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Comparison of three remote radiolabelling methods for long-circulating liposomes

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ABSTRACT

Long-circulating liposomes (LCL) are often used as a drug carrier system to improve the therapeutic index of water-soluble drugs. To track these LCL *in vivo*, they can be radiolabelled with ^{111}In-oxine. For this labelling method, generally DTPA is encapsulated in the aqueous phase of LCL (DTPA-LCL). Alternatively, LCL can be labelled with $^{111}InCl_3$ after incorporation of DTPA-conjugated DSPE in the lipid bilayer (DTPA-DSPE LCL). Here, we compared the *in vitro* properties of DTPA-DSPE LCL with those of DTPA LCL and empty LCL. Additionally, we compared the *in vivo* performance of DTPA-DSPE LCL with those of DTPA LCL in mice.

DTPA LCL (88 nm) and empty LCL (84 nm) were labelled with ^{111}In-oxine, and DTPA-DSPE LCL (83 nm) were labelled with $^{111}InCl_3$. Labelling efficiency at increasing specific activity was determined. *In vitro* stability of ^{111}In-labelled LCL was determined in human serum at 37 °C. The *in vivo* properties of ^{111}In-labelled LCL were determined in mice with a *Staphylococcus aureus* infection in the thigh muscle. Image acquisition, blood sampling and biodistribution studies were performed 1, 4 (blood sampling only), 24, 48 and 72 h p.i. of ^{111}In-labelled LCL. DTPA-DSPE LCL could be labelled efficiently at a much higher specific activity compared to DTPA LCL and empty LCL: >90% at 15 GBq/mmol, >90% at 150 MBq/mmol and 60–65% at 150 MBq/mmol, respectively. ^{111}In-labelled DTPA-DSPE LCL and DTPA LCL were stable in human serum, regarding label retention, for at least 48 h at 37 °C (>98% retention of the radiolabel). In contrast, only 68% radiolabel was retained in empty LCL after 48 h. *In vivo* targeting of ^{111}In-DTPA-DSPE LCL to the abscess was comparable to targeting of ^{111}In-DTPA LCL (3.5 ± 0.9%ID/g and 3.4 ± 0.9%ID/g abscess uptake respectively, 48 h p.i.).

In conclusion, labelling of DTPA-DSPE LCL with $^{111}InCl_3$ represents a robust, easy and fast procedure which is preferred over the more laborious conventional labelling of DTPA-LCL with ^{111}In-oxine.

1. Introduction

Liposomes are small lipid vesicles with an aqueous core, which have been extensively studied since their discovery in the early 60s [1,2]. Coating with polyethylene glycol (PEG) sterically hinders binding of proteins that could act as opsonins, which enables them to escape recognition by macrophages of the reticuloendothelial system, thereby increasing their blood circulation time if given intravenously [3]. These long-circulating liposomes (LCL) have been extensively studied as a drug carrier system to increase the therapeutic index of drugs and reduce their systemic side-effects [4–6]. By virtue of increased permeability of local vascular endothelium and their small size (80–120 nm in diameter), liposomes preferentially access pathological sites, such as inflamed, infectious or cancerous tissue, a phenomenon referred to as 'passive targeting' [7–9]. Retention of liposomes and drug delivery at target sites can also be enhanced by the attachment of antibodies, small molecules or peptides to the surface of the liposomes ('active targeting') [10–13]. To evaluate the pharmacokinetics and biodistribution of liposomes and their content *in vivo*, non-invasive imaging methods, such as positron emission tomography (PET) and single photon emission computed tomography (SPECT), are used [7,14]. For this end, liposomes have been radiolabelled with ^{111}In, ^{99m}Tc, ^{89}Zr, ^{67}Ga and ^{64}Cu and several remote labelling methods have been described [15–19]. Radioactivity is sequestered in the aqueous core, the lipid bilayer or on the surface of the liposomes. Entrapment of the radionuclides in the aqueous core and insertion in the lipid bilayer may

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require higher temperatures [20,21]. Because this might influence the liposomal properties and because the related labelling procedures are more laborious, surface labelling is the most convenient method to efficiently radiolabel liposomes, although firm attachment of the incorporated radionuclide after surface labelling has not always been determined. Since the half-life of ^{111}In (2.8 days) is sufficient for tracking the radiolabelled formulations during several days and the blood half-life of liposomes is also in the order of days (typically about 48 h), the use of ^{111}In is preferred in our experiments. To allow surface labelling with $^{111}InCl_3$, DTPA can be derivatized with a phospholipid that can be inserted in the lipid bilayer. Here, we used DTPA conjugated to 1,2-distearoyl-phosphatidylethanolamine (DSPE), which was incorporated in the lipid bilayer. Conventional ^{111}In-labelling of liposomes was achieved by the entrapment of ^{111}In in the aqueous core. In this method the soluble chelating agent diethylenetriamine pentaacetate (DTPA) is encapsulated in the liposomes and allows entrapment of the radionuclide by incubating the liposomes with the lipophilic chelate ^{111}In-oxine, that can pass through the lipid bilayer.

In the present study we determined the labelling efficiency, maximal specific activity and the stability of radiolabelling of 3 types of ^{111}In-labelled LCL: (1) DTPA-DSPE LCL surface-labelled with $^{111}InCl_3$ were compared with those of conventionally labelled liposomes containing (2) DTPA in their aqueous core and (3) empty LCL (without DTPA) labelled with ^{111}In-oxine. Additionally, we compared the *in vivo* performance of ^{111}In-DTPA-DSPE LCL with those of ^{111}In DTPA LCL in female NMRI mice with a focal *Staphylococcus aureus* infection.

2. Material and methods

2.1. Preparation of liposomes

DTPA-DSPE LCL, DTPA LCL and empty LCL (without DTPA) were prepared by injection of an ethanolic lipid solution into an aqueous dispersion medium (water for injection or saline), followed by extrusion, as described previously [22]. Briefly, dipalmitoylphosphatidylcholine (DPPC), 1,2-distearoyl-phosphatidylethanolamine-methyl-polyethyleneglycol conjugate-2000 (mPEG2000-DSPE) (both from Lipoid GmbH, Ludwigshafen, Germany) and cholesterol (BUFA, Uitgeest, The Netherlands) (1.85: 0.15: 1 M ratio) were dissolved in ethanol by heating to 70 °C with continuous stirring. For the preparation of DTPA-DSPE LCL also 1.3 mM DTPA-DSPE (Avanti Polar lipids, Alabaster, AL, USA) was dissolved in the ethanolic lipid solution. In the case of DTPA LCL, 6 mM DTPA (Sigma Aldrich, St. Louis, MO) was dissolved in saline (B. Braun, Melsungen, Germany) as an aqueous dispersion medium. After dispersion of the ethanolic lipid solution in the aqueous solution the resulting coarse dispersion was passed through polycarbonate filter membranes with pore sizes of 200, 100 and 50 nm, to adjust the size of the liposomes to 100 nm diameter. Size distribution and polydispersity were determined by dynamic light scattering (triplicate measurements) on a Malvern 4700 system (Malvern Ltd., Malvern, UK). Ethanol was removed by dialysis against saline using Slide-A-Lyzer dialysis cassettes (Thermo Fisher Scientific, Etten-Leur, The Netherlands) with a molecular weight cutoff of 10 kDa and repeated changing of the dialysis medium (phosphate buffered saline (PBS)).

2.2. Radiolabelling procedure

Surface labelling of DTPA-DSPE LCL was performed by incubation of the liposomes (24.7 pmol total lipid–24.7 μmol total lipid) with 3.7 MBq $^{111}InCl_3$ (Mallinckrodt Pharmaceuticals, 's Hertogenbosch, The Netherlands) in 0.5 M 2-(N-morpholino)ethanesulphonic acid buffer (MES buffer, twice the volume of $^{111}InCl_3$), pH 5.4, for 30 min at RT. The amount of activity that was added during the labelling procedure ranged from 0.15–15,000 GBq/mmol total lipid. After incubation, 50 mM EDTA was added to a final concentration of 5 mM to chelate unincorporated ^{111}In. Labelling efficiency was determined using instant thin layer chromatography (ITLC) on ITLC-SG strips (Agilent Technologies, Amstelveen, The Netherlands), using 0.1 M citrate buffer, pH 6.0, as mobile phase and gel filtration on PD-10 Columns (GE Healthcare Life Sciences, Buckinghamshire, United Kingdom).

Labelling of DTPA-DSPE LCL and empty LCL was performed by incubation of the liposomes (247 nmol total lipid–24.7 μmol total lipid and 2,47 μmol total lipid–24.7 μmol total lipid, respectively) with 3.7 MBq ^{111}In-oxine (Mallinckrodt Medical B.V., Petten, The Netherlands) in 0.2 M Tris buffer (50% of the total volume), pH 8.0, for 30 min at RT. So specific activity ranged from 0.15–150 GBq/mmol total lipid. After incubation, 50 mM EDTA was added to a final concentration of 5 mM. The labelling efficiency was determined by gel filtration on a disposable PD-10 column.

For the *in vivo* imaging and biodistribution experiments both liposomal preparations were labelled with 350 MBq ^{111}In, in order to obtain preparations with a specific activity of 150 MBq/mmol total lipid. The radiochemical purity of both radiolabelled preparations used in this experiment exceeded 95%.

All radiolabelling procedures were performed under metal-free conditions to prevent the interference of contaminating metals with DTPA.

2.3. In vitro stability of the radiolabelled liposomes

To examine the *in vitro* stability regarding label retention, radiolabelled liposomal preparations, DTPA-DSPE LCL were labelled with $^{111}InCl_3$ and DTPA LCL were labelled with ^{111}In-oxine with a specific activity of 150 MBq/mmol total lipid. The radiolabelled preparations were purified by gel filtration chromatography on a PD-10 column. These samples, with a radiochemical purity of 100%, were incubated in human serum (1:1) at 37 °C. At 24 h and 48 h, retention of the radiolabel to the liposomal preparations was determined by gel filtration chromatography on a PD-10 column. In case of ^{111}In-DTPA-DSPE LCL, retention of the radiolabel was also determined using instant thin layer chromatography (ITLC) on ITLC-SG strips, using 0.1 M citrate buffer, pH 6.0, as mobile phase.

2.4. DTPA — challenge assay

DTPA-DSPE LCL, DTPA LCL and empty LCL were radiolabelled with ^{111}In (specific activity of 150 MBq/mmol total lipid), diluted to a volume of 500 μl with saline and subsequently 500 μl DTPA solution, pH 7.4 was added. The final concentration of DTPA in the solutions was 10^{-5}, 10^{-4}, 10^{-3} and 10^{-2} M. Samples were incubated at 37 °C for 24 h and analysed by ITLC (^{111}In-DTPA-DSPE LCL) and by gel filtration chromatography on a PD-10 column (^{111}In-empty LCL and ^{111}In-DTPA LCL).

2.5. Animals

Female NMRI Mice, 4–6 weeks of age were purchased form Harlan (Horst, The Netherlands). Mice were housed in individually-ventilated cages (IVC) under standard laboratory conditions (temperature, 20–24 °C; relative humidity, 50–60%; 12 h light–dark cycle) and food (SNIFF Voer, Soest, The Netherlands) and water were available *ad libitum*. All animals were accustomed to the environment for at least one week before experiments were initiated. All *in vivo* experiments were approved by the institutional animal welfare committee of the Radboud University, Nijmegen, and were conducted in accordance with the principles laid out by the revised Dutch Act on Animal Experimentation (1997).

2.6. Murine thigh muscle infection model

To compare the *in vivo* properties of ^{111}In-DTPA-DSPE LCL with those of ^{111}In-DTPA LCL, both preparations were injected i.v. in mice with an intramuscular *S. aureus* abscess in the thigh muscle [23]. To induce the infection, mice were kept under anaesthesia (1–3% isoflurane in O_2), shaved and injected i.m. with 50 μl of bacterial suspension into the left