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^{125}I Labelled Inulin: a Convenient Marker for Deposition of Liposomal Contents In Vivo

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The utility of an iodinated derivative of inulin (^{125}I-tyraminyl-inulin, ^{125}ITI) for reporting in vivo tissue distributions of liposomal contents is described. It is shown, employing a rat model, that this probe satisfies the criteria that the free form is rapidly cleared from the circulation and excreted, whereas ^{125}ITI encapsulated in large unilamellar vesicle (LUV) systems and subsequently taken up in various tissues exhibits a long (>3 days) retention time. Further, high specific activities (>1 μCi per μl) are easily achievable, allowing low LUV dose levels (≤2.5 μmole phospholipid/kg body weight) to be employed. Minimal tissue workups for quantitation of ^{125}ITI distributions are required. It is concluded that from criteria of sensitivity, expense and simplicity, ^{125}ITI is a most convenient probe for characterizing liposome deposition in vivo.

Liposomes have important potential as targeted vehicles for drug delivery in vivo. However, in order to evaluate the efficacy of any targeting protocol, a convenient procedure for assaying delivery of encapsulated material to particular tissues is required. A variety of entrapped radiolabelled compounds including metals [1], lipid soluble labels [1,2] and proteins [3,4,5] have been employed for such studies. Unfortunately, most of these agents do not satisfy the criteria necessary for probe deposition to truly reflect the tissue distribution of liposome contents. These demands include rapid clearance (and subsequent excretion) of the free probe from the circulation, minimal leakage from the liposome in the absence of lytic processes and a long retention time once the encapsulated probe is taken up by the cells of a particular tissue. Inulin (^{14}C or ^{3}H labelled) fulfills these criteria [6,7]. However, such compounds are expensive and require tedious tissue workups for quantitation.

In this work we describe the (rapid) synthesis of an iodinated version of inulin (^{125}I-tyraminyl-inulin, ^{125}ITI) and characterize the properties of

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this probe as a reporter of liposome deposition. It is shown that ^{125}ITI satisfies the necessary requirements for monitoring liposome tissue distribution with the advantages of low cost, minimal tissue workup and high specific activities. This latter property allows tissue distributions to be ascertained for lower liposome doses than previously possible.

Materials and Methods

Chemicals: Inulin, periodic acid, sodium-m-arsenite, tyramine, G-25 Sephadex, sodium cyanoborohydride, sodium borohydride, and cholesterol were obtained from Sigma. Ultrogel Ac34 was obtained from Pharmacia, carrier free Na^{125}I (100 mCi/ml) was supplied by Amersham and iodogen was obtained from Pierce. Phosphatidylcholine was purified from egg yolks by standard techniques. All other chemicals were of analytical grade.

Synthesis of tyraminyl-inulin: Inulin (1.0 g) was dissolved in 90.0 ml distilled H_2O and cooled to 4°C, 10 ml (fresh) 0.1 M periodic acid was added and the solution was incubated at 4°C for 15 minutes in the dark. Periodate consumption was assayed by the arsenite method (8) indicating approximately two oxidations per inulin molecule. The reaction was terminated by neutralization with $Ba(OH)_2$ and the periodate and iodate salts were removed by centrifugation. To the supernatant 4.3 g NaH_2PO_4 and 0.55 g tyramine were added and the pH was adjusted to 7.5 with 1.0 M HCl. Subsequently, $NaBH_3CN$ (0.25 g) was added and the solution was stirred for 4 hr at room temperature. Remaining aldehyde groups were reduced by careful addition of 0.2 g $NaBH_4$ and the solution was stirred for another hour at 27°C. Aliquots (25 ml) were degassed under reduced pressure and applied to a 1.5 x 80 cm Sephadex G-25 column previously equilibrated with H_2O at 4°C. The flow rate was adjusted to 10 ml/hr and 4 ml fractions were collected. The fractions were assayed for tyramine by monitoring the absorbance at 279 nm and for sugar by employing the anthrone reagent technique (9). The sugar containing fraction eluted in the void volume and had a constant tyramine:inulin mole ratio of 0.6. The adduct was completely separated from the free tyramine and other salts as determined by rechromatography on Sephadex G-25. The peak fractions were lyophilyzed giving an 80% yield, based on inulin.

Iodination of the tyramine-inulin adduct: 2.5 mg of the tyramine-inulin adduct were dissolved in 0.2 ml HEPES (20 mM), NaCl (145 mM) pH 7.4 (HEPES buffered saline; HBS) and placed in a 1.5 ml stoppered vial in which 40 µg iodogen had been previously deposited from 300 µL $CHCl_3$. Then carrier free Na^{125}I (4 mCi, 100 mCi/ml) was added and the reaction allowed to procede for 45 min at room temperature. The solution was then transferred to a vessel containing 10 µl 0.1 M $Na_2S_2O_5$, 0.05 M KI which was then applied to a G-25 column (1x20 cm) equilibrated with HBS. Fractions (0.5 ml) were collected and the ^{125}I containing fractions eluting in the void volume (2.5 ml) were pooled. The resulting ^{125}I-tyramin-inulin (^{125}ITI) solution routinely contained 1 µCi/µL ^{125}I, where <0.01% was in the free iodide form (<0.01% was $CHCl_3$ extractable when made to 1.2% H_2O_2 and 0.4% KI) and over 99% of the material eluted as one peak in the void volume on re-chromatography employing Sephadex G-25. In all studies the material was used within 2 weeks of production. For injection as free ^{125}ITI the stock solution was diluted to 5 µCi/ml.

Preparation of large unilamellar vesicles: The liposomes employed were in the form of large unilamellar vesicles (LUVETS) prepared by an extrusion technique as previously described (10). Briefly, 30 µmol egg phosphatidylcholine (EPC) and 30 µmol cholesterol were dried down from $CHCl_3$. The resulting

lipid film was dispersed in 1 ml HBS containing 1 mCi ^{125}ITI by vortex mixing. The multilamellar systems thus produced were then extruded 10 times through two (stacked) polycarbonate Nucleopore filters (0.1 μm pore size) under N_2 pressure (200-400 psi). Aliquots (0.1 ml) of the LUVETS were applied to an Ultrogel Ac34 column (1 ml) previously equilibrated with HBS. The lipid containing fractions were pooled and rechromatography indicated that >97% of the ^{125}ITI was 'trapped' in the vesicles. The resulting liposome preparation had a trap volume of 0.9 μl/μmol phospholipid as calculated from lipid phosphate analysis (11) and entrapped ^{125}I. The average radius of these vesicles is 700 Å (10). The LUVETS containing ^{125}ITI were diluted to 0.5 μmol phospholipid in 200μL of HBS, stored at 4°C and used within 2 days of preparation.

In vivo experiments: Female Wistar rats (150-200 g) were obtained from the UBC animal care unit and fed ad libitum prior to and during experiments. They were lightly anesthetized with ether, weighed, and 200 μL HBS containing either ~0.5 μCi ^{125}ITI encapsulated in LUVETS (0.5 μmol phospholipid) or 1 μCi free ^{125}ITI was injected via the tail vein. The rats were allowed to recover in metabolic cages where the urine and feces were collected. At various times post injection the rats were anesthetized with ether and sacrificed by exsanguination via vena cava. Blood was collected in a syringe containing 200 μL 200 mM EDTA and recovery was approx. 85% assuming 4.9 ml blood/100 g rat. The heart, liver, lung, spleen and kidney were removed and the urine remaining in the bladder was collected. The carcass was then dissolved in 200 ml 9 M NaOH at 70°C overnight. Aliquots of carcass digest and samples of tissues were then assayed for the presence of ^{125}I.

Results and Discussion

Experiments were first conducted to determine the clearance of free ^{125}III from the circulation and subsequent excretion. Thus rats were injected with 1 μCi free ^{125}ITI and clearance from the blood, uptake into various tissues, and appearance in the urine assayed. As shown in Figure 1,

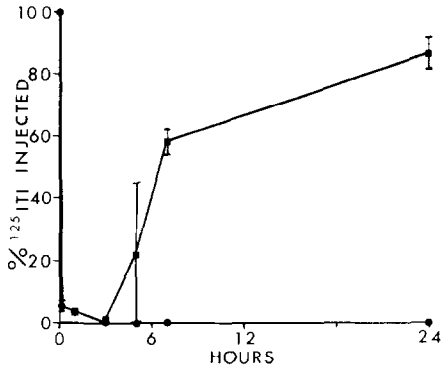

Figure 1. Clearance of free ^{125}I-tyraminyl-inulin (^{125}ITI) from the (rat) circulation (●) and subsequent excretion in the urine (■). Female Wistar rats were injected via the tail vein with 1 μCi ^{125}ITI in 200 μl HBS. Urine was collected in metabolic cages. The animals were sacrificed at the times indicated. Recoveries of blood averaged 80% assuming 4.9 ml blood per 100 g rat. Results are expressed as percentages of the total ^{125}ITI injected ±s.e. (n = 3).

Table I. The tissue distribution of free ^{125}ITI *in vivo* at various times post injection: Tissues were harvested from rats employed to obtain the data of Figure 1. Results are expressed as percentages of total ^{125}ITI injected.

Tissue	1 Hr	3 Hr	5 Hr	7 Hr
Kidney	2.7 ± 0.4	1.4 ± 0.4	0.9 ± 0.4	1.5 ± 0.7
Liver	0.22 ± 0.06	0.09 ± 0.01	0.05 ± 0.004	0.07 ± 0.02
Spleen	0.14 ± 0.09	0.02 ± 0.01	0.03 ± 0.02	0.02 ± 0.005
Heart	0.15 ± 0.03	0.02 ± 0.003	0.01 ± 0.003	0.01 ± 0.0005
Lung	0.32 ± 0.08	0.05 ± 0.01	0.02 ± 0.003	0.03 ± 0.01

clearance from the blood is extremely rapid, as only 4% remains after 10 min. The ^{125}ITI is eventually found in the urine, with recovery of 80% or more of the label within 24 hr. This indicates that free ^{125}ITI in the rat circulation is rapidly cleared from blood and excreted. As shown in Table 1, this is consistent with results obtained for other tissues. No significant ^{125}ITI tissue uptake was observed except for the kidney which transiently accumulated approximately 3% of the injected label at 1 hr. This can be explained by the excretion of ^{125}ITI into the urine. It is important to note that the liver and spleen take up less than 1 % of the label at any time post injection. Thus we conclude that ^{125}ITI in the free form is cleared and excreted rapidly and is not taken up into body tissue.

The next set of experiments were designed to determine the *in vivo* tissue distribution of the contents of LUV systems when introduced into the circulation. An associated objective was to demonstrate an ability to detect the fate of relatively low doses of LUV systems consistent with the levels required for possible therapeutic applications. In previous work we have demonstrated that drugs such as adriamycin can be trapped in LUV systems at concentrations of 125 mM or more [12]. Thus a "clinical" dose of adriamycin (2.5 μmol/kg body weight) for a 200 gm rat would require a trap volume of 4 μL. The trap volume of the EPC-cholesterol (1:1) LUV's employed here is 0.9 μl/μmol phospholipid, leading to an LUV dose of ∿5 μmol phospholipid. Any ability to "target" these carriers should appreciably

reduce the drug levels required, and we have therefore chosen to demonstrate an ability to follow the fate of LUV dose levels corresponding to 0.5 μmol phospholipid. An advantage of such low dose levels concerns reduced saturation ("blockade") of uptake processes [7].

The clearance from the circulation of ^{125}ITI encapsulated in EPC-cholesterol (1:1) LUV's and the subsequent appearance of inulin in the urine is illustrated in Fig. 2. In contrast to free ^{125}ITI, the encapsulated material in the circulation is initially rapidly reduced to ∿40% of the injected levels, and thereafter decays with a much longer half-life (∿ 3 hr). Further, only 30% of the injected dose is eventually found in the urine even after 3 days. This latter result clearly indicates tissue uptake and retention of LUV encapsulated ^{125}ITI. The actual tissue distributions are shown in Fig. 3 where ∿50% of the *in vivo* ^{125}ITI is accumulated by the liver, ∿10% by the spleen and the rest is found in the carcass. Less than 3% ^{125}ITI was found in the heart, lung and kidney at any

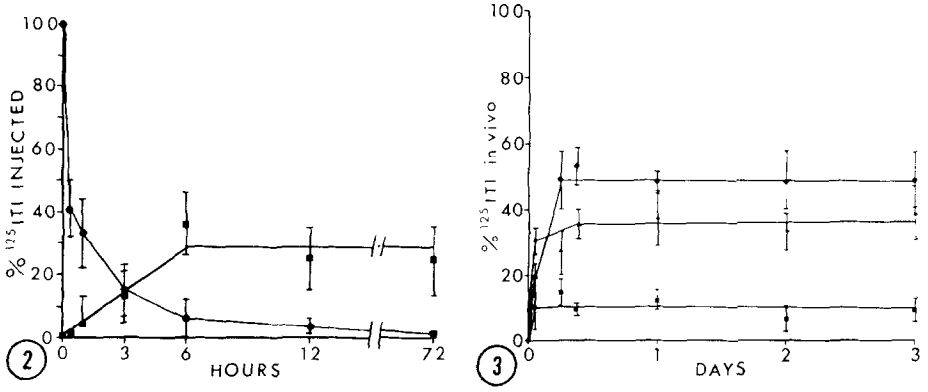

Figure 2. Clearance of ^{125}ITI entrapped in EPC-cholesterol (1:1) LUV systems from the (rat) circulation (●) and subsequent excretion in the urine (■). The LUV's were prepared as indicated in Methods and were injected into the tail vein of 150-175 g female Wistar rats at a dose level of 0.5 μmol phospholipid in 100 μl HBS. Urine was collected in metabolic cages. Blood was withdrawn and the animals sacrificed at indicated times and the total amount of ^{125}ITI in the blood calculated assuming 4.9 ml blood per 100 g rat. Results are expressed as percentages of the total ^{125}ITI injected ±s.e. (n = 4).

Figure 3. Tissue distribution of ^{125}ITI entrapped in EPC-cholesterol (1:1) LUV systems after injection of doses corresponding to 0.5 μmol phospholipid in 100 μl HBS. The symbols correspond to liver (●); carcass (▲) and spleen (■). The tissues were harvested as indicated in Methods. Results are expressed as percentages of total ^{125}ITI *in vivo* (total ^{125}ITI injected minus amount excreted) ±s.e. (n = 4).

time post injection (data not shown). The tissue distributions for the liposomal contents are similar to previous observations [13] and any differences may be attributed to differences in liposome dose, lipid composition and size, as well as the animal model employed. The important point is that once the encapsulated ^{125}ITI is associated and presumably endocytosed by the cells of a particular tissue, long retention times are observed. Such characteristics clearly support the utility of ^{125}ITI as a label for the deposition of liposomal contents.

In summary, ^{125}I-tyraminyl-inulin has been demonstrated to satisfy the criteria required of a marker for the in vivo fate of liposomal systems as it is cleared rapidly in the free form and exhibits long retention times when accumulated into tissues. These characteristics are similar to ^{14}C-methoxy-inulin [13], and indicate that addition of ~0.6 tyraminyl groups per inulin does not markedly alter the properties of inulin. Advantages of ^{125}ITI include a rapid and straightforward synthesis, high specific activities, low cost and excellent quenching properties.

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