Chapter 7

IN VIVO BIODISTRIBUTION STUDIES

7.1 Introduction

One of the most suitable methods for studying the biodistribution is to label these NPs with radioisotopes to measure the biodistribution of radioactivity in various organs at predetermined time periods [Arulsudar et al. 2003; Babbar et al. 1991; Bhatnagar et al. 1997]. Reddy and colleagues (2004) reported the biodistribution of radiolabeled etoposide loaded lipid NPs and doxorubicin incorporated into PBCA nanocapsules [Reddy et al. 2004]. Biodistribution and anti-tumor efficacy for doxorubicin loaded NPs reported recently by Yu and colleagues (2007) showed the highest accumulation of NPs preferentially in the tumor [Yu et al. 2007]. Assessment of biodistribution of drugs and drug delivery system is very important to understand the fate of delivery system *in-vivo*. In last few years, radiolabelling has also been used to recognize the biodistribution of various delivery systems. Reports from the literature indicated the usefulness of radiolabeled formulation to study biodistribution in animals.

Among all the radioisotopes, 125-Iodine (^{125}I) can be used for the biodistribution studies and for medical diagnostic purposes due to its relatively longer half life and requires relatively low radiation dose. Examples of the half lives of some radioisotopes are given in the Table 7.1.

Table 7.1: Approximate half life of various radioisotopes.

Radioisotope	Half life (approx.)
^{81m}Kr	13 seconds
^{99m}Tc	6 hours
^{131}I	8 days
^{51}Cr	30 days
^{125}I	60 days
^{137}Cs	30 years
^{241}Am	462 years
^{226}Ra	1620 years
^{238}U	4.51×10^9 years

Radioactive isotopes of iodine have proved to be appropriated for labeling both large and small biomolecules, proteins and peptides. Several therapeutic radioisotopes used for labeling many monoclonal antibodies are available, beta emitters like iodine-131, yttrium-90, rhenium-186, rhenium-188, cupper-67 and alpha emitters like bismuth-212

and astatine-211. The choice and use of these radioisotopes are based on their physical properties, suitable chemistry, advantages and disadvantages [Weadock et al. 1990]. The preference for radioiodine, mainly iodine-131, is sustained by its well-known chemistry, easy availability and low cost.

^{125}I due to its ideal nuclear characteristics ($t_{1/2}$: 59.40 d, γ : 27.5 KeV (76.5 abundance), 35.5 KeV (6.7) for in vitro assay and studies was used for radiolabeling antibody to study pharmacokinetics of radiolabelled NPs. Radioiodination with ^{125}I and purification of ER antibody was carried out. α-methyl Tyrosine ester (TME) was radioiodinated to serve as control. Radioiodination of biomolecules mainly proteins and peptides involve electrophilic substitution reaction at tyrosyl, histidyl or tryptophan residues. Iodine should be in +1 oxidation state to displace the H from ortho position of the phenolic ring. Oxidised species of iodine may be obtained by oxidizing iodide with agents like iodine monochloride, chloramine T or by using enzyme like HRP. In aqueous solutions, Chloramine T releases hypochlorous acid which oxidizes iodide.

$$I_2 + H_2O: \; H_2O\, I^{++} I^-$$

There are many methods of radiolabeling proteins with radioiodine, mostly oxidative reactions, as described by many authors [Weadock et al. 1990]. However, Chloramine-T, a mild oxidizing agent, can alter protein structure (denaturation) if exposure is prolonged. Using this procedure, the oxidizing process is terminated by addition of a reducing agent, sodium metabisulphite.

Labeling of the NPs with radioisotopes was carried out by tagging with suitable gamma emitting radioisotope. Two approaches for radiolabeling have been reported one is by attachment of the label to the polymer component prior to NP preparation and another way is to radiolabel the NP after manufacturing (Richardson et al., 1978). Radiolabeled NP have been successfully used for preclinical evaluation of pharmacokinetic parameters of nanocarrier delivery system as this can be administered to animals by different routes and its uptake in various organs can be estimated with time. Radiolabeled NP preparations have also been used successfully for imaging tumor, abscesses, ischemia and infracted region.

The procedure for biodistribution studies mainly involves attachment of ^{125}I with the antibody or α-methyl tyrosine ester which can be further conjugated to the delivery system under examination. The effective binding of the radio-labeled complex is assessed by quality control tests. Radio-labeled complex is then administered to the

animal depending on the mode of administration and the target organ. After administration of the radio-labeled complex, biodistribution can be studied either by sacrificing the animal at various time points and measuring the radioactivity in different organs.

7.2 Materials

α-methyl Tyrosine ester and chloramine T was obtained from Sigma Aldrich, Mumbai, India. PD10 columns were procured from GE healthcare. Mumbai, India.

7.3 Methods

7.3.1 Radioiodination of antibody

Radioiodination was carried out by adding 0.05 M phosphate buffer pH 7.5 (30 µl) to antibody solution (25 µl) followed by addition of 18.5 MBq of ^{125}I. Chloramine T was used as oxidizing agent (10 µl, 2 mg/ml in 0.05 M phosphate buffer, pH 7.5). After addition of chloramine T, reaction was stopped after 90 sec by addition of sodium metabisulphate (50 µl, 2 mg/ml in 0.05 M phosphate buffer, pH 7.5). To the above solution, KI (100 µl, 1 mg/ml in 0.05 M phosphate buffer) was added and mixed properly. Similarly radioiodination of α-methyl tyrosine ester (5 µl, 1 mg/ml in methanol) was carried out.

7.3.2 Determination of labeling efficiency by electrophoresis

Electrophoresis was carried out to check radioiodination of antibody and TME to calculate the yield of reaction. Whatman paper strips (3 mm) were first equilibrated with 0.025 M phosphate buffer pH 7.5. Reaction mixture for radiolabelled antibody and radiolabelled TME was spotted at the centre of paper strips and electrophoresis was carried out for 1 h at 240 V.

7.3.3 Purification of radiolabeled antibody

Commercially available PD10 column was equilibrated with 4 times column volume of 0.05 M phosphate buffer. Reaction mixture (250 µl) was loaded on the column. Purified antibody was eluted with 0.05 M phosphate buffer containing 0.5% BSA. Fractions of 1 ml/tube up to 20 tubes were collected and counted in NaI (Tl) counter. About 99% of activity was observed in fraction 5 and 6 while free iodide was observed at 12-15 fractions 1%. Electrophoresis of fraction 6 and 7 was carried out to ensure purity of column fractions.

7.3.4 Conjugation of iodinated antibody and TME on NPs

Iodinated antibody and TME was surface functionalized on NPs as described in chapter 5 (Section 5.3.3).

7.3.5 Stability of radiolabelled conjugates

Stability of the conjugated ^{125}I to antibody and tyrosine was determined after 24 h by electrophoresis. Electrophoresis was carried out as described previously (Section 7.3.2).

7.3.6 Biodistribution studies

All experiments conducted on animals were approved by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India, New Delhi, India. Balb/c mice (aged 4 to 5 months), weighing between 25 to 30 g were selected for the study on the basis of randomization technique. Three mice were used at each time point for each formulation. The mice were divided into four groups. Group 1 received ^{125}I-Tyr PLGA NPs, group 2 received ^{125}I-ER Ab PLGA NPs, group 3 received ^{125}I-Tyr PCL NPs and group 4 received ^{125}I-ER Ab PCL NPs administered intravenously via tail vein. The mice were sacrificed at different time intervals of 3 and 24 h post-administration, and blood was collected via cardiac puncture. Different organs including heart, lungs, liver, spleen, kidney, stomach, intestine, uterus, muscle and bone were dissected and weighed. The activity associated with organs/tissues was measured in a flat type NaI (Tl) scintillation counter (ECIL, India). The mean of this radioactivity was used to obtain the total injected radioactivity in each animal. The radioactivity present in each organ/tissue was interpreted as percentage of activity per gram of the tissue (%A/g).

Injection dose at time (t) = [(Total activity before inj. – total activity of empty syringe) – background counts]

$$\% \text{ Activity per gram of the tissue} = \frac{(\text{Organ counts} \times 100)}{(\text{Organ weight} \times \text{injected dose})}$$

7.3.7 Statistical Analysis

All data are reported as mean ± SD (standard deviation) and the difference between the groups were tested using Student's t-test at the level of $p < 0.05$. Statistical evaluation were compared using ANOVA and differences greater at $p < 0.05$ were considered significant.

7.4 Results and Discussion

7.4.1 Stability of iodinated complexes

Results of electrophoresis indicates the initial binding capacity of ^{125}I to tyrosine and antibody of 100% and 99.67%, respectively. After 24, the stability studies of 125I with tyrosine and antibody conjugated to NPs was performed. The results showed more than 90% stability of the conjugates indicating high binding capacity (Table 7.2).

Table 7.2 Stability of iodinated complexes after 24 h.

	Initial		After 24 h			
	Ab	Tyr	PLGA Tyr	PLGA Ab	PCL Tyr	PCL Ab
Bound fractions	144047	446657	26834	5158	10152	3081
	537840	949848	72965	22798	71367	19655
	107955	687611	4150	10697	23921	9004
	41338	59693	344	5481	324	3755
	19022	6150	145	1386	229	896
	17703	4334	97	190	117	137
Unbound fractions	1995	28	93	70	223	1693
	716	20	1795	2299	2183	1454
	158	25	1451	1101	634	674
	39	26	65	215	15	13
% Bound	99.67	100.00	96.85	92.54	97.20	90.50
% Unbound	0.33	0.00	3.15	7.46	2.80	9.50

7.4.2 Biodistribution studies

Biodistribution of ^{125}I-Tyr PLGA NPs, ^{125}I-ER Ab PLGA NPs, ^{125}I-Tyr PCL NPs and ^{125}I-ER Ab PCL NPs following i.v. administration in swiss mice were performed and the radioactivity was estimated at predetermined time point for 3 and 24 h. The results obtained are shown in table 7.3 for PLGA NPs and table 7.5 for PCL NPs. The concentration of formulation in each organ/tissue/blood following i.v. injection of NPs was shown in figure 7.1 for PLGA NPs and figure 7.2 for PCL NPs in bar graph. The ratios

of bio-distribution in liver, blood, and uterus were calculated and tabulated in table 7.4 for PLGA NPs and table 7.6 for PCL NPs.

7.4.2.1 Biodistribution of ^{125}I-Tyr PLGA NPs and ^{125}I-ER Ab PLGA NPs

The biodistribution data reveals higher initial rapid uptake by liver, which was 1.77 ± 0.32% for PLGA NP and 2.51 ± 0.44% for immunoNPs after 3 h post injection. ImmunoNPs were available more in circulatory system as compared to non-targeted NPs (5 times and about 8 times after 3 and 24 h, respectively). Non-targeted NPs are not retained in uterus as %ID/g decreased from 0.58 ± 0.34% to 0.10 ± 0.18% which is about 6 times ($p < 0.05$), whereas in case of immunoNPs %ID/g value was increased by more than 2 times from 0.63 ± 0.13% to 1.29 ± 0.28% after 3 and 24 h respectively ($p < 0.05$). Distribution of immunoNPs was increased by more than two times after 24 h as compared to 3 h. When distribution of targeted immunoNPs was compared with non-

Table 7.3 Biodistribution of ^{125}I labeled Tyrosine and ER antibody conjugated PLGA NPs and the radioactivity was measured after 3 and 24 h post injection. The values represented as mean ± SD. Radioactivity is expressed as percent of administered dose per gram of tissue or organ.

Organ	% Dose/g of organ			
	PLGA-NPs Tyr (3 h)	PLGA-NPs Tyr (24 h)	PLGA-NPs ER Ab (3 h)	PLGA-NPs ER Ab (24 h)
Liver	1.77 ± 0.32	0.31 ± 0.02	2.51 ± 0.44	1.57 ± 0.29
GIT	1.51 ± 0.29	0.09 ± 0.01	1.65 ± 0.14	0.35 ± 0.08
Stomach	1.91 ± 0.52	0.07 ± 0.03	17.98 ± 3.02	0.44 ± 0.15
Kidneys	2.60 ± 0.96	0.12 ± 0.10	2.54 ± 0.47	0.97 ± 0.15
Heart	1.60 ± 0.31	0.01 ± 0.10	1.32 ± 0.25	0.41 ± 0.15
Lungs	1.09 ± 0.15	0.06 ± 0.07	2.28 ± 1.18	0.84 ± 0.04
Spleen	0.49 ± 0.90	0.14 ± 0.14	2.02 ± 0.50	0.61 ± 0.16
Uterus	0.58 ± 0.34	0.10 ± 0.18	0.63 ± 0.13	1.29 ± 0.28
Blood	0.84 ± 0.62	0.23 ± 0.17	4.40 ± 0.75	1.82 ± 0.52
Muscle	8.99 ± 4.3	1.09 ± 0.70	7.13 ± 1.76	3.23 ± 0.57
Bone	6.64 ± 6.95	5.06 ± 4.82	7.71 ± 2.05	4.15 ± 2.36

targeted formulations, the uterus uptake was found to be slightly but not significantly higher which later increased to 13 times after 24 h which was a very significant increase. This result shows a clear active targeting of prepared immunoNPs which was found absent in case of long circulatory pegylated NPs.

Table 7.4 Different ratio between the tissue/organ of ^{125}I labeled Tyrosine and ER antibody conjugated PLGA NPs.

Ratio of % Dose/g of organ	PLGA-NPs Tyr (3 h)	PLGA-NPs Tyr (24 h)	PLGA-NPs ER Ab (3 h)	PLGA-NPs ER Ab (24 h)
Liver to blood ratio	2.11	1.35	0.57	0.86
Blood to uterus ratio	1.45	2.3	6.98	1.41

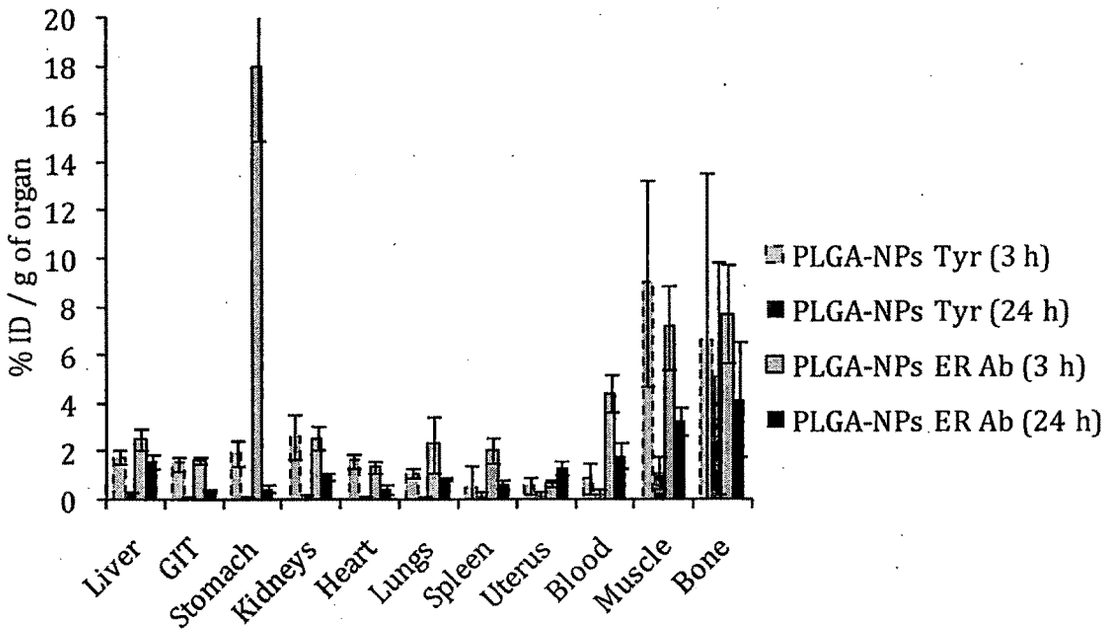

Figure 7.1 Biodistribution of ^{125}I labeled Tyrosine and ER antibody conjugated PLGA NPs and the radioactivity was measured after 3 and 24 h post injection. The values represented as mean ± SD. Radioactivity is expressed as percent of administered dose per gram of tissue or organ.

From the results of liver to blood distribution ratio, significant difference between the two formulations can be observed. Value of liver to blood ratio of above 1 signifies that

higher liver uptake of NPs as compared to circulating particles. Targeted NPs showed liver to blood ratio below 1 at both the time points indicating more NPs in circulation than uptaken by liver. Blood to uterus ratio of the injected NPs was found to be lower in case of non-targeted NPs indicating non-targetability of the NPs without antibody conjugation. The higher values of this ratio after 3 h is possibly because of more number of immunoNPs are present in circulation. The overall %ID/g activity present in uterus was 13 times more as discussed previously which clearly demonstrates the active targeting of the immunoNPs.

7.4.2.2 Biodistribution of ^{125}I-Tyr PCL NPs and ^{125}I-ER Ab PCL NPs

The biodistribution data of ^{125}I labeled Tyrosine and ER antibody conjugated PCL NPs reveals lower uptake by liver, which was 3.79 ± 0.46% and 1.44 ± 0.30% for immunoNPs ($p < 0.01$) and 7.50 ± 0.68% and 3.20 ± 1.22% for non-targeted NPs after 3

Table 7.5 Biodistribution of ^{125}I labeled Tyrosine and ER antibody conjugated PCL NPs and the radioactivity was measured after 3 and 24 h post injection. The values represented as mean ± SD. Radioactivity is expressed as percent of administered dose per gram of tissue or organ.

Organ	% Dose/g of organ			
	PCL-NPs Tyr (3 h)	PCL-NPs Tyr (24 h)	PCL-NPs ER Ab (3 h)	PCL-NPs ER Ab (24 h)
Liver	7.50 ± 0.68	3.20 ± 1.22	3.79 ± 0.46	1.44 ± 0.30
GIT	6.43 ± 0.95	0.62 ± 0.24	1.30 ± 0.32	0.53 ± 0.03
Stomach	1.67 ± 0.60	0.79 ± 0.32	15.28 ± 6.97	0.89 ± 0.13
Kidneys	1.44 ± 0.40	1.06 ± 0.39	2.62 ± 0.24	1.10 ± 0.14
Heart	1.37 ± 0.27	0.93 ± 0.04	1.20 ± 0.06	1.17 ± 0.27
Lungs	1.51 ± 0.31	0.76 ± 0.12	2.62 ± 0.30	1.19 ± 0.52
Spleen	4.81 ± 1.06	2.98 ± 1.51	3.30 ± 0.51	1.91 ± 0.37
Uterus	0.32 ± 0.19	0.28 ± 0.19	0.38 ± 0.11	3.49 ± 0.55
Blood	4.21 ± 0.79	1.59 ± 0.56	5.15 ± 0.96	1.75 ± 0.18
Muscle	6.14 ± 2.80	5.18 ± 0.88	5.30 ± 1.24	5.79 ± 1.92
Bone	6.52 ± 1.63	9.17 ± 2.63	8.07 ± 0.39	11.62 ± 4.99

and 24 h ($p < 0.01$), respectively post injection. The overall distribution of immunoNPs in liver was low at both the time points. Along with liver uptake other RES organ, i.e. spleen also showed less distribution of immunoNPs (3.30 ± 0.51%, 3 h and 1.91 ± 0.37%, 24 h) than non-targeted NPs (4.81 ± 1.06%, 3 h and 2.98 ± 1.51, 24 h) ($p < 0.05$) after both the time points. Similar to distribution of PLGA NPs, more concentration of immunoNPs was present in circulation after both the time points, as shown in table 7.4. No significant difference in uterus uptake was observed after 3 h (1.2 times higher uptake of immunoNPs as compared to non-targeted nanoparticulate system), which incrased to about 12.5 folds after 24 h.

Table 7.6 Different ratio between the tissue/organ of ^{125}I labeled Tyrosine and ER antibody conjugated PCL NPs.

Ratio of % Dose/g of organ	PCL-NPs Tyr (3 h)	PCL-NPs Tyr (24 h)	PCL-NPs ER Ab (3 h)	PCL-NPs ER Ab (24 h)
Liver to blood ratio	1.78	2.01	0.74	0.82
Blood to uterus ratio	13.16	5.68	13.55	0.5

From the results of liver to blood distribution ratio, significant difference between the two formulations can be observed. Value of liver to blood ratio of above 1 signifies that higher liver uptake of NPs as compared to circulating particles. Targeted NPs showed liver to blood ratio below 1 at both the time points indicating more NPs in circulation than uptaken by liver.

No significant difference was found in distribution of NPs in uterus after 3 h. However, blood to uterus ratio of the injected NPs was found to be lower in case of non-targeted NPs indicating non-targetability of the NPs without antibody conjugation. The higher value of this ratio after 3 h is possibly because of more number of immunoNPs are present in circulation and not reached the organ. After 24 h, blood to uterus ratio was 0.5 indicating higher concentration of immunoNPs in uterus than in blood. The overall %ID/g activity present in uterus was more than 12 times which clearly demonstrates the active targeting of the immunoNPs.

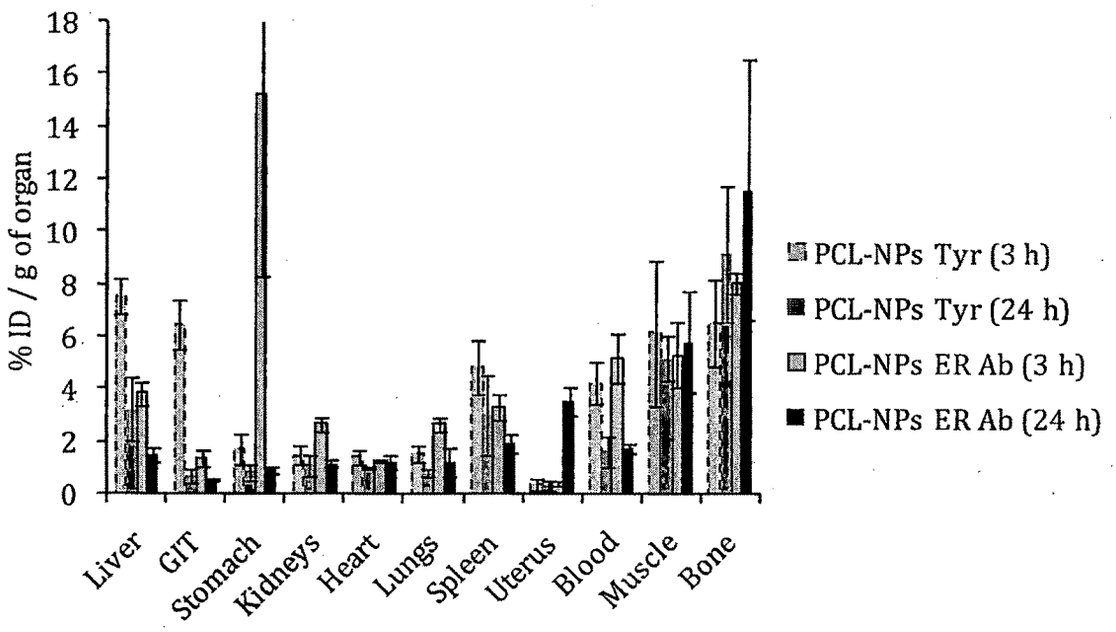

Figure 7.2 Biodistribution of ^{125}I labeled Tyrosine and ER antibody conjugated PCL NPs and the radioactivity was measured after 3 and 24 h post injection. The values represented as mean ± SD. Radioactivity is expressed as percent of administered dose per gram of tissue or organ.

7.5 References

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