

## Preparation and preliminary studies of [<sup>64</sup>Cu]-antiMUC-1 for breast cancer targeting

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### Abstract

PR81 is a monoclonal antibody that binds with high affinity to MUC1 that over expressed on breast tumors. PR81 is considered a suitable targeting molecule that was radiolabeled using Cu-64 for positron imaging studies. The monoclonal antibody was conjugated with DOTA moiety and after purification was evaluated for radiochemical purity, immunoreactivity, cell toxicity and structure integrity as well as biodistribution study in normal rats. The radiolabeled antibody prepared with acceptable radiochemical purity ( $> 93.2 \pm 0.6\%$ , ITLC; specific activity;  $4.6 \mu\text{Ci}/\mu\text{g}$ ), protein structure integration, significant cytotoxicity and significant immunoreactivity retention was assessed by radioimmunoassay (RIA). Animal biodistribution of the <sup>64</sup>Cu-DOTA-PR81 was consistent with other radiolabeled antibodies. The results showed that <sup>64</sup>Cu-DOTA-PR81 may be considered for tumor imaging for ultimate diagnosis and follow-up of MUC1 expression in oncology.

**Keywords:** Copper-64, PR81, MUC1, tumor, biodistribution

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### Introduction

Monoclonal antibodies (mAb) have been applied for radioimmunoscintigraphy of breast cancer (1) and are within the important categories of molecules used in targeted therapy of cancers (2). An ideal antibody for radioimmunoscintigraphy should target an antigen that is tumor-specific, generously expressed on all the cancer cells and is not shed into the blood circulation (3). MUC1 is high molecular weight glycoprotein as an antigen that is expressed on the apical membrane of the epithelial cells in the ducts and acini of the

breast tissue (4,5). Cancerous MUC1 is structurally different from normal MUC1, exposing novel regions of the protein core (6). MUC1 expression causes anchorage independent growth and tumor formation, and is a useful marker for the prognosis of the patients with carcinoma (7,8). This antigen is aberrantly overexpressed in 80% of the breast cancers and is an attractive target for radioimmunotherapy and radioimmunoscintigraphy. Recently several anti-MUC1 mAbs against the human breast carcinoma has been reported for

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radioimmunosciintigraphy and radioimmunotherapy (9,10). PR81 is a new murine anti-MUC1 mAb that reacts with the membrane extracts of several human breast cancerous tissues and the cell surface of many MUC1 positive cell lines (11). Breast cancer radioimmunosciintigraphy targeting MUC1 expression has been proposed by different research groups. PR81 as an intact anti MUC1 mAb and its fragments have been radiolabeled and used in the imaging and therapy of MUC1-positive tumors using  $^{99m}\text{Tc}$  (12,13),  $^{131}\text{I}$  (14),  $^{177}\text{Lu}$  (15).  $^{64}\text{Cu}$  ( $t_{1/2} = 12.7$  h), with both  $\beta^+$  and  $\beta^-$  emissions, allows for both PET imaging and possibly radionuclide therapy (16).  $^{64}\text{Cu}$  labeled antibodies have attracted considerable interest in the field of targeted radionuclide therapy and diagnosis (17). 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) ligand has already shown acceptable biological performance when used in antibody conjugation of various radioisotopes such as Ac-225 (18), Lu-177 (19), lead radioisotopes (20), and  $^{64}\text{Cu}$  (21) and has been important in theranostic applications. In the present study, we labeled PR81 with  $^{64}\text{Cu}$  via DOTA-NHS reaction and performed the necessary quality control tests and *in vivo* studies in normal rats to evaluate the complex for radioimmunosciintigraphy of breast cancer.

### Materials and methods

Production of  $^{64}\text{Cu}$  was performed at AMIRS 30 MeV cyclotron (IBA, Cyclone-30). Enriched zinc-68 oxide of > 90% purity was commercially available; DOTA-NHS was purchased from Macrocylics (NJ, USA). PR81 hybridoma cell line was

obtained from the original supplier (Department of Biotechnology, School of Medical Sciences, Tarbiat Modarres University, Tehran, Iran). PD10 de-salting column was purchased from Amersham Pharmacia Biotech (now owned by GE). Vivaspin-2 filters (30,000 MWCO PES) was purchased from Sartorius stedim biotech (Göttingen, Germany), The other chemicals were purchased from Sigma chemical company (St Louis, MO, USA). SDS-Polyacrylamide Gel Electrophoresis and Transfer equipment (14 × 16 cm) Laemmli gels run in a Model SE600 vertical slab gel unit with a model PS500X power supply, Model TE42 transfer unit with a Model TE51 power supply; Hoeffler Scientific Instruments, Germany). Radiochromatography was performed by using a Bioscan AR-2000 radio TLC scanner instrument (Bioscan, Paris, France). A high purity germanium (HPGe) detector coupled with a Canberra™ (model GC1020-7500SL) multichannel analyzer and a dose calibrator ISOMED 1010 (Dresden, Germany) were used for counting distributed activity in rat and mice organs. Calculations were based on the 511 keV peak for  $^{64}\text{Cu}$ . Animal studies were performed in accordance with the United Kingdom Biological Council's Guidelines on the Use of Living Animals in Scientific Investigations, 2nd edn. The ethical permission was obtained from Tarbiat Modares University ethics committee of treating experimental animal.

### *Production and quality control of copper-64 chloride*

The <sup>68</sup>Zn target was electroplated on a gold-coated copper backing plate and irradiated at an angle of 6 degrees toward the proton beam, in order to achieve a higher production yield. The optimum energy for the production of <sup>64</sup>Cu via <sup>68</sup>Zn (p, αn) <sup>64</sup>Cu reaction is 20-35 MeV (22), however the highest available proton energy is 30 MeV at the AMIRS cyclotron. Therefore, the target had to be thick enough to reduce the energy of the incident protons from 30 MeV to about 20 MeV (23). Our results showed 100 μm of the target material was electroplated on the copper backing when 20 MeV energy was used at proper angle. For this purpose, <sup>68</sup>ZnO was dissolved in 0.05 N HCl to prepare a zinc cation-containing solution at the optimized conditions electrodeposition was performed and resulted in a 100 μm zinc layer on the gold-coated copper backing after 3.5 hours. In order to prepare the gold supporting layer, a gold containing bath was prepared according to a previously reported method with slight modifications (24). The irradiated target was dissolved by 10 N HCl (15 mL, 20 μL of H<sub>2</sub>O<sub>2</sub> added) and the solution was passed through a cation exchange resin (AG 50 W×8, H<sup>+</sup> form; mesh 200-400) (h:10 cm, Ø:1.3 cm) that was preconditioned by passing 25 ml of 9 N HCl. The column was then washed by 25 mL of 9 N HCl at a rate of 1 ml/min to elute copper and zinc ion contents. To the latter elute was added 30 mL of DDH<sub>2</sub>O. The mixture was passed through another anion exchange resin (Dowex 1X8, Cl<sup>-</sup> form; mesh: 100-200) (h:25 cm; Ø:1.7 cm), preconditioned with 100 mL of 6 N HCl. In order to elute copper-64 ions, the column was washed by 50 mL of 2 N HCl. The column was finally

eluted by 0.05 N HCl (150 mL), in order to recover of precious zinc-68 contents. The whole chemical separation process took about 105 min. Gamma spectroscopy of the final sample was carried out using an HPGe detector coupled to a Canberra<sup>TM</sup> multi-channel analyzer for 300 seconds. The presence of zinc and copper cations were checked by polarographic methods. The resulting high-purity [<sup>64</sup>Cu]CuCl<sub>2</sub> solution was used directly in the labeling step .

#### *Production and immunoreactivity of monoclonal antibody*

PR81 hybridoma cell line was obtained from the original supplier (Department of Biotechnology, School of Medical Sciences, Tarbiat Modarres University, Tehran, Iran) and monoclonal antibody was prepared according to the reported protocol as 10 mg of pure protein per week (25). The native cancerous antigen (MUC1) was purified from ascetic fluid of a patient with aggressive small-cell lung carcinoma and metastasis to peritoneum by an antibody-sepharose affinity (immunoaffinity) column which is explained elsewhere (26). Purified MUC1 was coated onto the wells of microtiter plates (0.6 μg/well) at 37 °C overnight. Bovine serum albumin (BSA) was used as negative control. The contents of the wells were emptied, washed and blocked with a 4% solution of skimmed milk in PBS (10 mM, pH 7.2) for 1 h at 37 °C. At the end of incubation time, wells were washed and added with dilutions of mAb PR81, and was incubated at 37 °C for 2 h. The content of the wells were emptied, wells were washed, added with proper

dilution of rabbit anti mouse conjugated to HRP and incubated at 37 °C for 1 h. Finally, wells were washed, added with 50 µL of substrate tetramethylbenzidine (TMB) (Roche Diagnostics) and incubated for 10 min. The reaction was stopped by adding 50 µL of 1 M H<sub>2</sub>SO<sub>4</sub> per well and were read at 450 nm in the ELISA auto reader .

#### *Conjugation of DOTA-NHS with the PR81*

In the first step, the purified antibody concentration was measured using a biophotometer (Eppendorf) at OD 280 nm, then PR81 (3.3 mg in 1 mL) bicarbonate buffer (0.2 M Na<sub>2</sub>CO<sub>3</sub>, pH. 9.2) was added to different molar ratios of DOTA-NHS to mAb (50, 80 and 120) in 400 µL bicarbonate buffer 0.2 M, pH 9.2), separately in a borosilicate vial and mixed gently for 20 times by pipetting. The mixtures were gently shaken and incubated at room temperature for 2 h followed by 4 °C for 10 h. Each mixture was then transferred on Vivaspine-2 cut-off filter (30 kDa) separately and centrifuged at 2.684 g for 15 min. In order to terminate the conjugation step and provide the suitable radiolabeling pH, the upper filter fractions were washed thoroughly using ammonium acetate buffer (0.2 M, pH 5.5) three times in order to remove excess of DOTA-NHS. To the upper fraction ammonium acetate buffer (1 mL) was added and the mixture was pipetted 10-20 times for immunoconjugate dissolution. The filter was then centrifuged upside-down at 2.684 g for 5 min. Finally the antibody conjugate concentrations were measured using a biophotometer (Eppendorf) at OD 280 nm .

#### *Preparation of radiolabelled monoclonal antibody*

Typically, 37.5-58 MBq of [<sup>64</sup>Cu]CuCl<sub>2</sub> (in 0.2 M HCl) was added to a conical vial and dried under a flow of nitrogen. To the copper-containing vial was added with acetate buffer (500 µL, pH. 5.5) and vortexed for 10 min. The conjugate containing fraction (250 µg) in acetate buffer with the measured protein content was added to the vial and mixed gently for 5 min using pipetting (10-20 x). The mixture was then incubated at 40 °C for 60 min followed by testing the radiochemical purity by ITLC (Instant Thin Layer Chromatography) using a radio TLC scanner (Whatman No.2, 1mM DTPA). Finally ETDA solution (5 - 10 µL, 10 mM) was added to the labeling mixture and incubated for 10 min in order to scavenge the excess unlabelled Cu cation. The mixture was then passed through the disposable PD10 de-salting column in order to further increase the radiochemical purity of the mixture. The solution was then passed through a 0.22 micron biological filter and stored at 4 °C for animal studies .

#### *SDS-polyacrylamide gel electrophoresis*

The radioimmunoconjugate was analysed for integrity by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The radiolabeled mAb was evaluated without reduction by 2-mercaptoethanol. Approximately 10 µg of each preparation was applied per lane and the 4-20% polyacrylamide were run according to the method of Laemmli (27).

#### *Reactivity of radiolabeled mAb PR81 towards MUC1 antigen by RIA*

After labelling of mAb with <sup>64</sup>Cu, the reactivity of radiolabeled mAb PR81 towards

MUC1 was considered by RIA. Purified MUC1 antigen was coated onto the wells of microtiter plates (0.2 µg/well) at 37 °C overnight, same concentration of BSA was used as negative control. The contents of the wells were emptied, washed and blocked with a 4% solution of skim milk for 1 h at 37 °C. Finally, wells were washed and added with 30,000 cpm of <sup>64</sup>Cu-DOTA-PR81 and was incubated at 37 °C for 2 h. The content of the wells were emptied, wells were washed and the radioactivity of wells were measured by γ-counter.

#### *Cell toxicity*

MCF7 and CHO cells were harvested and re-suspended at 1×10<sup>4</sup> cells/200 µL in RPMI 1640 medium and cultured in 96-well plate. After 24 h, 0–2500 ng/ml of the conjugate was added to the wells and incubated for 2 h at 37 °C. The wells were aspirated, and 200 µL of the medium was added to each well. In different times, at 6, 12, 24 and 48 h after reaction, the viability of MCF7 and CHO cells were determined by MTT assay using an ELISA reader (microreader, Hyperion) at 540 nm and considered as a percentage of viability (28). The same procedure was repeated for <sup>64</sup>Cu alone and unmodified PR81 separately as control studies.

#### *Biodistribution of <sup>64</sup>Cu-DOTA-PR81 and <sup>64</sup>CuCl<sub>2</sub> in normal rats*

For biodistribution studies, <sup>64</sup>Cu-DOTA-PR81 and <sup>64</sup>CuCl<sub>2</sub> were administered to normal Sprague Dawley rats separately. A volume (50-100 µL) of final radioactive solution containing 150 ± 5.5 µCi radioactivity and 30 ± 5 µg of PR81 was injected intravenously to rats through their tail

vein. The total amount of radioactivity injected into each rat was measured by counting the 1 mL syringe before and after injection in a dose calibrator with a fixed geometry. Biodistribution studies were performed using groups of three rats sacrificed at 2, 6, 12 and 24 hours after injection of each radiolabeled mAb PR81. At each time point, rats were sacrificed using CO<sub>2</sub> gas and normal organs (lungs, stomach, small and large intestine, spleen, blood, heart, kidneys, bone, feces, skin and liver) were excised. Organs were weighed and gamma counted with an HPGe detector counting for the area under the curve of the 511 keV peak. The percentage of injected dose of radioisotope per gram (%ID/g) organ was calculated (after correcting for radioactive decay using an aliquot of the injected at each time point). Mean values and standard errors of radioactive counting for each tissue and time point was plotted.

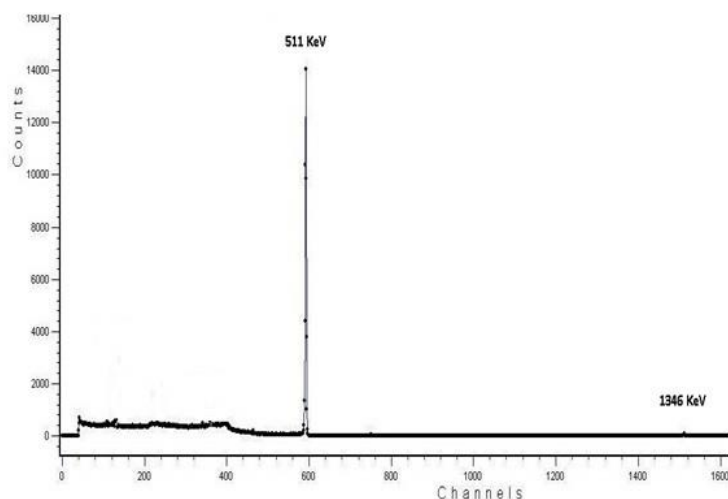
#### *Statistical analysis*

All values were expressed as mean ± standard deviation (Mean ± SD) and the data were compared using student's T-test following statistical significance defined as P < 0.05.

## **Results**

#### *Radionuclide production*

The radionuclide was prepared in a 30 MeV cyclotron according to the reported methods with a range of no-carrier-added specific activity for radiolabeling use, after counting the samples on an HPGe detector for 5 min, two major photons (511 keV; %35 and 1346 keV; %1 intensity) were observed as reported previously (29) (Fig 1).



**Figure 1** Gamma spectrum of  $^{64}\text{CuCl}_2$  solution obtained in this study using Canberra<sup>TM</sup> multi-channel analyser equipped by HPGe detector in 300 seconds.

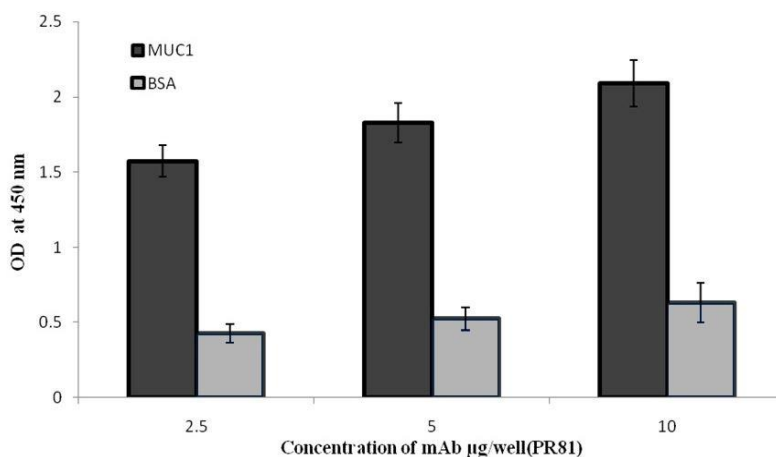
The radioisotope was dissolved in acidic media as a starting sample and was further diluted and evaporated for obtaining the desired pH and volume followed by sterile filtering. The radiochemical purity of the  $^{64}\text{Cu}$  solution was checked in two solvent systems, in 1 mM DTPA and 10% ammonium acetate:methanol mixture.

#### *Reactivity of mAb PR81 towards MUC1 by ELISA*

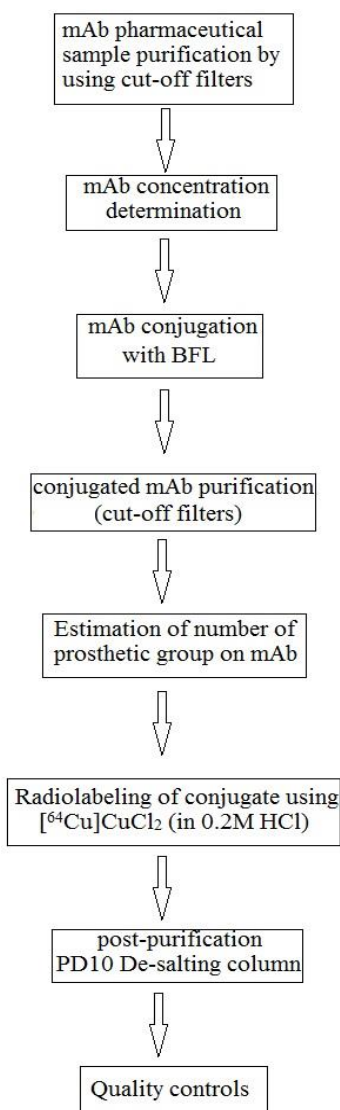
Figure 3 shows the reactivity of mAb PR81 towards the native cancerous MUC1 purified from metastasis ascites fluid, following the ELISA procedure represented here. mAb PR81, produced from hybridoma cell line, showed high immunoreactivity towards MUC1 protein. The highest optical density difference from that of the nonspecific (reactivity of anti MUC1 with corresponding concentration of BSA) was found at an antibody concentration of 10  $\mu\text{g}$  (Fig 2).

#### *Conjugation and radiolabeling of the antibody with $^{64}\text{Cu}$*

In order to overcome the effect of non-antibody DOTA-NHS conjugation and producing appropriate pH for conjugation step the pharmaceutical sample was purified by ultra-filtration using cut-off filters followed by determination of the antibody concentration using spectrophotometry. In order to improve the conjugation step alkaline pH is necessary, thus bicarbonate buffer was used to reconstitute the antibody. In each step biophotometric assay as well as structure integrity test using SDS-PAGE performed in order to guarantee the quantity and the quality of the antibody. The use of polymer tubes and other synthetic materials in the conjugation and labeling step interfered with the conjugation reaction, while borosilicate vials were the appropriate vessels. In order to remove the leftover of DOTA-NHS in the reaction and concentrate the antibody, the cut-off filter was used once more (30 kDa) (Fig 3).



**Figure 2** Reactivity of mAb PR81 ( 2.5, 5 and 10 µg/well) towards cancerous MUC1 (0.6 µg/well) purified from ascetic fluid and BSA (0.6 µg/well). Assays were performed in triplicate.



**Figure 3** Schematic flow-diagram for the monoclonal antibody conjugation and radiolabelling; (BFL: Bi-Functional Ligand).

*Radiochemical purity determination*

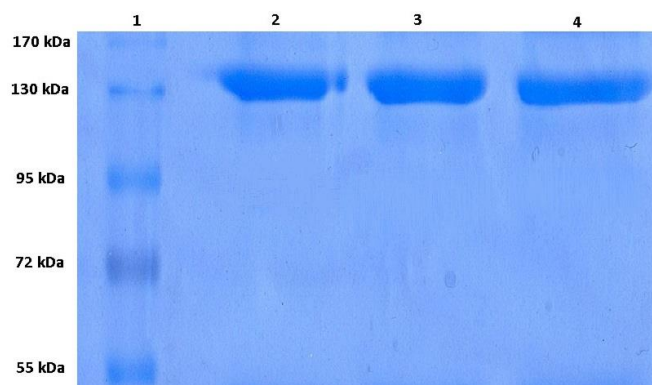
ITLC using various mobile and stationary phases was performed in order to ensure the existence of only the desired radiolabeled antibody. Two different solvent systems with two stationary phases were tested. In all tests, radiolabeled antibody retained at the origin while other species migrated to other retention factors ( $R_f$ s) depending on the mobile phase used. The  $R_f$ s of the possible occurring chemical species in chromatography of the reaction steps are summarized in Table 1 ( $n = 5$ ).

*Protein integrity test using SDS-polyacrylamide gel electrophoresis*

In order to demonstrate the integrity of the protein after residulation and radiolabeling, gel electrophoresis was performed on the SDS PAGE gels using 16% bisacrylamide gel. The loaded samples were PR81 mAb sample, DOTA-PR81 and radiolabeled antibody samples three weeks after the experiment while kept at 4 °C. Gels were stained with Coomassie Blue. The samples were showed to have similar pattern of migration in the gel electrophoresis reported previously (30) (Fig 4).

**Table 1** The chromatography details of  $^{64}\text{Cu}$ -DOTA-PR81 and related species

Chromatography system	Chemical species	Mobile phase	Stationary phase	$R_f$
1	$^{64}\text{Cu}$ -DOTA-PR81	10% Ammonium acetate:methanol (1:1)	Silicagel	0.0
	$^{64}\text{Cu}$ -DOTA	//	//	0.3
	$^{64}\text{Cu}$	//	//	0.0
2	$^{64}\text{Cu}$ -DOTA-PR81	1 mM DTPA (pH. 5)	Whatman No. 2	0.0
	$^{64}\text{Cu}$ -DOTA	//	//	0.0
	$^{64}\text{Cu}$	//	//	0.8



**Figure 4.** SDS-PAGE of the  $^{64}\text{Cu}$ -DOTA-PR81 (Lane 2), conjugated DOTA-PR81 (Lane 3) and the purified PR81 (Lane 4) monoclonal antibodies, ( Lane 1) standard high molecular mass protein marker with molecular weights of 170, 130, 95, 72 and 55 kDa; all samples showed a molecular mass related to the whole IgG is 150 kDa.



*Reactivity of radiolabeled PR81 towards MUC1 antigen by RIA*

Reactivity of radiolabelled mAb PR81 towards the MUC1 antigen (0.2  $\mu\text{g}/\text{well}$ ) and BSA (0.2  $\mu\text{g}/\text{well}$ ) was shown in Figure 5, The  $^{64}\text{Cu}$ -DOTA-PR81 showed high immunoreactivity towards MUC1 antigen. This result also showed that the chemical binding of  $^{64}\text{Cu}$  to mAb had no adverse effects on the immunoreactivity of antibody towards MUC1 antigen.

*Cell toxicity of MCF7 cell line*

Cytotoxicity of  $^{64}\text{Cu}$ -DOTA- PR81 towards MCF-7 and CHO cells proved to be negligible and concentration dependent, these results suggested that  $^{64}\text{Cu}$ -DOTA-PR81 conjugate can target and kill about only 20-25% of MCF-7 cells *in vitro* using medium concentration 2.5 ng/ml at different times of study. Also our radioimmunoconjugate inhibited only 25-30% growth of the MCF-7 cultured cell lines *in vitro* in a 25 to 250 ng/ml concentration. At the maximum dose of 2500 ng/ml of  $^{64}\text{Cu}$ -DOTA-PR81 the cell survival was

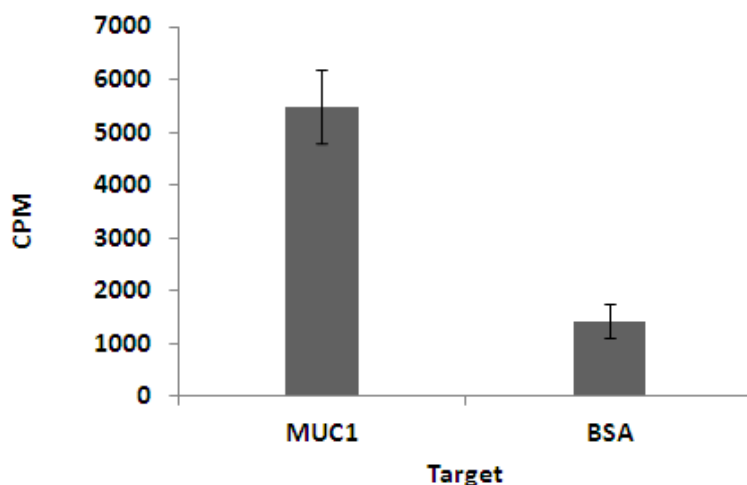
65–70% at 12-24 h, while those treated with nonspecific  $^{64}\text{Cu}$  and PR81 mAb was about 82%, and 78% respectively at 12-24 h (Fig 6).

*$^{64}\text{Cu}$ -free biodistribution in normal Sprague-Dawley rat tissues*

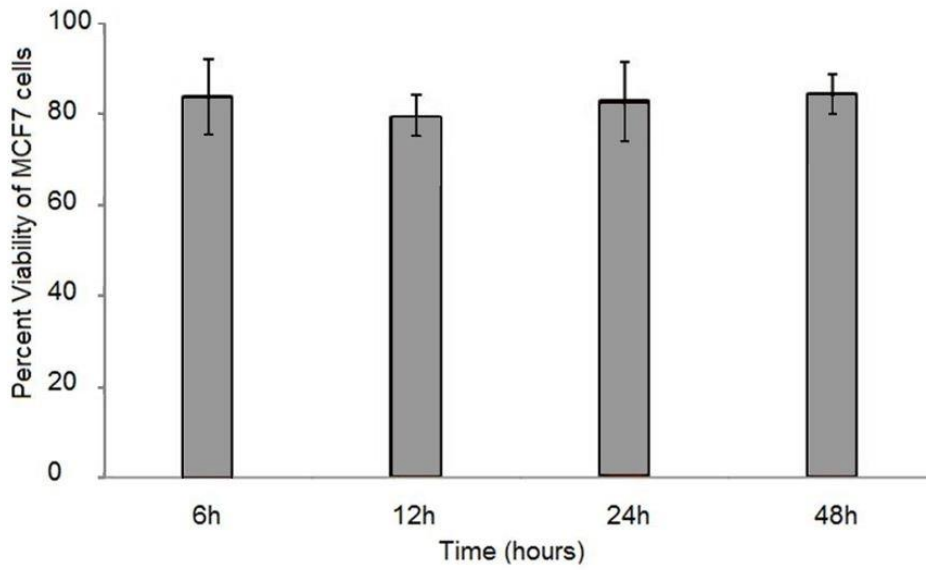
The liver uptake of the cation is comparable to many other radio-metals accumulation reported in other works. In this study about 20-40 % of the activity accumulates in the liver after 24 h (Fig 7).

*$^{64}\text{Cu}$ -DOTA-PR81 biodistribution in normal Sprague-Dawleyrat tissues*

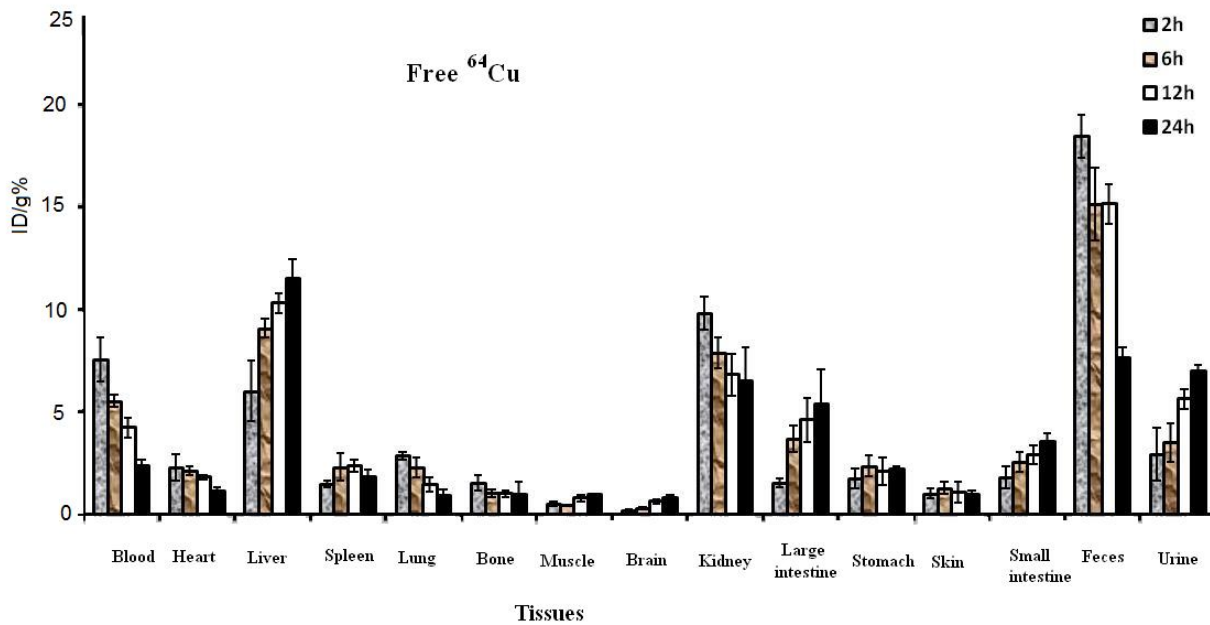
As shown in Figure 8, biodistribution studies in adult normal Sprague-Dawely rats after injection of  $^{64}\text{Cu}$ -DOTA-PR81 demonstrated significant uptake in stomach, small intestine, liver and lung organs during the study time. Also high uptake in kidney organ during the study observed which is due to the presence of MUC1 antigens in mentioned tissues leading to high colon and feces activity content, which is in agreement with the other findings.



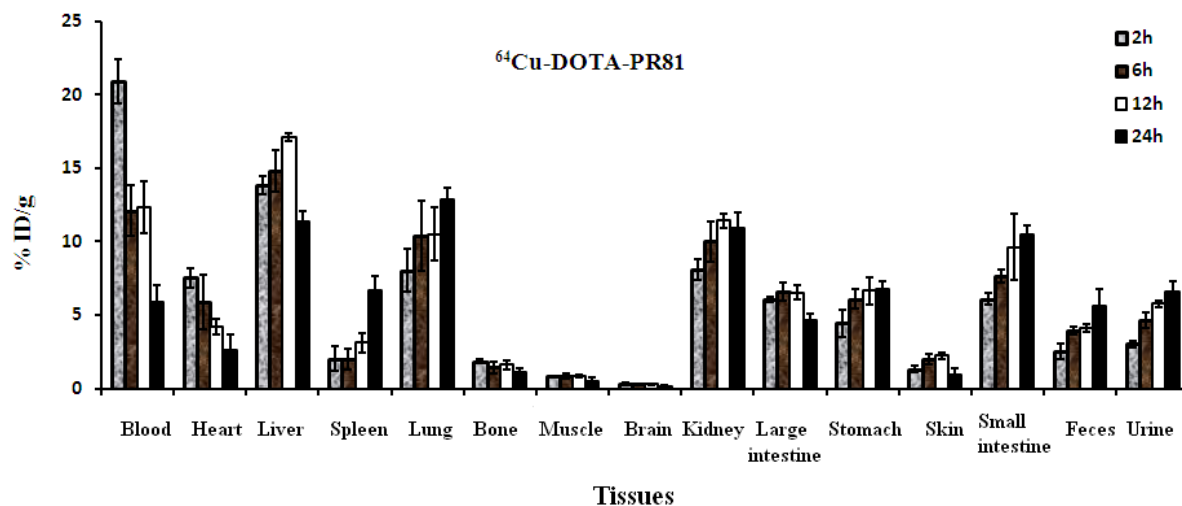
**Figure 5.** Reactivity of  $^{64}\text{Cu}$ -DOTA-PR81 (5 $\mu\text{g}/\text{well}$ ) towards MUC1 (0.2 $\mu\text{g}/\text{well}$ ) and BSA (0.2 $\mu\text{g}/\text{well}$ ). All assay performed in triplicate. Error bars represent standard error of the mean.



**Figure 6** The results of MTT colorimetric assay after 6, 12, 24 and 48 h of treatment with equivalent concentration of  $^{64}\text{Cu}$  (radioactivity) that is same with  $^{64}\text{Cu}$ -DOTA-PR81 in 2500 ng/ml.



**Figure 7** Percentage of injected dose per gram (% ID/g) of  $^{64}\text{Cu}$ -free in normal Sprague–Dawley rat tissues at 2, 6, 12 and 24 h post-injection (n = 3).



**Figure 8**  $^{64}\text{Cu}$ -DOTA-PR81 biodistribution results (ID/g %) in adult normal Sprague-Dawley rat tissues at 2, 6, 12 and 24 h post-injection (n = 3).

### Discussion

The radiochemical purity of the  $^{64}\text{Cu}$  solution was checked in two solvent systems, in 1 mM DTPA,  $\text{Cu}^{2+}$  cation is converted to more lipophilic Cu-DTPA form and migrates to higher Rf (0.8) while any small radioactive fraction remaining at the origin could be related to other Cu species, not forming Cu-DTPA complex, such as  $[\text{CuCl}_4]^{2-}$  and/or colloids (not observed).

On the other hand, 10% ammonium acetate:methanol mixture was also used to determine radiochemical purity. The fast eluting species was possibly the ionic  $^{64}\text{Cu}$  cations other than  $\text{Cu}^{2+}$  (not observed) and the remaining fraction at Rf. 0 was a possible mixture of  $\text{Cu}^{2+}$  and/or colloids. The difference in values of impurity in two solvent systems is possibly due to the presence of colloidal impurity in the sample, considering the purities of both chromatograms copper cation is the only radiochemical species present.

The highest optical density difference from that of the nonspecific (reactivity of anti

MUC1 with corresponding concentration of BSA) was found at an antibody concentration of  $10\ \mu\text{g}$ . The specific binding of the monoclonal antibody was at least 4 times of the unspecific binding of the BSA in all treatments. The hybridoma cell line that produces a mAb PR81 was stable from the year 2000 and destabilizing factors such as chromosomal deletion or rearrangement in cell division were not occurred during this period. It was previously found that IgG antibodies of reasonably high affinity ( $1 \times 10^8\ \text{M}^{-1}$ ) are optimal for both imaging and therapy (31). For example, the anti MUC1 monoclonal antibodies, BC2 and hCTMO1, is reported to have a high affinity towards MUC1 ( $1.36 \times 10^7\ \text{M}^{-1}$  and  $2.6 \times 10^7\ \text{M}^{-1}$  respectively). The two mentioned antibodies are in clinical trial for breast cancer therapy (32). In this way, mAb PR81, which exhibited high affinity ( $2.19 \times 10^8\ \text{M}^{-1}$ ) towards MUC1 antigen and some MUC1 positive cell line (MCF-7, BT20 and T47D) may be suitable for *in vivo* tumor

targeting, imaging and therapy (33). In order to remove the leftover of DOTA-NHS in the reaction and concentrate the antibody, the cut-off filter was used once more (30 kDa). At this stage, a pH 5.0 buffer was used to recover the antibody in order to terminate the conjugation step and provide the suitable radiolabeling pH, and finally the quantity of the antibody was measured at OD 280 nm. Each conjugated DOTA-PR81 fraction was mixed with  $^{64}\text{CuCl}_2$  solution at the appropriate acidity in acetate buffer at 40 °C for 30 min separately followed by testing the radiochemical purity by ITLC. By the addition of EDTA to the above solution for 5 min the radiolabeling reaction was terminated as well as production of Cu-EDTA complex is permitted, which can be better removed by size exclusion method. The EDTA scavenging time was shown to be critical in order to maintain the appropriate radiochemical purity, increased EDTA incubation time led to the decomposition of radioimmunoconjugate and the reduction of radiochemical purity. As shown in the table-1, for  $^{64}\text{Cu}^{2+}$  detection, the best elution systems is chromatographic protocol 2 resulting in Rf 0.9. However for  $^{64}\text{Cu}$ -DOTA detection, system 1 as mentioned can be used (Rf 0.3).  $^{64}\text{Cu}$ -DOTA-PR81 remains at the origin in all systems used due to the size and charge of the protein ( $\approx 150,000$  Da). By direct radiolabeling of the kit in a simple and rapid manner, the radioimmunoconjugate was easily prepared in radiochemical purity of higher than 90%. Alternatively, the mixture can be further purified using de-salting column to reach at least 95% radiochemical purity. The DOTA-PR81 conjugate,

conjugated by molar chelator to mAb ratio of 50/1 was selected for current study, was labeled with  $^{64}\text{Cu}$  at specific activity as high as 4.6  $\mu\text{Ci}/\mu\text{g}$ . The radiolabeled mAb exhibited high *in vitro* serum stability and minimal loss of immunoreactivity.

There was a little difference in the survival values *in vitro* for the test  $^{64}\text{Cu}$ -DOTA-PR81 compared with  $^{64}\text{Cu}$ , cold mAb. In this study the highest possible dose of Cu-64 (3000 cps) was used as non specific control equivalent to the Cu-DOTA coupled to antibody activity (2500 ng/ml) in order to adjust the radioactivity of specific and non specific ligand. It is known that  $^{64}\text{Cu}$  is a  $\beta$ -emitter radionuclide theoretically to be used as a therapeutic radionuclide in proper concentrations. In another study (16) when  $^{131}\text{I}$ -PR81 was used as a therapeutic radionuclide at an activity of 3000 cps equivalent to 2500 ng/ml of  $^{131}\text{I}$ -PR81, 40% of the cells remained viable in 48 h while at the same conditions for  $^{64}\text{Cu}$ -DOTA-PR81 more than 70% of the cells remained viable in 48 h. These results suggested that the radio nuclide prepared in this study is suitable for imaging purposes and exhibits minimum adverse effects.

Mohammadnejad et al in 2010 working on cytotoxicity of PR81 mAb as well as  $^{131}\text{I}$ -PR81 on MCF-7 cell demonstrated 50% cell growth inhibition by the radiolabeled antibody at 250-2500 ng/ml (16). At the maximum dose in 48 h only 35-40% of cells survived. In our studies using  $^{64}\text{Cu}$ -DOTA-PR81 however in 48 h the survival ratio of the cells was 65-70% while other results could be obtained if the dose was increased. Using  $^{131}\text{I}$ -PR81 after 96 h the survival ratio was 10-20% while at the same

conditions using I-131 in free form as well as cold antibody the survival was 78 and 80% respectively. Thus the MCF7 cell death ratio of 131I-PR81 in 48h was 3-4 times of the control samples (i.e. free I-131 and cold antibody), while for <sup>64</sup>Cu-DOTA-PR81 in 48 h the ratio is less than 2 times. These data demonstrates that 131I-PR81 efficiently destroys MCF-7 cells while <sup>64</sup>Cu-PCTA-PR81 effect is lower possibly due to the ratio of beta emission of Cu-64 compared to I-131 (beta energies; 182 keV for I-131 and 71 keV for Cu-64).

Salouti et al. studied the cell toxicity of <sup>177</sup>Lu-DOTA-PR81 on MCF-7 cells in 2011 and showed that the cell survival ratios of free Lu-177 and cold antibody was 80 and 85% while at 250-2500 ng/ml in 24h and 48 h, 50 and 20-30% of the cells survive respectively (34). The cytotoxicity of 22% obtained by the cold antibody on MCF-7 cells might be a result of participation of MUC-1 antigens in cell growth, however factors such as Ab:Ag affinity, antigen amount can also play important roles in cell death (16).

Song et al. using <sup>213</sup>Bi-C595 radiimmunoconjugate against MUC1 in pancreas cancer on 6cCAPAN-1,CFPAC-1,PANC-1 cell lines, demonstrated that using 10 microcuries of the complex just 10% of the cells survived (35), while in our case 60-65% of the cells survived. These data also suggests the difference of particle irradiation effects on the cells and <sup>64</sup>Cu-DOTA-PR81 therapeutic effects are minor and this radioimmunoconjugate is possibly considered a diagnostic probe.

The transferin-metal complex uptake and final liver delivery showing the possible

route of accumulation. The blood content of free <sup>64</sup>Cu is low at all time intervals (less than 25%) and rapidly decreases during the study time, showing the rapid removal of activity for the circulation. Lung, muscle, bones and also skin do not demonstrate significant uptake of free <sup>64</sup>Cu, this is the same pattern as other cations accumulation. A 30% kidney uptake is observed for the cation due to water solubility. Intestine and feces activity demonstrates significant uptake due to the hepatobiliary excretion.

This has already been shown by other groups, working with anti-MUC1 probes (14,16,17). Lacunza et al showed that different tissues express MUC1 antigen in normal rat tissues including stomach, small intestine, pancreas, lung, liver and kidney of normal rats during late gestation, neonates and adulthood (36).

A significant kidney uptake of radiolabeled mAb PR81 is observed all time intervals. This effect is possibly observed due to the presence of MUC1 antigen as mentioned before. However, it is possible that the free cation (Cu-64) is also in part excreted through urinary tract leading to the accumulation reduction up to 24 h.

*In vivo* experiments in rats by Bass et al demonstrated that <sup>64</sup>Cu was incorporated into SOD (Super Oxide Dismutase) in the hepatocytes of rats administered with <sup>64</sup>Cu-labeled somatostatin analogues. In the case of <sup>64</sup>Cu-DOTA-PR81, increased transchelation of <sup>64</sup>Cu to intracytoplasmic proteins such as SOD could enhance hepatic accumulation. On the other hand the accumulation in liver and spleen, reflect the uptake of macromolecule to the reticuloendothelial cells of these organs. In

our study, uptake level of radiolabeled mAb PR81 increased in the liver up to 12 h and decreased later on (up to 24 h). The same behavior is also observed for free copper cation. Liver activity slightly increases by the time for the cation due to natural copper-ceruloplasmin uptake in the liver as the reservoir organ. This behavior has been already shown to be consistent with half-life of intact mAb in circulation like other radiolabeled mAb anti-MUC1 probes.

### Conclusions

Radiolabeling, stability testing, radiochemical purity, structural integrity, immunoreactivity and cell cytotoxicity of  $^{64}\text{Cu}$ -DOTA-PR81 were determined followed by biodistribution studies (tissue counting and imaging) in normal Sprague-Dawley rat tissues. The radioimmunoconjugate was prepared with a radiochemical purity of higher than  $93.2 \pm 0.6\%$  (ITLC). The  $^{64}\text{Cu}$ -DOTA-PR81 *in vitro* stability of more than 89% in PBS and  $78 \pm 0.5\%$  in human serum over 48 h. RIA studies

showed also high retention of immunoreactivity. The cell toxicity study showed that the complex inhibited 25-30% growths of MCF-7 cells at a concentration of 2500 ng/ml after 24 h. The biodistribution studies showed high blood and liver content as reported for many radiolabeled monoclonal antibodies. Further studies on the application of  $^{64}\text{Cu}$ -DOTA-PR81 in MUC1+ tumor bearing models is essential. Preliminary binding as well as distribution studies for  $^{64}\text{Cu}$ -DOTA-PR81 suggests a potential promising compound for PET imaging in diagnosis and follow-up of MUC1 expression in breast cancer imaging.

### Conflict of interest

The authors declared no potential conflict of interest with respect to the authorship, and/or publication of this study.

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