. Mirzadeh and colleagues found that 36% of  $^{212}\text{Bi}$  was released from the DOTA-chelator when  $^{212}\text{Pb}$  decayed and they claimed the breakup of the complex was due to internal conversion from  $\gamma$ -rays emitted from excited  $^{212}\text{Bi}$  nuclei [26]. We have found no corresponding examination for the retention of  $^{212}\text{Bi}$  by the TCMC-chelator when  $^{212}\text{Pb}$  decays, and therefore made an estimation based on our data. A sample of radiolabeled TCMC-trastuzumab was mixed with EDTA solution and stored until less than 5% of the  $^{212}\text{Bi}$  originally complexed to TCMC had decayed. We thereafter determined the  $^{212}\text{Bi}$  to  $^{212}\text{Pb}$  ratio in fraction 4 of the PD-10 purified product

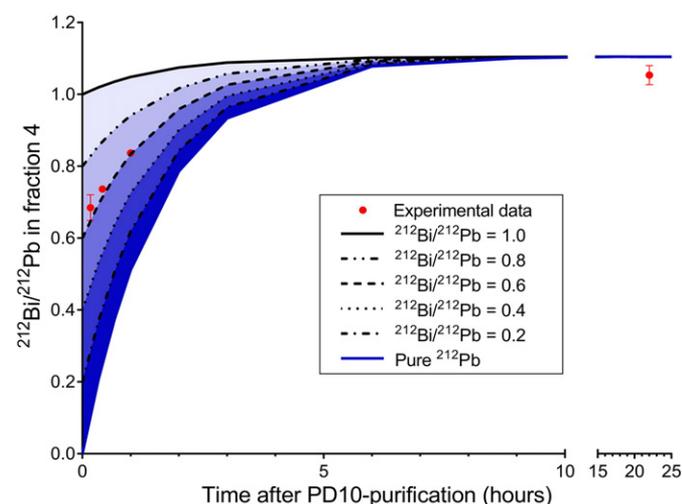


**Fig. 3.** The measured activity in the energy ranges 520–640 keV (A and B) and 60–110 keV (C and D) in each of the seven collected fractions after PD-10 purification of trastuzumab (not TCMC-conjugated) radiolabeled with  $^{212}\text{Pb}$  using a  $^{224}\text{Ra}$  solution. The bar graphs illustrate the presence of  $^{212}\text{Bi}$  by an indirect assessment from the  $^{208}\text{Tl}$   $\gamma$  in the 520–640 keV window with (B) and without (A) use of EDTA to quench the reaction mixture prior to loading it on the PD-10 column. Similarly, the presence of  $^{212}\text{Pb}$  is assessed from the measurements in the 60–110 keV window with (D) and without (C) EDTA present. The activity is displayed as the decay corrected percentage of the total activity loaded on the column at different time points after finalizing the PD-10 purification. Each bar represent data from two parallel samples.

at different time points after finalizing the purification. These ratios are plotted in Fig. 4 together with theoretical  $^{212}\text{Bi}$  to  $^{212}\text{Pb}$  ratios as a function of time for a few selected, initial  $^{212}\text{Bi}$  to  $^{212}\text{Pb}$  relationships. Depending on the initial amount of  $^{212}\text{Bi}$  present, the  $^{212}\text{Bi}/^{212}\text{Pb}$  ratio increases to varying degrees before it reaches a maximum plateau where the nuclides are in transient equilibrium. Under the assumption that all activity in fraction 4 is bound to the antibody-conjugate straight after purification and by taking into account ingrowth of  $^{212}\text{Bi}$  from  $^{212}\text{Pb}$  during the time from finalizing the purification to the time of measurement, it was estimated that no less than about 60% of the  $^{212}\text{Bi}$  was associated with the TCMC-chelator after  $^{212}\text{Pb}$  decay. This is seen in Fig. 4, where the red filled circles representing the experimental data follows the shape of the curve that corresponds to a  $^{212}\text{Bi}$  to  $^{212}\text{Pb}$  ratio of 0.6 at time zero. We also determined the retention of  $^{212}\text{Bi}$  by the TCMC chelator after  $^{212}\text{Pb}$  decay with a different method. A sample of radiolabeled TCMC-trastuzumab was stored in an EDTA solution overnight, and the RCP was analyzed. The  $^{212}\text{Bi}$  to  $^{212}\text{Pb}$  ratio in the lower half of the ITLC strip was determined and all the activity measured here was assumed to be complexed to TCMC-trastuzumab. This ratio was compared with the  $^{212}\text{Bi}$  to  $^{212}\text{Pb}$  ratio in a reference sample of  $^{224}\text{Ra}$  in equilibrium in daughters. With this method it was found that approximately 70% of the  $^{212}\text{Bi}$  remained associated with the TCMC-chelator after  $^{212}\text{Pb}$  decay. The values we found are in good agreement with each other and the previously mentioned data on  $^{212}\text{Bi}$  retention by the DOTA-chelator [26]. It has been claimed that the four N-donor and four O-donor atoms of the TCMC-chelator will provide for good binding abilities with bismuth [27], and thus the relatively high retention of  $^{212}\text{Bi}$  in the TCMC-chelator is not unlikely.

#### 3.4. Evaluation of possible radiolytic problems at elevated radiation doses

As mentioned previously in the discussion, the current study was carried out with relatively low activity levels and radiolytic problems may arise when higher, clinical relevant activity of  $^{224}\text{Ra}$ -solution is used. A potential drawback of using  $^{224}\text{Ra}$  in equilibrium with  $^{212}\text{Pb}$  instead of pure  $^{212}\text{Pb}$  in the radiolabeling reaction, is the elevated radiation dose to the reaction solution due to increased  $\alpha$ -particle activity. The radiation exposure to the antibody-conjugate is likely to be at its



**Fig. 4.** The relationship between the  $^{212}\text{Bi}$  and  $^{212}\text{Pb}$  activity in a sample plotted as a function of time and compared with the experimentally determined ratios in fraction 4 of the PD-10 purified product (filled red circles). The solid and dotted lines represent ratios determined theoretically from a few selected, initial  $^{212}\text{Bi}/^{212}\text{Pb}$ -ratios, ranging from a sample of pure  $^{212}\text{Pb}$  without any  $^{212}\text{Bi}$  present (solid blue line in the bottom) to a sample where the activity ratio is equal to one (solid black line on top). Ingrowth of  $^{212}\text{Bi}$  from  $^{212}\text{Pb}$  were calculated using a universal decay calculator (<http://www.wiseuranium.org/rcc.html>). The filled red circles represent data from 4, 2, 3 and 3 parallel experiments for the 10, 20, 60 min and 22 h time points respectively.

highest during the radiolabeling procedure. At that time  $^{224}\text{Ra}$  and all progeny will contribute to the dose, whereas after purification it will mainly be dose delivered from  $^{212}\text{Pb}$  and daughters. The total decay energy (excluding photons) from  $^{224}\text{Ra}$  and progeny is 27.8 MeV, compared to only 8.8 MeV released from decay of  $^{212}\text{Pb}$  and daughters. To expose the antibody to a high radiation dose, after the incubation period of 30 min, we continued to store the  $^{212}\text{Pb}$ -labeled TCMC-trastuzumab in the  $^{224}\text{Ra}$ -solution in equilibrium until a dose of approximately 700 Gy was achieved. Analysis with size-exclusion HPLC showed a peak including 96% of the total radioactivity at a time consistent with intact TCMC-trastuzumab, with less than 1.3 and 2.9% associated with higher and lower molecular weight compounds respectively. The  $^{212}\text{Pb}$ -TCMC-trastuzumab exposed to 700 Gy was also compared with unlabeled TCMC-trastuzumab with detection of absorption at 280 nm. The results showed a peak of molecular weight compounds lower than the IgG when the radiolabeled antibody was analyzed. This peak comprised approximately 11% and was not seen with the unlabeled TCMC-trastuzumab and is therefore likely caused by radiolytic degradation of the protein. The amount of high molecular species was similar between the two samples (less than 1.6%). However, the apparent radiolytic damage to a fraction of the antibodies did not seem to significantly influence the immunoreactive fraction of the product. Two samples of  $^{212}\text{Pb}$ -labeled TCMC-trastuzumab exposed to 100 and 700 Gy, was purified with a PD-10 column and the immunoreactivity of fraction 4 was determined to be 60 and 57% respectively, with low (less than 3%) non-specific binding in both cases. This is in accordance with results from the literature, where exposure of up to 1000 Gy was tolerated without significantly reducing the cell binding fraction of a radiolabeled antibody [29].

Altogether, the examination of possible radiolytic effects at higher radiation doses indicate that the radiation dose to the antibody should be kept significantly below 700 Gy. In the phase I study of intraperitoneally administered  $^{212}\text{Pb}$ -TCMC-trastuzumab the highest dose the patients received was 27.4 MBq/m<sup>2</sup> [5]. By using an average body surface area of 1.79 m<sup>2</sup> found in a study of adult cancer patients [28] this dose corresponds to 49 MBq per patient. To prepare a patient dose with this activity it is reasonable to assume that an activity of 100 MBq  $^{224}\text{Ra}$  should be sufficient as it corresponds to about the double of the highest dose of  $^{212}\text{Pb}$ -TCMC-trastuzumab administered to patients in the phase I trial. The radiation dose to the antibody solution during a 30 min labeling with 100 MBq of  $^{224}\text{Ra}$  in a reaction volume of 1.5 mL, which is compatible with the PD-10 gel exclusion purification format, was estimated to be 534 Gy. If a further decrease in dose should be necessary, it is possible to reduce the labeling time as we have shown that the reaction between  $^{212}\text{Pb}$  and TCMC is very fast and similar yields could be achieved after 5 and 30 min when 4 mg/mL TCMC-trastuzumab was used. Based on the calculations, it is predicted that the method described herein could be useful also in a clinical setting where high activity levels are used.

#### 3.4.1. Discussion of the feasibility of the proposed method

The current study demonstrates that a TCMC-conjugated monoclonal antibody can be efficiently labeled with  $^{212}\text{Pb}$  from solutions of  $^{224}\text{Ra}$  in equilibrium with progeny. When a concentration of 4 mg/mL of TCMC-trastuzumab was used, the labeling with  $^{212}\text{Pb}$  was close to quantitative after only 5 min (Table 3). At this concentration it was also observed that the major part of the  $^{212}\text{Bi}$  would be chelated by the antibody conjugate. Subsequently, the radiolabeled conjugate can be separated from the generator nuclide  $^{224}\text{Ra}$  using desalting gel exclusion separation. Fig. 2 shows a flow chart of our proposed process compared to the method presented by Baidoo et al. [19]. In contrast to the current ion exchange based generators, which may be eluted several times, the liquid  $^{212}\text{Pb}$  generator described here is designed for preparation of a single dose only. With our proposed method, a ready to use  $^{224}\text{Ra}$  solution can be shipped from a centralized supplier to the end user. This is beneficial both from a logistic point of view and because

the work required by the end user is reduced and simplified. We believe it is an advantage with our method that the steps involving handling and evaporation of concentrated acid solutions with high radioactivity levels can be completely avoided in the hospital or radiopharmacy setting. An additional benefit with eliminating the acid digestion procedures is that the total preparation time at the hospital will be shorter, since it is only the actual antibody labeling and purification that needs to be performed. Baidoo et al. reported that this part of the process required only 80 min of the total preparation time of an injectable dose of about 210 min [19]. A shorter preparation time reduces the activity loss caused by decay and will therefore lead to a higher amount of  $^{212}\text{Pb}$  administered to the patients. This is beneficial both to limit the amount of free daughter nuclides in the product at time of injection and to minimize the risk of possible problems with radiolysis of the antibody.

On evaluation of our proposed method for radiolabeling of antibodies, it is also important to address the radiation safety requirements. As with all procedures involving open sources of  $\alpha$ -emitting radionuclides, precautions must be followed to avoid inhalation or ingestion. All handling should therefore be performed in either a biosafety bench or in a glove-box under negative pressure to protect the worker. This is especially important when handling the  $^{224}\text{Ra}$ -series, because  $^{220}\text{Rn}$  is one of the daughters. The working space also need to be appropriately shielded. One of the  $^{224}\text{Ra}$ -daughters,  $^{208}\text{Tl}$ , has a highly energetic  $\gamma$ -ray of 2.6 MeV in 36% abundance relative to  $^{224}\text{Ra}$ , that will determine the thickness of the shielding required. Baidoo et al. have described appropriate shielding for activities up to 740 MBq of  $^{224}\text{Ra}$  to be approximately 15 cm of lead. The dose rate at 30 cm distance from a point source of  $^{224}\text{Ra}$  with this activity in equilibrium with daughters will be reduced from approximately 1600 to 3  $\mu\text{Sv/h}$  when 15 cm lead shielding is used. Because the  $^{212}\text{Pb}$ -generator solution we have presented here is intended for preparation of a single patient dose, we do not see it as plausible that the shielding requirements will exceed what has been described by Baidoo et al. Even if  $^{224}\text{Ra}$  is present until purification with our method, as opposed to only on the column when working according to the method presented by Baidoo et al., this will not alter the shielding requirements because 99% of the  $\gamma$ -activity in the series originate from  $^{212}\text{Pb}$  and daughters, and especially the previously mentioned high energy  $\gamma$ -ray from  $^{208}\text{Tl}$  that will dictate the shielding requirement. The evaporation steps included in the method described by Baidoo et al. should be performed in a glove-box or in some sort of closed system where the acid vapor is collected, and will therefore require dedicated equipment for this process. With the liquid generator system proposed in this study, the risk associated with acid vapor is eliminated.

#### 3.4.2. Acceptable product purity vs. generator nuclide in an injectate

The purity of  $^{212}\text{Pb}$  vs.  $^{224}\text{Ra}$  is an important quality parameter for the  $^{212}\text{Pb}$ -labeled radioimmunoconjugate. For the *in situ* labeling method proposed in this paper to be a feasible alternative to the existing protocol, careful consideration must be given to define acceptable limits of  $^{224}\text{Ra}$  in the end product. Fortunately,  $^{224}\text{Ra}$  has been extensively studied both in animals and in humans, and the toxicity profile is well-known. As with other radium-isotopes, after intravenous injection,  $^{224}\text{Ra}$  is mainly deposited in bone. Because of its natural bone seeking properties, it was introduced as a palliative treatment of ankylosing spondylitis already in the 1940s [30]. It was in use for several decades, until about 1990 [31], and then briefly re-introduced for the same indication by a different manufacturer from 2000 to 2005 [32,33]. Dosimetric calculations, performed according to the model proposed by the International Commission on Radiological Protection, showed that the absorbed doses after intravenous injection of  $^{224}\text{Ra}$ -dichloride was highest on the bone surface and red bone marrow [34]. Since introduction, weekly injections of 1 MBq, up to a total of ten injections, has been used as a treatment regimen in adult patients [30,35,36]. Reports including around 1000 patients who received this dose have shown that such amounts of  $^{224}\text{Ra}$ -dichloride can be administered without

considerable bone marrow toxicity [30,36,37]. These historical data indicate that a 1 MBq of  $^{224}\text{Ra}$  per dosing, or a total of 10 MBq cumulative, might be acceptable in adult patients as long as the  $^{212}\text{Pb}$  product by itself does not produce a high degree of bone marrow toxicity.

In the phase I study of intraperitoneally administered  $^{212}\text{Pb}$ -TCMC-trastuzumab, no significant myelosuppression was found [5,6]. From the results obtained here, when EDTA was used to quench the reaction mixture before PD-10 purification, the remaining amount of  $^{224}\text{Ra}$  in the end product could be kept below 1%. This corresponds to 0.5 MBq of  $^{224}\text{Ra}$  administered to a patient given 50 MBq of a  $^{212}\text{Pb}$ -based product. A patient dose of approximately 49 MBq was the highest dose of  $^{212}\text{Pb}$ -TCMC-trastuzumab (27.4 MBq/m<sup>2</sup>) administered in the previously mentioned phase I trial [5]. Altogether, these estimations indicate that a sufficient purity of  $^{212}\text{Pb}$  vs.  $^{224}\text{Ra}$  in the end product of a  $^{212}\text{Pb}$ -labeled radioimmunoconjugate prepared from a  $^{224}\text{Ra}$  solution could be achieved, under the assumption that up to 1 MBq of  $^{224}\text{Ra}$  per dosing is a tolerable amount.

## 4. Conclusions

The current work demonstrates the feasibility of using a  $^{224}\text{Ra}$ -solution as a shippable generator solution for producing  $^{212}\text{Pb}$ -based radioimmunoconjugates, which may be easier to execute and less time-consuming for the end user in comparison with existing ion exchange based methods.

## Acknowledgments

The study was supported by the Norwegian Research Council (grant number 237661) and the private Norwegian companies Oncoinvent AS, Sciencons AS and Nordic Nanovector ASA.

## References

- [1] Wilson JM, Parker C. The safety and efficacy of radium-223 dichloride for the treatment of advanced prostate cancer. *Expert Rev Anticancer Ther* 2016;16:911–8.
- [2] Kim YS, Brechbiel MW. An overview of targeted alpha therapy. *Tumour Biol* 2012;33:573–90.
- [3] Lechner PK. Radiation dosimetry of monoclonal antibodies: Practical considerations. In: Henkin RE, Boles MA, Dillehay GL, Halama JR, Karesh SM, Wagner RH, et al, editors. *Nuclear medicine*. 1st ed. St. Louis: Mosby; 1996. p. 558–62.
- [4] Meredith RF, Torgue J, Azure MT, Shen S, Saddekni S, Banaga E, et al. Pharmacokinetics and imaging of  $^{212}\text{Pb}$ -TCMC-trastuzumab after intraperitoneal Administration in Ovarian Cancer Patients. *Cancer Biother Radiopharm* 2014;29:12–7.
- [5] Meredith RF, Torgue JJ, Rozgaja TA, Banaga EP, Bunch PW, Alvarez RD, et al. Safety and outcome measures of first-in-human intraperitoneal  $\alpha$  Radioimmunotherapy with  $^{212}\text{Pb}$ -TCMC-Trastuzumab. *Am J Clin Oncol* 2016 Nov 30, Published ahead of print.
- [6] Meredith R, Torgue J, Shen S, Fisher DR, Banaga E, Bunch P, et al. Dose escalation and dosimetry of first-in-human  $\alpha$  Radioimmunotherapy with  $^{212}\text{Pb}$ -TCMC-trastuzumab. *J Nucl Med* 2014;55:1636–42.
- [7] Milenic DE, Garmestani K, Brady ED, Albert PS, Abdulla A, Flynn J, et al. Potentiation of high-LET radiation by gemcitabine: targeting HER2 with Trastuzumab to treat disseminated peritoneal Disease. *Clin Cancer Res* 2007;13:1926–35.
- [8] Milenic DE, Garmestani K, Brady ED, Baidoo KE, Albert PS, Wong KJ, et al. Multimodality therapy: potentiation of high linear energy transfer radiation with paclitaxel for the treatment of disseminated peritoneal Disease. *Clin Cancer Res* 2008;14:5108–15.
- [9] Milenic DE, Baidoo KE, Shih JH, Wong KJ, Brechbiel MW. Evaluation of platinum chemotherapy in combination with HER2-targeted  $\alpha$ -particle radiation. *Cancer Biother Radiopharm* 2013;28:441–9.
- [10] Milenic DE, Garmestani K, Brady ED, Albert PS, Ma D, Abdulla A, et al.  $\alpha$ -particle Radioimmunotherapy of disseminated peritoneal Disease using a  $^{212}\text{Pb}$ -labeled Radioimmunoconjugate targeting HER2. *Cancer Biother Radiopharm* 2005;20:557–68.
- [11] Kasten BB, Azure MT, Schoeb TR, Fisher DR, Zinn KR. Imaging, biodistribution, and toxicology evaluation of  $^{212}\text{Pb}$ -TCMC-trastuzumab in nonhuman primates. *Nucl Med Biol* 2016;43:391–6.
- [12] Milenic DE, Molinolo AA, Solivella MS, Banaga E, Torgue J, Besnainou S, et al. Toxicological studies of  $^{212}\text{Pb}$  intravenously or intraperitoneally injected into mice for a phase 1 trial. *Pharmaceuticals (Basel)* 2015;8:416–34.
- [13] Zucchini GL, Friedman AM. Isotopic generator for  $^{212}\text{Pb}$  and  $^{212}\text{Bi}$ . *Int J Nucl Med Biol* 1982;9:83–4.
- [14] Hassfjell S. A  $^{212}\text{Pb}$  generator based on a  $^{228}\text{Th}$  source. *Appl Radiat Isot* 2001;55:433–9.
- [15] Hassfjell SP, Hoff P. A generator for production of  $^{212}\text{Pb}$  and  $^{212}\text{Bi}$ . *Appl Radiat Isot* 1994;45:1021–5.

- [16] Atcher RW, Friedman AM, Hines JJ. An improved generator for the production of  $^{212}\text{Pb}$  and  $^{212}\text{Bi}$  from  $^{224}\text{Ra}$ . *Int J Rad Appl Instrum A* 1988;39:283–6.
- [17] Chappell LL, Dadachova E, Milenic DE, Garmestani K, Wu C, Brechbiel MW. Synthesis, characterization, and evaluation of a novel bifunctional chelating agent for the lead isotopes  $^{203}\text{Pb}$  and  $^{212}\text{Pb}$ . *Nucl Med Biol* 2000;27:93–100.
- [18] Yong K, Brechbiel MW. Towards translation of  $^{212}\text{Pb}$  as a clinical therapeutic; getting the lead in! *Dalton Trans* 2011;40:6068–76.
- [19] Baidoo KE, Milenic DE, Brechbiel MW. Methodology for labeling proteins and peptides with lead-212 ( $^{212}\text{Pb}$ ). *Nucl Med Biol* 2013;40:592–9.
- [20] Larsen RH, inventor; Sciencons AS, assignee. Radiopharmaceutical Solutions with Advantageous Properties. US 2016/0250360 A1; 2016.
- [21] Westrøm S, Bønsdorff TB, Abbas N, Bruland ØS, Jonasdottir TJ, Mælandsmo GM, et al. Evaluation of CD146 as target for Radioimmunotherapy against osteosarcoma. *PLoS One* 2016;11:e0165382.
- [22] Fodstad Ø, Brøgger A, Bruland Ø, Solheim OP, Nesland JM, Pihl A. Characteristics of a cell line established from a patient with multiple osteosarcoma, appearing 13 years after treatment for bilateral retinoblastoma. *Int J Cancer* 1986;38:33–40.
- [23] Milenic DE, Baidoo KE, Brechbiel MW. Bench to bedside: stability studies of GMP produced Trastuzumab-TCMC in support of a clinical trial. *Pharmaceuticals (Basel)* 2015;8:435–54.
- [24] Vosjan MJWD, Perk LR, Visser GWM, Budde M, Jurek P, Kiefer GE, et al. Conjugation and radiolabeling of monoclonal antibodies with zirconium-89 for PET imaging using the bifunctional chelate p-isothiocyanatobenzyl-desferrioxamine. *Nat Protoc* 2010;5:739–43.
- [25] Murray A, Simms MS, Scholfield DP, Vincent RM, Denton G, Bishop MC, et al. Production and characterization of  $^{188}\text{Re}$ -c595 antibody for Radioimmunotherapy of transitional cell bladder cancer. *J Nucl Med* 2001;42:726–32.
- [26] Mirzadeh S, Kumar K, Gansow OA. The chemical fate of  $^{212}\text{Bi}$ -DOTA formed by  $\beta^-$  decay of  $^{212}\text{Pb}(\text{DOTA})^{2-}$ . *Radiochim Acta* 1993;60:1–10.
- [27] Lloyd G, Luckay R. 1,4,7,10-Tetrakis(carbamoylmethyl)-1,4,7,10-tetraazacyclododecane. *Acta Crystallogr Sect E Struct Rep Online* 2005;61:o3405–7.
- [28] Sacco JJ, Botten J, Macbeth F, Bagust A, Clark P. The average body surface area of adult cancer patients in the UK: a multicentre retrospective study. *PLoS One* 2010;5:e8933.
- [29] Larsen RH, Bruland ØS. Radiolysis of radioimmunoconjugates. Reduction in antigen-binding ability by  $\alpha$ -particle radiation. *J Label Compd Radiopharm* 1995;36:1009–18.
- [30] Koch W. Indication for  $^{224}\text{Ra}$ -therapy in ankylosing spondylitis (Morbus Struempell-Bechterew-Marie). In: Muller W, Ebert H, editors. Biological effects of  $^{224}\text{Ra}$  - benefits and risks of therapeutic application - proceedings of the second symposium at Neuherberg/München, September 20–21, 1976. The Hague/Boston: Martinus Nijhoff Medical Division; 1978. p. 21–9.
- [31] Wick RR, Nekolla EA, Gaubitz M, Schulte TL. Increased risk of myeloid leukaemia in patients with ankylosing spondylitis following treatment with radium-224. *Rheumatology (Oxford)* 2008;47:855–9.
- [32] Kommission Pharmakotherapie. Stellungnahme der Deutschen Gesellschaft für Rheumatologie zur Therapie der ankylosierenden Spondylitis (AS) mit Radiumchlorid ( $^{224}\text{SpondylAT}$ ). *Zeitschrift für Rheumatologie* 2001;60:84.
- [33] Eckert & Ziegler. Eckert & Ziegler stoppt klinische Entwicklung von SpondylAT®. Available from: [http://www.ezag.com/de/startseite/presse/pressemeldungen/detail/?tx\\_ttnews%5Btt\\_news%5D=425&cHash=e69ca5ac3dcd82b7ed0efd648b88c474](http://www.ezag.com/de/startseite/presse/pressemeldungen/detail/?tx_ttnews%5Btt_news%5D=425&cHash=e69ca5ac3dcd82b7ed0efd648b88c474); 2006. [cited 05 Feb 2017].
- [34] Lassmann M, Nosske D, Reiners C. Therapy of ankylosing spondylitis with  $^{224}\text{Ra}$ -radium chloride: dosimetry and risk considerations. *Radiat Environ Biophys* 2002;41:173–8.
- [35] Wick R, Gössner W. History and current uses of  $^{224}\text{Ra}$  in ankylosing spondylitis and other diseases. *Environ Int* 1993;19:467–73.
- [36] Tiepolt C, Grüning T, Franke WG. Renaissance of  $^{224}\text{Ra}$  for the treatment of ankylosing spondylitis: clinical experiences. *Nucl Med Commun* 2002;23:61–6.
- [37] Alberding A, Stierle H, Brandt J, Braun J. Wirksamkeit und Verträglichkeit von Radiumchlorid in der Behandlung der ankylosierenden Spondylitis-Ergebnisse einer Anwendungsbeobachtung. *Z Rheumatol* 2006;65:245–51.