**90Y/177Lu-labelled Cetuximab immunoconjugates: radiochemistry optimization to clinical dose formulation**

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Radiolabelled monoclonal antibodies (mAbs) are increasingly being utilized in cancer theranostics, which is a significant move toward tailored treatment for individual patients. Cetuximab is a recombinant, human–mouse chimeric IgG1, mAb that binds to the epidermal growth factor receptor with high affinity. We have optimized a protocol for formulation of clinically relevant doses (~2.22 GBq) of 90Y-labelled Cetuximab and 177Lu-labelled Cetuximab by conjugation of the mAb with a suitable bifunctional chelator, N,N′-[trans-(S,S)-cyclohexane-1,2-diamine]-N,N′,N″,N″-pentaacetic acid (CHX-A′-DTPA). The radioimmunoconjugates demonstrated reasonably high specific activity (1.26 ± 0.27 GBq/mg for 90Y-CHX-A′-DTPA-Cetuximab and 1.14 ± 0.15 GBq/mg for 177Lu-CHX-A′-DTPA-Cetuximab), high radiochemical purity (>95%) and appreciable in vitro stability under physiological conditions. Preliminary biodistribution studies with both 90Y-CHX-A′-DTPA-Cetuximab and 177Lu-CHX-A′-DTPA-Cetuximab in Swiss mice bearing fibrosarcoma tumours demonstrated significant tumour uptake at 24-h post-injection (p.i.) (~16%ID/g) with good tumour-to-background contrast. The results of the biodistribution studies were further corroborated by ex vivo Cerenkov luminescence imaging after administration of 90Y-CHX-A′-DTPA-Cetuximab in tumour-bearing mice. The tumour uptake at 24 h p.i. was significantly reduced with excess unlabelled Cetuximab, suggesting that the uptake was receptor mediated. The results of this study hold promise, and this strategy should be further explored for clinical translation.

**Keywords:** cancer; cetuximab; EGFR; lutetium-177; theranostics; yttrium-90

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**Introduction**

Over the last several years, there is an increasing thrust towards use of radiolabelled monoclonal antibodies (mAbs) with high affinity towards tumour-associated antigens for cancer-specific imaging and therapy. 1,2 One of the most promising targets for this purpose is the family of epidermal growth factor receptors (EGFRs). 2,3 Overexpression of EGFR is the characteristic of many human tumours, such as head and neck squamous cell carcinoma (HNSCC), gastrointestinal and abdominal carcinomas, lung carcinomas, soft tissue carcinomas, breast carcinomas, carcinomas of the reproductive tract, melanomas, glioblastomas and thyroid carcinomas. 2,3 In several cases, EGFR overexpression may act as a prognostic indicator, predicting poor survival and/or more advanced disease stage.

Cetuximab (IMC-C225), an EGFR antagonist, is a chimeric human-mouse mAb commercially available as Erbitux® (Merck KGaA, Darmstadt, Germany). 2 This mAb has been approved by the United States Food and Drug Administration for treatment of colorectal cancer or HNSCC. Over the last decade, there have been numerous reports on preparation of radiolabelled Cetuximab conjugates for cancer management. 2,4 Among various radionuclides studied, 90Y (t1/2 = 64.1 h) and 177Lu (t1/2 = 6.7 days) are the most frequently used radionuclides in radioimmunotherapy (RIT) of tumours. 8–10 Yttrium-90 is a high-energy β- emitter (Emax = 2.28 MeV) that gives higher dose rate because of its shorter half-life. Its long emission range can penetrate to tissues farther away from the target tissue and kill the adjoining cancerous tumour cells that may not have bound the radiolabelled Cetuximab. This radioisotope is especially advantageous in larger tumours and in tumours with heterogeneous receptor distribution. On the other hand, 177Lu has lower energy and smaller particle range (emits β- particles with Emax of 497 keV (78.6%), 384 keV (9.1%) and 176 keV (12.2%)), leading to better absorption in smaller tumours. Additionally, this radioisotope emits 208 keV γ-radiation which is suitable for single photon emission computed tomography (SPECT) imaging and also enables dosimetry during RIT.

In order to enable radiolabelling with a metallic radioisotope such as 90Y or 177Lu, Cetuximab must be conjugated with a suitable bifunctional chelator (BFC). The selection of the BFC can significantly affect both the radiochemical and biological...
features of the prepared radiolabelled mAb. Among various BFCs studied, the derivatives of 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) and diethylentriamine pentaacetate (DTPA) are most commonly used for labelling mAbs with $^{90\text{Y}}$Y or $^{177\text{Lu}}$Lu and their coordination chemistry is well established.\textsuperscript{11,12} Despite availability of extensive volume of literature on radiolabelling Cetuximab conjugates, it is pertinent to mention that most of the reported studies have employed tracer level of activity in preclinical settings.\textsuperscript{2} From the perspective of real clinical utility, it is essential to optimize a procedure for preparation of clinically relevant doses of the radiolabelled immunoconjugate, which can be administered in human subjects. Herein, we report a procedure for preparation of clinically relevant doses of $^{90\text{Y}}$Y-labelled Cetuximab and $^{177\text{Lu}}$Lu-labelled Cetuximab immunoconjugates. The radiolabelling procedure was optimized, and the suitability of the radiolabelled agent for clinical use was evaluated. The biological efficacy of the radiolabelled agents was established by biodistribution studies in Swiss mice bearing fibrosarcoma tumours, which was further substantiated by Cerenkov luminescence imaging (CLI).

**Experimental**

**Materials and equipment**

Lutetium oxide (84.6% enriched in $^{176\text{Lu}}$Lu, spectroscopic grade, > 99.99% pure) used as the target for the production of $^{177\text{Lu}}$Lu in nuclear reactor was procured from Trace Science International, Canada. Erbitux$^\text{a}$ (Cetuximab) was obtained from Merck, Germany. The p-isothiocyanato benzyl derivatives of BFCs such as CHX-A$^\text{b}$-DTPA-NCS and DOTA-NCS were purchased from Macro cyclics, USA. Suprapure hydrochloric acid was purchased from Merck, Germany. MilliQ water (resistivity > 18.2 MΩ·m) was used for all the studies. All other chemicals used in the experiments were of analytical reagents grade and supplied by reputed chemical manufacturers.

Flexible silica plates (coating thickness 0.25 mm) from J. T. Baker Chemical Company, USA, were used for thin-layer chromatography (TLC) studies. Paper chromatography (PC) strips (3MM Chr, 20 mm width) were purchased from Whatman International Limited, United Kingdom. PD-10 desalting columns were procured from G.E. Healthcare, Germany. Amicon Ultra-15 centrifugal filter units with Ultracel-30 membrane (30 K centrifugal filters) of 15 mL capacity were obtained from Millipore (Merck, Germany).

Assay of $^{90\text{Y}}$Y activity obtained from the generator was carried out by using a liquid scintillation counter (Model–Tricarb 2100TR-Packard Instrument Co., USA). Gamma ray spectra of $^{177\text{Lu}}$Lu were recorded using an HPGe detector (Canberra Eurusys, France) coupled to a 4 K multichannel analyser system for specific activity and radionuclidic purity assay. A $^{15\text{Eu}}$Eu reference source, obtained from Amersham Inc., USA, was used for energy and efficiency calibration of the HPGe detector. Other radioactivity measurements were carried out by using a well-type NaI (TI) scintillation counter (Mucha, Raytest, Germany). CLI of the animals after administration of the radiolabelled agents were recorded using a Photon Imager (Biospace Lab, France).

Sterility of the radiolabelled formulation was tested in tryptic soya broth media and fluid thioglycollate media using Himedia Labs sterility test kits. Pyrogenicity of the preparation was checked by point-of-use test system (Charles River, USA).

**Production of $^{177\text{Lu}}$Lu**

Lutetium-177 was produced following direct neutron activation route in the Dhruva research reactor at Bhabha Atomic Research Centre, India, by irradiation of isotopically enriched (84.6% in $^{176\text{Lu}}$Lu) Lu$_2$O$_3$ target at a thermal neutron flux of $\sim$1.2 $\times$ 10$^{14}$ n cm$^{-2}$ s$^{-1}$ for a period on 21 days, as per the procedure reported by us earlier.\textsuperscript{13,14} After end of irradiation (EOI), the irradiated target was cooled for 6 h and then dissolved in 0.01 mol L$^{-1}$ suprapure HCl by heating at 80 °C under reflux for a period of 15 min.

Assay of the total activity of $^{177\text{Lu}}$Lu produced and determination of the radionuclidic purity of the radioisotope were carried out by gamma ray spectrometry using HPGe detector coupled to a 4 K multichannel analyser system. In order to determine the specific activity of $^{177\text{Lu}}$Lu produced, the accurate mass of Lu in an aliquot of processed $^{177\text{Lu}}$Lu$_3$ solution was determined by inductively coupled plasma–atomic emission spectroscopy technique. The specific activity of $^{177\text{Lu}}$Lu at EOI was determined by dividing the total activity of $^{177\text{Lu}}$Lu with the actual mass Lu present after irradiation. The radiochemical purity of the processed $^{177\text{Lu}}$Lu$_3$ solution was analysed by PC using 0.9% NaCl (w/v) in 0.02 M HCl as the eluting solvent.

**Production of $^{90\text{Y}}$Y**

Yttrium-90 used in this investigation was obtained from a $\sim$5 GBq electrochemical $^{90\text{Sr}}$/90Y generator developed in-house. A HNO$_3$ solution (0.01 mol L$^{-1}$) containing $^{90\text{Sr}}$ in equilibrium with $^{90\text{Y}}$Y was used as an electrolyte, and $^{90\text{Y}}$Y was selectively electrodeposited on a platinum cathode following the procedure reported earlier by our group.\textsuperscript{15–17} The $^{90\text{Y}}$Y activity deposited on the electrode was subsequently retrieved in 0.25 mL of 0.1 mol L$^{-1}$ HCl solution.

Assay of $^{90\text{Y}}$Y activity obtained from the electrochemical $^{90\text{Sr}}$/90Y generator was carried out by using a liquid scintillation counter. The presence of trace level of $^{90}\text{Sr}$ impurity in $^{90\text{Y}}$Y was evaluated adopting the extraction PC technique, as reported earlier.\textsuperscript{16} The radiochemical purity of the processed $^{90\text{Y}}$YCl$_3$ solution was analysed by PC using 0.9% NaCl (w/v) in 0.02 mol L$^{-1}$ HCl as the eluting solvent.

**Conjugation of Cetuximab with BFCs and characterization of immunoconjugates**

Conjugation of BFCs (CHX-A$^\text{b}$-DTPA or DOTA-NCS) with Cetuximab was carried out at as per the procedure reported by Cai et al.\textsuperscript{19} The original solution of Cetuximab was concentrated by ultrafiltration using an Amicon-15 centrifugal device (molecular weight cutoff: 30 kDa, volume capacity 15 mL). Concentrations of the mAb were determined by UV spectrophotometry at 280 nm. For conjugation with BFC, 10 mg of Cetuximab in 0.01 mol L$^{-1}$ phosphate buffer (pH 7.2) was mixed with ten-fold molar excess of the respective BFC previously dissolved in phosphate buffer and the final pH was adjusted to 8.5 with 1 mol L$^{-1}$ NaOH solution. Afterwards, the reaction mixture was incubated at room temperature (25 °C) overnight. The immunoconjugates were purified by size-exclusion chromatography using PD-10 desalting columns, pre-equilibrated with PBS. The immunoconjugates were eluted between 3.5 and 4.5 mL. The immunoconjugates were then concentrated by ultrafiltration using an Amicon-15 centrifugal device, and their concentrations were determined by UV spectrophotometry at 280 nm.

The immunoconjugates were characterized by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 5% stacking gel and 8% resolving gel; non-reducing conditions) using Coomassie brilliant blue R-250 staining, as per the reported procedure.\textsuperscript{20}

**Optimization of radiolabelling conditions of immunoconjugates at tracer level**

Cetuximab conjugates (CHX-A$^\text{b}$-DTPA-Cetuximab or DOTA-Cetuximab) were radiolabelled with $^{90\text{Y}}$Y in the following way. The respective immunoconjugate solution (4 μg L$^{-1}$) in MilliQ water was mixed with 0.25 mol L$^{-1}$ ammonium acetate buffer (pH ~5.5), and 100 μL of $^{90\text{Y}}$Y ($\sim$185 MBq, 5 μCi in 0.1 mol L$^{-1}$ HCl medium) was added to it. The pH of the resulting mixture was adjusted to ~5, by adding 0.1 mol L$^{-1}$ HCl solution. The volume of the final reaction mixture was made up to 1 mL by addition of MilliQ water.

Labelling of Cetuximab conjugates (CHX-A$^\text{b}$-DTPA-Cetuximab or DOTA-Cetuximab) with $^{177\text{Lu}}$Lu was carried out by adopting similar
procedure as described earlier for labelling with ⁹⁰Y. The only difference in this case was that 20 μL of ¹⁷⁷Lu (~185 MBq, 5 mCi in 0.1 mol L⁻¹ HCl medium) was added to the reaction mixture instead of ⁹⁰Y.

For both the radioisotopes, different sets of reaction mixtures were prepared by varying the amount of immunocomplex (20, 40, 100 and 200 μg) while keeping other reaction parameters constant. The reaction mixtures were incubated at room temperature (25 °C) or 37 °C for 1 h, and the radiolabelling yields were determined.

**Determination of radiolabelling yields of immunocomplexes**

The radiolabelling yields of the radioimmunocomplexes were determined by radio-TLC, as per the reported procedure.²¹ For this purpose, 5 μL of radiolabeled immunocomplex was applied on a TLC plate at 1.5 cm from the bottom. The chromatogram was developed in 10 mM DTPA solution (pH 3). Subsequently, the TLC plate was dried and cut into 1 cm pieces, and activity of each piece was determined using a NaI(Tl) counter. The radiochemical purity of ⁹⁰Y-labelled Cetuximab or ¹⁷⁷Lu-labelled Cetuximab was expressed as the percentage of the total activity, which remained at the point of application (Rₑ = 0–0.1). As control experiments, radio-chromatograms of ⁹⁰YCl₂, ¹⁷⁷LuCl₃, ⁹⁰⁰Y-DOTA, ⁹⁰⁰Y-CHX-A-DTPA, ¹⁷⁷Lu-DOTA and ¹⁷⁷Lu-CHX-A-DTPA solutions were also developed in 10 mM DTPA solution, as described before. For this purpose, ⁹⁰⁰Y-DOTA, ⁹⁰⁰Y-CHX-A-DTPA, ¹⁷⁷Lu-DOTA and ¹⁷⁷Lu-CHX-A-DTPA complexes were prepared as per the reported procedure.¹²,²²

**Purification of radioimmunocomplexes**

The radiolabelled agent was purified using PD-10 columns, with PBS as the mobile phase. The radioactive fractions containing radioimmunocomplexes (which typically elute between 3.5 and 4.5 mL) were collected and used for further studies.

**In vitro stability of radioimmunocomplexes in PBS and mouse serum media**

In vitro stability of the radioimmunocomplexes in PBS and mouse serum media was investigated by radio-TLC assay. For this purpose, an aliquot of the radioimmunocomplex was added to 10 times excess volume of PBS or mouse serum. The radio-TLC pattern was developed adopting the procedure as described earlier. The stability of the radioimmunocomplexes in PBS or mouse serum (at 37 ± 0.1 °C) media over a period of 72 h was analysed by radio-TLC assays at different time intervals.

**Clinical dose formulation of ⁹⁰⁰Y-CHX-A-DTPA-Cetuximab and ¹⁷⁷²⁶⁰Lu-CHX-A-DTPA-Cetuximab and their quality control**

For preparation of clinically relevant doses of ⁹⁰⁰Y-CHX-A-DTPA-Cetuximab, 0.5 mL solution of CHX-A-DTPA-Cetuximab in MilliQ water (~4 μg/mL concentration) was mixed with 0.3 mL of 0.25 mol amonomium acetate buffer (pH 5) and 0.2 mL of ⁹⁰⁰Y solution (~3.7 GBq, 100 mCi in 0.1 mol L⁻¹ HCl medium) was added to it. The pH of the resulting mixture was adjusted to 5, by adding 0.1 mol L⁻¹ HCl solution.

Similarly, for preparation of clinically relevant doses of ¹⁷⁷²⁶⁰Lu-CHX-A-DTPA-Cetuximab, 0.5 mL solution of CHX-A-DTPA-Cetuximab in MilliQ water (~4 μg/mL concentration) was mixed with 0.3 mL of 0.25 mol L⁻¹ ammonium acetate buffer (pH 5) and 0.2 mL of ¹⁷⁷²⁶⁰Lu solution (~3.7 GBq, 100 mCi in 0.1 mol L⁻¹ HCl medium) was added to it. The pH of the resulting mixture was adjusted to 5, by adding 0.1 mol L⁻¹ HCl solution.

In both the cases, the reaction mixtures were incubated at room temperature (25 °C) for 1 h and the radiolabelling yields were determined as described before. The radioimmunocomplex was purified using PD-10 columns, with PBS as the mobile phase. The radioactive fractions containing radioimmunocomplex were collected and passed through 0.2 μm syringe filter before in vivo studies.

The radiochemical purity of the radioimmunocomplexes (after passing through PD-10 column) was determined by radio-TLC assay as described before. In order to ascertain the stability of the radioimmunocomplexes, the radiochemical purities were also determined at different time intervals over a period of 72 h. Additionally, in vitro stability of radioimmunocomplexes were assessed in PBS and mouse serum media over a period of 72 h, as described before. The sterility and apyrogenicity of the radioimmunocomplexes were established by following the reported procedure.²³

**Biodistribution studies in tumour bearing mice**

All animal studies were conducted under a protocol approved by the Bhabha Atomic Research Centre Animal Ethics Committee. The biological behaviour of the radioimmunocomplexes was studied in Swiss mice bearing fibrosarcoma tumours. Fibrosarcoma tumours were raised in Swiss mice by injecting ~1 × 10⁶ fibrosarcoma cells (ATCC® CRL-2295™) suspended in 200 μL of phosphate-buffered saline (PBS) subcutaneously into the right thigh of each mouse weighing 20–25 g. The animals were observed for visibility of tumours and allowed to grow for about 2 weeks. Animals having tumours with a mean diameter of ~8 mm (range, 6–9 mm) were used in the biodistribution experiments.

The radioimmunocomplex was diluted in 0.9% NaCl medium, and 100 μL (3.7–5.5 MBq) solution was injected into each animal through a lateral tail vein. The animals were sacrificed by cardiac puncture post-anaesthesia at different time points post-injection (p.i.). Four animals were used at each time point. Various organs, tissues and tumours were excised after sacrifice, washed with physiological saline, dried and the radioactivity associated with each organ and tissue was determined using a flat-type NaI(Tl) counter. The weight of each organ and tumour was also determined by using an analytical balance. The percent injected activity (%ID) in various organs, tissues and tumour was calculated from the earlier data and expressed as percentage injected activity per gram (%ID/g) of organ/tissue.

For ⁹⁰⁰Y-CHX-A-DTPA-Cetuximab, in addition to ex vivo biodistribution studies, Cerenkov luminescence images of the excised organs and tumour were also acquired in a Photon Imager at 24 and 72 h.p. of the radioimmunocomplex in fibrosarcoma tumour-bearing mice.

Saturation studies were also performed to determine whether the uptake of the radioimmunocomplexes in fibrosarcoma tumour is receptor (EGFR) mediated. For this, another group of tumour-bearing mice was used, and each animal was administered with 3.7–5.5 MBq of the respective radiotracer (¹⁷⁷²⁶⁰Lu-CHX-A-DTPA-Cetuximab and ⁹⁰⁰Y-CHX-A-DTPA-Cetuximab) along with 5 mg (200–250 mg kg⁻¹ of body weight) of ‘cold’ Cetuximab. Such a high dose of mAb was used to ensure that all the receptors are blocked. The animals were sacrificed at 24 p.i., and %ID/ g of organ/tissue was determined following the procedure mentioned before. The uptakes in different organs/tissue and tumour were compared with those obtained in the absence of Cetuximab.

**Results**

**Production of ¹⁷⁷²⁶⁰Lu**

The yield and specific activity of ¹⁷⁷²⁶⁰Lu produced by thermal neutron bombardment on isotopically enriched (84.6% in ¹⁷⁶Lu) for 21 days were found to be 693 ± 16 GBq and 92 ± 23 GBq/mg (n = 10), respectively, at the EOI. The radionuclidic purity of ¹⁷⁷²⁶⁰Lu as determined by gamma ray spectrometry was found to be 99.985 ± 0.005% 24 h post EOI.¹⁷⁷²⁶⁰Lu (t₁/₂ = 160.4 d, β⁺ + β⁻, Eᵣ = 128, 153, 228, 378, 414 and 418 keV) was found to be the sole radionuclidic impurity. No γ-ray peaks corresponding to any other radionuclide except ¹⁷⁷²⁶⁰Lu were observed even in the decayed ¹⁷⁷²⁶⁰Lu sample (50–70 days decay time). Because the radionuclidic impurity burden of ¹⁷⁷²⁶⁰Lu in ¹⁷⁷²⁶⁰Lu produced was <0.02% at EOI, the total burden of ¹⁷⁷²⁶⁰Lu in a patient administered with 1.85 GBq dose of ¹⁷⁷²⁶⁰Lu would be less than
37 kBq (1 μCi). This is not expected to have any adverse consequences as far as the therapeutic utility of $^{177}$Lu is concerned as the contribution to the absorbed dose from $^{177m}$Lu would be negligible.24

The radiochemical purity of $^{177}$Lu obtained after radiochemical processing of the irradiated target was found to be 99.2 ± 0.2%, as determined by paper chromatographic technique. Hydrated $^{177}$Lu$^{3+}$ ion, the desired radiochemical species of $^{177}$Lu, exhibited $R_f$ of 0.9–1.0. Any lutetium in colloidal/particulate form, if present as radiochemical impurity, remains at the point of spotting ($R_f = 0$).

Production of $^{90}$Y

In a typical batch, ~4.44 GBq (120 mCi) of no-carrier-added (NCA) $^{90}$Y could be availed from the electrochemical $^{90}$Sr/$^{90}$Y generator. The $^{90}$Y separation yield was always >80%, which indicates the highly reproducible performance of this generator. The $^{90}$Sr breakthrough was expressed as a percentage of the $^{90}$Sr activity in the separated $^{90}$Y at time of separation. The $^{90}$Sr/$^{90}$Y ratio in the separated product as estimated by extraction paper chromatograph was <8×10⁻⁷, which is well within the pharmacopeia limits (2×10⁻⁵).15

Radiochemical purity of $^{90}$Y obtained after electrochemical separation was found to be 99.4 ± 0.5% as determined by PC technique using 0.9% NaCl (w/v) in 0.02 M HCl as the eluting solvent. Hydrated $^{90}$Y$^{3+}$ ion, exhibited $R_f$ of 0.9–1.0, while yttrium in colloidal/particulate form, if present as radiochemical impurity remains at the point of spotting ($R_f = 0$).

Conjugation of Cetuximab with BFCs and characterization of immunoconjugates

The schematic for the conjugation of CHX-A$^{‴}$-DTPA and DOTA-NCS with Cetuximab is illustrated in Figure 1A. The purity of the CHX-A$^{‴}$-DTPA-Cetuximab and DOTA-Cetuximab was analysed by SDS-PAGE, which showed distinct bands at ~150 kDa, indicating the integrity of the mAb (Figure 1B). The concentration of the immunoconjugates ranged from 3.8 to 4.4 mg mL⁻¹ as determined by UV spectrometry.

Optimization of radiolabelling conditions of immunoconjugates at tracer level

The radiolabelling yields of the immunoconjugates were determined by radio-TLC assay. The radioimmunoconjugate remained at the point of application ($R_f = 0–0.1$), while all other radiochemical impurities migrated to the solvent front. Table 1 depicts the effect of varying amounts of immunoconjugates on the radiolabelling yields when the reaction was carried out at room temperature (25 °C) and 37 °C. It can be seen from the table that in case of CHX-A$^{‴}$-DTPA-Cetuximab, maximum radiolabelling yields (~70%) with both $^{90}$Y and $^{177}$Lu at room temperature could be achieved with 100 μg of the immunoconjugate. Further increase in the amount of immunoconjugate led to marginal increase in radiolabelling yield. The increase in radiolabelling yields was also not significant when the reaction mixtures were heated at 37 °C for 1 h.

The radiolabelling reaction of DOTA-Cetuximab with both $^{90}$Y and $^{177}$Lu showed similar trend. However, the radiolabelling yields in case of DOTA-Cetuximab were much lower than in case of CHX-A$^{‴}$-DTPA-Cetuximab (Table 1), which proved the superiority of CHX-A$^{‴}$-DTPA as a BFC for labelling with $^{90}$Y or $^{177}$Lu.

Purification of radioimmunoconjugates was carried out by size-exclusion chromatography using PD-10 column with >80% recovery yield.

In vitro stability of radioimmunoconjugates in PBS and mouse serum media

In vitro stabilities of all the radioimmunoconjugates prepared were assessed in PBS and mouse serum media after purification by passing through PD-10 column, and the results are depicted in Figure 2. It is evident from the figure that all the radioimmunoconjugates were stable (with marginal decrease of 4–5% in radiochemical purity) on incubation in excess volume of mouse serum and PBS over a period of 72 h.
Clinical dose formulation of radioimmunoconjugates and their quality control

Though all the radioimmunoconjugates demonstrated similar in vitro stability in PBS and mouse serum media, CHX-A*-DTPA-Cetuximab was used for preparation of clinically relevant doses of ⁹⁰Y-labelled mAb and ¹⁷⁷Lu-labelled mAb in view of the higher radiolabelling yields observed with this immunoconjugate. The specific activities of ⁹⁰Y-CHX-A*-DTPA-Cetuximab and ¹⁷⁷Lu-CHX-A*-DTPA-Cetuximab were determined to be 1.26 ± 0.27 and 1.14 ± 0.15 GBq mg⁻¹ respectively.

After preparation of the clinically relevant doses of ⁹⁰Y-CHX-A*-DTPA-Cetuximab and ¹⁷⁷Lu-CHX-A*-DTPA-Cetuximab their radiochemical purity was determined by radio-TLC assay and found to be >95% (Figure 3A). The radiochemical purity of ⁹⁰Y-CHX-A*-DTPA-Cetuximab and ¹⁷⁷Lu-CHX-A*-DTPA-Cetuximab remained almost consistent over a period of 168 h (Figure 3B). Even after 72 h of incubation of ¹⁷⁷Lu-CHX-A*-DTPA-Cetuximab and ⁹⁰Y-CHX-A*-DTPA-Cetuximab in excess volume of PBS or

<table>
<thead>
<tr>
<th>Immunoconjugate</th>
<th>Radioisotope</th>
<th>At room temperature (25 °C)</th>
<th>At elevated temperature (37 °C)</th>
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<tr>
<td></td>
<td></td>
<td>20 μg</td>
<td>40 μg</td>
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<tr>
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<td>18 ± 7</td>
<td>48 ± 5</td>
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<td>¹⁷⁷Lu</td>
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<td>⁹⁰Y</td>
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<td>8 ± 3</td>
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*For radiolabelling, 185 MBq (5 mCi) of ¹⁷⁷Lu or ⁹⁰Y was used. n = 5.*

Figure 2. Determination of stabilities of (A) ¹⁷⁷Lu-CHX-A*-DTPA-Cetuximab, (B) ⁹⁰Y-CHX-A*-DTPA-Cetuximab, (C) ¹⁷⁷Lu-DOTA-Cetuximab, and (D) ⁹⁰Y-DOTA-Cetuximab, when incubated with excess volume of mouse serum/PBS, at 37 °C.
mouse serum media, the radiochemical purities of the radioimmunoconjugates were found to be ~90%.

The radioimmunoconjugates after passing through 0.22 μm filter was found to be sterile. The endotoxins in all the decayed samples tested were found to be <4 EU mL⁻¹. Thus, the radiolabelled formulations were found suitable for human administration.

Biodistribution studies in tumour bearing mice

The uptake of ¹⁷⁷Lu-CHX-A⁺DTPA-Cetuximab and ⁹⁰Y-CHX-A⁺DTPA-Cetuximab in different organs/tissues of Swiss mice bearing fibrosarcoma tumours expressed as %ID/g at different p.i. times is shown in Figures 4A and 5A. The results of the biodistribution studies revealed significant uptake of both ¹⁷⁷Lu-CHX-A⁺DTPA-Cetuximab and ⁹⁰Y-CHX-A⁺DTPA-Cetuximab in fibrosarcoma tumour. For ¹⁷⁷Lu-CHX-A⁺DTPA-Cetuximab, tumour uptake of 9.7 ± 1.2%ID/g was observed within 4 h.p.i., which increased to 17.6 ± 1.8%ID/g at 24 h.p.i. The biodistribution of ⁹⁰Y-CHX-A⁺DTPA-Cetuximab was comparable, wherein tumour uptake of 8.6 ± 1.2%ID/g was observed within 4 h.p.i., which increased to 15.9 ± 2.8%ID/g at 24 h.p.i. Initial accumulation of activity was observed in various non-target organs, viz. liver, gastrointestinal track, stomach and lungs. However, with the progress of time, the uptake in non-target organs gradually reduced. The maximum tumour uptake (21.2 ± 2.9%ID/g in case of ¹⁷⁷Lu-CHX-A⁺DTPA-Cetuximab and 18.2 ± 3.3%ID/g in case of ⁹⁰Y-CHX-A⁺DTPA-Cetuximab) was observed at 72 h p.i.

The tumour-to-blood, tumour-to-muscle and tumour-to-liver ratios of ¹⁷⁷Lu-CHX-A⁺DTPA-Cetuximab and ⁹⁰Y-CHX-A⁺DTPA-Cetuximab at different time points p.i. are shown in Figures 4B and 5B respectively. For ¹⁷⁷Lu-CHX-A⁺DTPA-Cetuximab, the tumour-to-muscle ratio was observed to increase from 6.91 ± 0.62 at 4 h p.i. to 58.82 ± 5.23 at 72 h p.i., while the tumour-to-liver and tumour-to-blood ratios increased from 0.46 ± 0.21 to 2.83 ± 0.98 and 0.58 ± 0.11 to 15.45 ± 2.12, respectively, between the same time points. Similarly, for ⁹⁰Y-CHX-A⁺DTPA-Cetuximab, the tumour-to-muscle, tumour-to-liver ratio and tumour-to-blood ratios were observed to increase from 4.87 ± 1.12 to 44.34 ± 4.74, 0.37 ± 0.16 to 2.67 ± 0.64 and 0.57 ± 0.23 to 15.39 ± 1.86, respectively, between 1 and 72 h p.i. The results of the biodistribution studies were further corroborated by ex vivo CLI after administration of ⁹⁰Y-CHX-A⁺DTPA-Cetuximab in fibrosarcoma tumour-bearing mice (Figure 6).
On administration of a blocking dose of Cetuximab, the tumour uptakes of $^{177}$Lu-CHX-A*-DTPA-Cetuximab and $^{90}$Y-CHX-A*-DTPA-Cetuximab were significantly reduced to 5.9 ± 2.4 and 5.8 ± 1.1%ID/g, respectively, at 24 h p.i. (Figure 7), thereby demonstrating the specificity of the radioimmunoconjugates toward EGFR in vivo.

**Discussion**

The cornerstone for the success of a radiolabelled mAb from clinical perspective lies in the choice of the radioisotope and the BFC that binds the radioisotope to form a stable coordination complex so that it can be properly directed to a desirable location.
molecular target in vivo. Among various available radioisotopes, we have chosen 90Y and 177Lu for radiolabelling with mAb because synergistic utilization of these radioisotopes could potentially lead to the development of a ‘personalized cocktail’ approach in RIT, similar to the strategy adopted for peptide receptor radionuclide therapy of somatostatin receptor-expressing tumours. Both these radioisotopes can be produced in large quantities and distributed widely either as pure radionuclides (90Y and 177Lu) or as a generator (90Sr/90Y generator for availing 90Y).

Out of these two radioisotopes, when used at relatively lower dosage, 177Lu can also be used for cancer diagnosis because this radioisotope emits γ-radiation with energy suitable for SPECT imaging. The relatively long half-life of 177Lu is reasonably well suited for molecular imaging applications with mAbs with comparable biological half-lives. Though, utility of positron emission tomography (PET) radioisotopes such as 89Zr is comparable biological half-lives. Though, utility of positron emission tomography (PET) radioisotopes such as 89Zr (t½ = 3.3 days) is proposed to be a better choice for this purpose because of the obvious advantages of PET imaging over SPECT imaging, the prospective of 177Lu as a theranostic radioisotope cannot be ruled out in view of its cost-effective availability worldwide. The PET radioisotopes (such as 89Zr) can only be produced in cyclotrons, and therefore, their availability and clinical utility are restricted to few countries having excellent cyclotron facility for radiolabelling studies, the speculative 177Lu produced by indirect route, wherein an isotopically enriched 176Lu target undergoes (n, γ) reaction to produce 177Yb.

177Lu-CHX-A-DTPA-Cetuximab
24 h p.i.

A

B

Figure 7. Comparison of the biodistribution profile at 24 h p.i. of (A) 177Lu-CHX-A-DTPA-Cetuximab and (B) 90Y-CHX-A-DTPA-Cetuximab, with and without co-injection of a blocking dose (200–250 mg/kg) of Cetuximab.
The efficacy of radiolabelled Cetuximab for tumour targeting was evaluated by biodistribution studies after administration in mice bearing fibrosarcoma tumours. It is established that EGFR is highly expressed in many soft tissue sarcomas, including fibrosarcoma. The biodistribution pattern and tumour uptake for both 90Y-CHX-A-DTPA-Cetuximab and 177Lu-CHX-A-DTPA-Cetuximab were comparable. Significantly, high tumour uptake (~16%ID/g) for both the radiotracers was observed at 24 h p.i., with good tumour-to-background ratio. Subsequently, radiotracer uptake in most tissues declined significantly over time yet the tumour uptake remained prominent. However, high liver uptake (~10%ID/g) was observed even at 24 h p.i. in case of both 90Y-CHX-A-DTPA-Cetuximab and 177Lu-CHX-A-DTPA-Cetuximab. The high liver observed in case of radiolabelled mAbs is not unexpected as these bulky macromolecules (~150 kDa molecular weight) clear from the biological system mainly through the hepatic route. Moreover, it is well known that the liver displays relatively high levels of EGFR leading to increased uptake of radiolabelled Cetuximab in the liver.

Practically, it may be difficult to determine whether accumulation of radiolabelled Cetuximab in tumour is solely because of EGFR targeting. The enhanced permeability and retention effect (because of the leaky vasculature and lack of lymphatic drain in the tumour) may also lead to uptake of radiolabelled mAb in tumour. However, tumour uptake exclusively because of the enhanced permeability and retention effect has a characteristic pattern which reaches maxima at initial time points (the actual time fluctuates between different tumours), followed by steady reduction afterwards. For specific targeting attributed to mAb-antigen interaction, the tumour uptake increases rapidly over time and reaches a plateau and remains steady, such as the uptake observed for 90Y-CHX-A-DTPA-Cetuximab and 177Lu-CHX-A-DTPA-Cetuximab in the present study (Figures 4 and 5). Significant reduction in tumour uptake was observed in mice co-injected with excess Cetuximab (5 mg), which also corroborated that radiotracer uptake in the fibrosarcoma tumour was indeed EGFR specific (Figure 7). Detailed evaluation of therapeutic effectiveness of radiolabelled Cetuximab conjugates, and estimation of their radiation dosimetry in preclinical settings has been well reported earlier and hence were not considered essential from the perspective of the present study. Nevertheless, the preliminary biodistribution study carried out with 90Y-CHX-A-DTPA-Cetuximab and 177Lu-CHX-A-DTPA-Cetuximab in fibrosarcoma tumour-bearing mice confirmed in vivo avidity and specificity of the radioimmunoconjugates for EGFR.

Conclusions
In this study, we have optimized the procedure for preparation of clinically relevant doses of 90Y and 177Lu-labelled Cetuximab immunoconjugates. The radiolabelled agents were obtained with high radiochemical purity, radiolabelling yield, specific activity, and stability, with minimal loss of immunoreactivity. Preliminary biodistribution studies in fibrosarcoma tumour-bearing mice showed significant uptake of the radioimmunoconjugates in the tumour and reticuloendothelial organs. It is envisaged that this study might significantly expedite the process of clinical translation of radiolabelled mAbs to provide maximum benefit in management of various types of cancers.