Remote loading of liposomes with a 124I-radioiodinated compound and their in vivo evaluation by PET/CT in a murine tumor model

Engudar, Gokce; Schaarup-Jensen, Henrik; Fliedner, Frederikke P.; Hansen, Anders E.; Kempen, Paul; Jølck, Rasmus I.; Kjær, Andreas; Andresen, Thomas L.; Clausen, Mads H.; Jensen, Andreas Tue Ingemann

Published in:
Theranostics

Link to article, DOI:
10.7150/thno.26706

Publication date:
2018

Document Version
Peer reviewed version

Link back to DTU Orbit

Citation (APA):
Remote loading of liposomes with a $^{124}$I-radioiodinated compound and their in vivo evaluation by PET/CT in a murine tumor model

Gokce Engudar$^{1,5}$, Henrik Schaarup-Jensen$^{1,5}$, Frederikke P. Fliedner$^2$, Anders E. Hansen$^{2,3,5}$, Paul Kempen$^{3,5}$, Rasmus I. Jølck$^{3,5}$, Andreas Kjæer$^2$, Thomas L. Andresen$^{3,5}$, Mads H. Clausen$^{1,5}$, Andreas I. Jensen$^{4,5}$, and Jonas R. Henriksen$^{3,5*}$

$^1$Department of Chemistry, Technical University of Denmark, 2800 Lyngby, Denmark.
$^2$Cluster for Molecular Imaging, Dept. of Biomedical Sciences and Dept. of Clinical Physiology, Nuclear Medicine & PET, Rigshospitalet and University of Copenhagen, 2200 Copenhagen.
$^3$Department of Micro- and Nanotechnology, Technical University of Denmark, 2800 Lyngby, Denmark.
$^4$Center for Nuclear Technologies, Technical University of Denmark, 4000 Roskilde, Denmark.
$^5$Center for Nanomedicine and Theranostics, Denmark.

*Corresponding author: Jonas R. Henriksen, jhen@nanotech.dtu.dk

Graphical Abstract
Abstract

Long circulating liposomes entrapping iodinated and radioiodinated compounds offer a highly versatile theranostic platform. Here we report a new methodology for efficient and high-yielding loading of such compounds into liposomes, enabling CT/SPECT/PET imaging and $^{131}$I-radiotherapy.

Methods: The CT contrast agent diatrizoate was synthetically functionalized with a primary amine, which enabled its remote loading into PEGylated liposomes by either an ammonium sulfate or a citrate based pH transmembrane gradient. Further, the amino-diatrizoate was radiolabeled with either $^{124}$I ($t_{1/2} = 4.18$ days) for PET or $^{125}$I ($t_{1/2} = 59.5$ days) for SPECT, through an aromatic Finkelstein reaction.

Results: Quantitative loading efficiencies (>99%) were achieved at optimized conditions. The $^{124}$I-labeled compound was remote-loaded into liposomes, with an overall radiolabeling efficiency of $77 \pm 1\%$, and imaged in vivo in a CT26 murine colon cancer tumor model by PET/CT. A prolonged blood circulation half-life of 19.5 h was observed for the radiolabeled liposomes, whereas injections of the free compound were rapidly cleared. Lower accumulation was observed in the spleen, liver, kidney and tumor than what is usually seen for long-circulating liposomes.

Conclusion: The lower accumulation was interpreted as release of the tracer from the liposomes within these organs after accumulation. These results may guide the design of systems for controlled release of remote loadable drugs from liposomes.

Keywords: remote loading, pH-gradient liposomes, transmembrane ammonium sulfate, iodinated imaging agents, I-124, PET imaging

Introduction

Nanoparticles, and liposomes in particular, have been vigorously investigated due to their ability to improve the biodistribution of small molecular drugs. The multifunctional nature of liposomes has furthermore potentiated their use as theranostic agents and companion diagnostics. $^1$ Here, diagnostic liposomes are used for initial patient screening, while their therapeutic counterparts are used in follow-up treatment$^2$ - $^3$. The prolonged blood circulation and enhanced tissue accumulation of liposomes have also precipitated their use in imaging of the hepatic and cardiovascular system$^4$, and for clinical and preclinical assessment of the enhanced permeation and retention (EPR) effect in tumors$^5$ - $^6$. Alternative compositions such as nanoemulsions, micelles, polymeric and metal alloy nanoparticles are also being explored as reporters of the EPR effect$^7$ - $^9$. Several imaging modalities have been used in combination with liposomes for diagnostic purposes, including gadolinium based magnetic resonance imaging
Iodine has 37 isotopes and covers most of the above mentioned modalities. Non-radioactive iodine \( ^{127}\text{I} \) is radiodense and provides contrast in radiographic imaging agents, while various iodine radioisotopes are applied in molecular imaging for SPECT \( ^{123}\text{I}, ^{125}\text{I}, ^{131}\text{I} \) and PET \( ^{124}\text{I} \). Iodine radioisotopes are furthermore used as radioactive tracers in surgical guidance\(^{20,21} \), and for internal radiotherapy via beta irradiation \( ^{131}\text{I} \), photon irradiation \( ^{125}\text{I} \) and more recently in Auger therapy \( ^{125}\text{I} \). In recent years, \(^{124}\text{I} \) \( (T_{1/2} = 4.18 \text{ days}) \) has gained particular attention as a long-lived radioisotope for PET imaging. \(^{124}\text{I} \) has a positron branch of 23\% \( (E_{\text{average}} = 686 \text{ keV} \ (11\%) \) and 974 keV \( (11\%) \)) as well as several high-energy gamma emissions \( 511 \text{ keV} \ (46\%), \ 603 \text{ keV} \ (61\%), \ 723 \text{ keV} \ (10\%) \) and 1691 keV \( (11\%) \). \(^{23} \) Recent phase I/II studies have demonstrated \(^{124}\text{I} \) to be a valuable theranostic agent supporting current clinical \(^{131}\text{I} \) treatments of differentiated thyroid cancer, by improving staging, grading and lesion delineation using PET imaging. \(^{24} \) Taking advantage of the well-established iodine radiochemistry, labeling procedures can easily be adapted for each radioisotope. This gives access to either SPECT, PET or CT modalities for imaging and low energy beta-particle therapy. \(^{25} \) The combination of iodine and liposomes thus offers a highly versatile theranostic platform.

Visualization of the blood-pool and tumor vasculature has previously been investigated using liposomes labeled with iodine or radioiodine through either: i) passive encapsulation, ii) membrane incorporation of chemically modified iodo-lipids, or iii) remote-loading of iodine rich species. \(^{4,26,27} \) Passive encapsulation however, suffers from low encapsulation efficiencies and laborious preparation procedures \(^{28–31} \), while remote loading of liposomes using the well-known Bolton-Hunter reagent offers iodine loading efficiencies of only about 60\%. \(^{32} \) Accordingly, there is an unmet need to develop new methods for loading iodinated compounds into liposomes with quantitative loading efficiencies.

Transmembrane chemical potentials across lipid bilayers created by ion gradients can be used as a driving force for loading compounds into liposomes with high efficiency, as previously demonstrated for the clinically approved formulation Doxil™ \(^{33–36} \). In recent studies, similar approaches were applied to load radionuclides, such as the single photon emitter \(^{99m}\text{Tc} \), the therapeutic radioisotope \(^{186}\text{Re} \) and positron emitters \(^{64}\text{Cu} \) and \(^{52}\text{Mn} \). \(^{11,15,18,37–39} \)

In the present study, we report a new method for loading liposomes with an iodine-rich compound (Fig. 1). Diatrizoic acid, an iodinated CT contrast agent used clinically for visualization of e.g. the bowel \(^{40} \) and in digital subtraction angiography \(^{41} \), was functionalized to carry a primary amine enabling its remote loading into liposomes by a transmembrane pH gradient. While the unprotonated species may traverse the lipid membrane, protonation inside the liposome serves to trap it here. Remote loading was accomplished using either citrate or ammonium-sulfate based pH gradients, and was further optimized by varying the pH of the utilized buffers, the compound-to-lipid ratio, and the duration of the loading procedure. \(^{124}\text{I} \) radioiodinated...
diatrizoic acid analogues were obtained by an aromatic Finkelstein reaction\textsuperscript{42}, and were remote loaded into stealth liposomes. The biodistribution of the $^{124}\text{I}$-radiolabeled stealth liposomes and the free compound was evaluated by PET/CT imaging and \textit{ex vivo} gamma-counting in tumor-bearing mice. Radiolabeling of liposomes with $^{125}\text{I}$ via remote loading was furthermore conducted as part of the optimization procedure.

Such iodine-loaded liposomal theranostic agents are highly valuable for future biomedical applications, including the diagnosis and staging of the disease and for the evaluation of therapeutic responses.

**Figure 1.** Remote loading of the amino diatrizoic acid (ADA) imaging agent (A) into liposomes by transmembrane pH gradients. Possible iodine labels for SPECT, therapy, PET and CT are outlined. Equilibria governing the remote loading of amine functionalized compounds into liposomes via ammonium sulfate (B) or citrate based pH gradients (C) are shown.

**Materials and Methods**

**Materials**

For synthesis of the imaging agent, starting materials, reagents and solvents were purchased from commercial suppliers and used without further purification. All solvents beside 2-methoxy-ethanol were HPLC grade and dry solvents were obtained via an Innovative Technology PS-MD-7 puresolv system. $^{124}\text{I}]\text{NaI}$ and $^{125}\text{I}]\text{NaI}$ were purchased from PerkinElmer Life Science as solutions in aqueous NaOH (0.02 M and $10^{-5}$ M, respectively).

The lipid mixture, composed of Hydrogenated Soy L-\textalpha-phosphatidylcholine (HSPC), Cholesterol (CHOL) and 1,2-distearoyl-\textit{sn}-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG\textsubscript{2000}) in the molar ratio HSPC:CHOL:DSPE-PEG\textsubscript{2000} (56.5:38.2:5.3), was purchased from Lipoid
1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N (lissamine rhodamine B sulfonyl) (ammonium salt) (Rho-DPPE) was purchased from Avanti Polar Lipids. Reagents used in the ICP-MS experiments, nitric acid and tetramethylammonium hydroxide solution (25% in H₂O), were TraceSelect® and purchased from Fluka Analytical and Te, Ga, P standards and potassium iodide were purchased from Sigma-Aldrich. Ammonium sulfate, citric acid, trisodium citrate dihydrate, sodium phosphate monobasic dihydrate, sodium phosphate dibasic dihydrate, sodium chloride, sodium carbonate, HEPES ([4-(2-Hydroxyethyl)-piperazino]-1-ethanosulfonic acid) and HEPES sodium salt, Trizma® base (Tris(hydroxymethyl)aminomethane), MES (2-(N-morpholino) ethanesulfonic acid), and CHES (2-(Cyclohexylamino)ethanesulfonic acid), were purchased from Sigma-Aldrich. Water used for all experiments was collected from a Milli-Q system (Millipore).

Illustra™ MicroSpin™ G-50 columns and PD-10 desalting columns were from GE Healthcare. Slide-A-Lyzer® 10K Dialysis Cassettes were from ThermoScientific. Amicon® Ultra (4 and 0.5 mL - 30K and 100K) centrifugal filter devices were purchased from Millipore. Sep-Pak® Plus Silica cartridges (690 mg sorbent per cartridge, 55-105 µm particle size) were purchased from Waters. TLC plates (Silica gel 60 F₂₅₄) were purchased from Merck.

An isotonic HEPES buffer (25 mM HEPES, 150 mM NaCl) was prepared by mixing HEPES and HEPES sodium salt in Milli-Q water and adjusted to pH 7.4 with HCl (osmolality: 310 mOsm/kg). Phosphate buffer was prepared by mixing sodium phosphate monobasic dihydrate and sodium phosphate dibasic dihydrate to obtain pH 7.0 (25 mM phosphate, 150 mM NaCl, pH 7.0, 326 mOsm/kg). CHES buffer (25 mM CHES, 150 mM NaCl, pH 9.0, 310 mOsm/kg), MES buffer (25 mM MES, 150 mM NaCl, pH 6.0, 307 mOsm/kg) and TRIS buffer (25 mM TRIS, 150 mM NaCl, pH 8.0, 313 mOsm/kg) were prepared in Milli-Q water and the pH values were adjusted with HCl/NaOH to desired pH. Sodium citrate buffer was prepared by mixing citric acid and trisodium citrate dihydrate for the final pH of 4.0 (200 mM citrate, pH 4.0, 380 mOsm/kg).

Methods

Synthesis of amino diatrizoic acid (ADA)

Diatrizoic acid 1 was esterified by nucleophilic substitution of 2-(boc-amino)ethyl bromide (1.5 equiv.) in DMF at 50 °C by forming the corresponding carboxylate of 1 using K₂CO₃ (1.2 equiv). This afforded the ester 2 in fair yield (48%). The Boc-group of 2 was subsequently removed using TFA in CH₂Cl₂ yielding the TFA salt of 3 in quantitative yield (For detailed synthetic procedures and NMR spectra of final product, see Supporting Information data S1). Compound 3 is hereafter referred to as amino diatrizoic acid (ADA).
Scheme 1. Synthetic scheme of amino-functionalized diatrizoic acid (3) and radioiodination.

Synthesis of $[^{124}\text{I}]$ADA and $[^{125}\text{I}]$ADA

$[^{124}\text{I}]$ADA and $[^{125}\text{I}]$ADA were synthesized using an aromatic Finkelstein reaction (iodine-iodine exchange) modified from Aissi et al.\textsuperscript{42} Amino diatrizoic acid (ADA) (0.35 mg, 0.45 µmol, TFA salt) was dissolved in glacial acetic acid (240 µL) in a glass vial, followed by adding a dispersion of CuSO\textsubscript{4} in glacial acetic acid (120 µL, 1.0 mg/mL, 0.76 µmol). $[^{124}\text{I}]$NaI as an aqueous solution in 0.02 M NaOH (120 µL, 109 MBq) was added and the mixture was magnetically stirred in a closed vial at 130 °C for 2.5 h. After cooling to ambient temperature, ethyl acetate (720 µL) was added to the mixture and the resulting solution was applied to a Sep-Pak Plus Silica cartridge. The product was subsequently isolated by sequential elution of 10 fractions (F) according to the following scheme: F1: neat ethyl acetate (3 mL), F2: EtOH:EtOAc:AcOH (75:25:1) (2 mL), F3: EtOH:EtOAc:AcOH (90:10:1) (1 mL), F4: EtOH:EtOAc (90:10) (1 mL), F5: EtOH:25% aq. NH\textsubscript{4}OH (99:1) (1 mL), F6: EtOH:25% aq. NH\textsubscript{4}OH (98:2) (1 mL), F7: EtOH:25% aq. NH\textsubscript{4}OH (97:3) (1 mL), F8: EtOH:25% aq. NH\textsubscript{4}OH (96:4) (1 mL), F9: EtOH:25% aq. NH\textsubscript{4}OH (95:5) (1 mL), F10: EtOH:25% aq. NH\textsubscript{4}OH (95:5) (1 mL). Fractions 5-10 were pooled and evaporated to dryness at 70 °C under a stream of air for 35 minutes, furnishing the desired product. Likewise, to radiolabel ADA with $^{125}\text{I}$, ADA (1.0 mg, 1.3 µmol, TFA salt) was dissolved in glacial acetic acid (100 µL) and a dispersion of CuSO\textsubscript{4} in glacial acetic acid was added (100 µL, 1.0 mg/mL, 0.63 µmol). An aqueous solution of $[^{125}\text{I}]$NaI in 10⁻⁵ M NaOH (3 µL, 8.7 MBq) and water
(7 µL) was added to this mixture. The resulting solution was magnetically stirred at 130 °C for 30 minutes. After cooling to room temperature, ethyl acetate (400 µL) was added to the mixture. The product was isolated using a Sep-Pak Plus Silica cartridge and sequential elution in accordance with the above gradient scheme. Fractions F5-10 were evaporated to dryness at 60 °C under a stream of air for 45 minutes.

Preparation of liposomes with a transmembrane ammonium gradient
The lipid mixture composed of HSPC:CHOL:DSPE-PEG2000 (56.5:38.2:5.3 molar ratio) was mixed with Rho-DPPE in a tert-butanol:water (9:1) (0.2 mol% fluorescent lipid), and lyophilized overnight. Rhodamine B was included for UV-VIS detection of these liposomes. The freeze-dried lipid was hydrated in 150 mM ammonium sulfate (pH 5.3, 330 mOsm/kg) for 1 h at 65 °C and seven freeze-thaw cycles were conducted before sizing to large unilamellar vesicles (LUVs). To form LUVs, the lipid suspension was extruded through either 200 nm or 100 nm (for the in vivo PET study) pore sized polycarbonate membranes by an Avanti mini extruder at 65 °C. The hydrodynamic diameter and size distribution of the liposomes were determined by dynamic light scattering (ZetaSizer, Malvern Instruments) and the phosphorous content by ICP-MS (iCAP Q, Thermo Scientific).

The ammonium sulfate gradient was created using Slide-A-Lyzer® 10K Dialysis Cassettes (buffer volume was 300-fold the volume of the sample) for buffer exchange with buffers having different pH values. The dialysis buffer was replaced three times with new buffer to provide a successful buffer exchange. The external buffers were isotonic 25 mM phosphate buffer pH 7.0, TRIS pH 8.0, CHES pH 9.0 or MES pH 6.0 buffers all containing 150 mM NaCl. The average hydrodynamic diameter of the liposomes was 146 nm (PDI: 0.062).

Preparation of liposomes with a citrate based transmembrane pH gradient
Large unilamellar vesicles (LUVs) were prepared as previously described using a 200 mM sodium citrate buffer (C₆H₈O₇: Na₃C₆H₅O₇, pH 4.0, 380 mOsm/kg) for hydration. The liposome solution was incubated for 1 h at 65 °C, followed by seven freeze-thaw cycles. The transmembrane pH gradient was formed by increasing the external pH of liposomes to a desired pH of 7.0 by adding aq. sodium carbonate (1 M, pH 11.8), resulting in a final lipid concentration of 50 mM (volume ratio of base and liposomes V_base/V_liposome = 0.2). The hydrodynamic diameters of the liposomes extruded through 200 nm or 100 nm filters were 142 nm (PDI = 0.072) or 137 (PDI = 0.042) respectively.

Remote loading and quantification of ADA and [¹²⁴I]ADA
Having formed the ion gradient across the liposome membrane, the liposome dispersion was added to solid ADA. As an example, 2.2 mg ADA (2.9 µmole) was mixed with aq. 550 µL liposomes (50 mM lipid). For the ammonium sulfate gradient liposomes, a fixed ADA-to-lipid molar ratio of 0.1 was used for the method optimization in combination with varying the external pH (6-9) and incubation time (1-24 h). For the citrate gradient liposomes, ADA-to-lipid ratios from 0.1 to 1.0 were investigated at constant lipid concentration and as a function of the incubation time (1-24h). Initial screening
revealed 55 °C to be the optimal incubation temperature when judged by loading rate (data not shown). All samples were heated and continuously stirred at 55 °C.

**Quantification of the liposome loading efficiency of ADA**

The loading efficiency was quantified as the ratio of the ADA-to-lipid ratio before and after microcolumn separation of the free and liposome entrapped ADA. Briefly, a 25 µL liposome sample was applied to the Illustra MicroSpin G-50 columns (GE Healthcare) and centrifuged at 2000 g for 4 min. The ADA-to-lipid ratio (before and after column separation) was determined by UV-VIS spectroscopy (NanoDrop 2000C, ThermoScientific), where rhodamine labeled DPPE acted as a surrogate for the full lipid sample. Samples with ADA-to-lipid ratio of 0.1, 0.25, 0.5 or 1.0 were diluted either 150, 300, 600 or 1200-fold in ethanol containing 0.6 v/v % TFA respectively, prior to the UV-VIS measurement in order to dissolve the liposomes and assure protonation of ADA. The absorbance of ADA and Rho-DPPE (liposomes) were measured at 243 nm (λ<sub>max</sub>), and 560 nm (λ<sub>max</sub>) respectively, and the loading efficiency was calculated as:

\[
\% \text{LE} = \frac{A_{243,a}}{A_{243,b}} \cdot \frac{A_{560,b}}{A_{560,a}} \cdot 100\%
\]

Eq. 1

where \(A_{243,b}\) and \(A_{243,a}\) denotes the peak absorbance of ADA before and after column separation respectively. \(A_{560,b}\) and \(A_{560,a}\) denotes the peak absorbance of Rho-DPPE (liposome) before and after column separation respectively (see Supporting Information Fig. S3-S4).

Iodine and phosphor concentrations of the liposomes were in addition quantified by ICP-MS (Thermo Scientific, iCAP Q) to verify the loading efficiency determined by UV-VIS spectroscopy (see Supporting Information data S5).

**Preparation of [¹²⁴I]ADA in HEPES buffer for in vivo study**

Radiolabeled [¹²⁴I]ADA (45.3 MBq, 1.2 µmol) was dissolved in EtOH:H<sub>2</sub>O (7:1, 800 µL). From this solution, 110 µL (5.24 MBq) was evaporated to dryness under argon flow for 5 minutes at 70°C. The residue was redissolved in isotonic HEPES buffer (500 µL) and used directly for the in vivo study. The final activity concentration was 9.0 MBq/mL at the time of injection.

**Preparation of [¹²⁴I]ADA loaded liposomes for in vivo study**

Citrate based transmembrane pH-gradient liposomes for the in vivo study was prepared as described above resulting in liposomes with hydrodynamic diameters of 137 nm (PDI = 0.04). Radiolabeled [¹²⁴I]ADA (45.3 MBq, 1.2 µmol) was dissolved in EtOH:H<sub>2</sub>O (7:1, 800 µL), whereafter 39 MBq (690 µL) was transferred to an HPLC vial and evaporated to dryness under argon flow for 5 minutes at 70 °C. Citrate based transmembrane pH-gradient liposomes (50 mM, 510 µL) were added to the dry [¹²⁴I]ADA (39 MBq) and incubated for 3 h at 55°C with continuous stirring. The initial
ADA-to-lipid molar ratio was 0.04. After loading, non-encapsulated $[^{124}\text{I}]$ADA and external citrate buffer were removed by exchanging the external medium with HEPES buffer (25 mM, 150 mM NaCl, pH 7.4, 310 mOsm/kg) using PD-10 desalting columns (GE Healthcare). The final lipid and activity concentration of the collected liposomes were 10.2 mM and 20.5 MBq/ml at the time of injection.

**Quantification of the $[^{124}\text{I}]$ADA loading efficiency**

$[^{124}\text{I}]$ADA-loaded liposomes and free $[^{124}\text{I}]$ADA were separated using PD-10 desalting columns (GE Healthcare). The sample was applied to the column and 500 µL fractions were collected by eluting with isotonic HEPES buffer. The radioactivity of the collected fractions was measured using a dose-calibrator (CRC-55tR, Capintec, Inc.). The loading efficiency was calculated as the ratio of the radioactivity of $^{124}\text{I}$ in the liposome fraction to the total radioactivity in the collected fractions. Furthermore, the PD-10 purified $^{124}\text{I}$-liposomes were analyzed by thin layer chromatography (TLC) in order to determine the radiochemical purity. 2 µL of purified $[^{124}\text{I}]$ADA-liposomes were spotted on a TLC plate (Silica gel 60 F$^\text{254}$) and ethanol:25% aq. NH$_4$OH (99:1) was used as eluent. The radio-TLC was measured using a phosphor imager (Cyclone Plus, PerkinElmer) and the $[^{124}\text{I}]$ADA compound was identified at Rf = 0.35, which was identical to the non-radioactive reference (ADA).

**Cryo-TEM imaging**

3 µL of liposome solution was placed on a lacy carbon 300 mesh copper TEM grid, blotted and plunge frozen in liquid ethane using a FEI Vitrobot Mark IV. Samples were imaged using a FEI Tecnai G2 20 TWIN transmission electron microscope operated at 200 keV in low dose mode with a FEI High-Sensitive (HS) 4k x 4k Eagle camera.

**Animal model**

Tumors were established by subcutaneous injection of $3\times10^5$ CT26 murine colon cancer cells (CRL-2638, American Type Culture Collection, ATCC, VA, USA) suspended in 100 µL of culture medium over the thigh/flank of 7 weeks old female Balb/c mice (Janvier Labs, France). Cells were grown in vitro in culture flasks using RPMI medium (Gibco, Thermo Fisher, Waltham, Massachusetts, USA) supplemented with Bovine Serum Albumin (Gibco, Thermo Fisher, Waltham, Massachusetts, USA) and Pencillin Streptomycin (Pen Strep, (Gibco, Thermo Fisher, Waltham, Massachusetts, USA)) before inoculation in mice. Cells were tested for murine virus, human virus, mycoplasma, and lastly tested for cell line authentication to exclude cross-contamination during in vitro growth. Tumors were allowed to grow for 16 days before study start. The National Animal Experiments Inspectorate approved all study procedures (license no. 2016-15-0201-00920).

**MicroPET/CT imaging procedure**

PET/CT imaging was performed on an Inveon® small animal PET/CT system (Siemens Medical Systems, PA, USA). Mice were anesthetized by inhalation of anesthesia (~3% sevoflurane) and either 3.46 ± 0.04 MBq/mouse of $[^{124}\text{I}]$ADA -
lipoosomes (n = 5) or 1.39 ± 0.05 MBq/mouse of [124I]ADA in HEPES (n = 3) was injected into the tail vein. The mice receiving liposomes were each administered with 1.3 mg lipid and 71 µg ADA, whereas the mice administered with the free compound received 29 µg ADA each. Decay corrected injected activities were determined by gamma counting syringes before and after injections. [124I]ADA -lipoosomes or [124I]ADA was allowed to distribute for 18 minutes before commencing a 5 minute PET scan (Scan1) and a corresponding CT scan. Additional PET/CT scans were performed after a distribution period of 1.5 h (Scan2) (5-minute acquisition), 20 hours (Scan3) (10-minute acquisition), 48 hours (Scan4) (15-minute acquisition) and 70 hours (Scan5) (15-minute acquisition). Emission data were corrected for dead time and decay and attenuation correction was performed based on the corresponding CT scan. PET scans were reconstructed using a maximum a posteriori (MAP) reconstruction algorithm (0.815×0.815×0.796 mm). Image analysis was performed using Inveon® software (Siemens Medical Systems, PA, USA). 3D regions of interest (ROIs) were manually constructed and decay corrected data (%injected dose per gram tissue (%ID/g)) were reported.

**Gamma counting**

For post mortem analysis, the following tissues were collected and transferred into pre-weighed gamma counter tubes; blood, liver, spleen, kidney, tumor and thyroid gland (complete larynx region). All tubes were well counted using an 124I setup and decay correction (Wizard gamma counter, Perkin Elmer, Waltham, MA, USA). The specific activity is reported as a %ID/g, and is based on the total injected activity and the measured activity and weight of the tissue samples. The activity of the thyroid gland was determined from the overall activity in the excised complete larynx region, which was not further dissected to avoid damaging the thyroid. The measured activity was assumed to be primarily from the thyroid gland and activity is therefore reported as injected dose in the thyroid gland (%ID/thyroid).

**Results and Discussion**

**Synthesis and characterization of ADA**

A diatrizoic acid derivative (ADA) was synthesized by esterification with 2-(boc-amino)ethyl bromide. This introduced an amino group to enable remote loading into liposomes via a transmembrane pH gradient. The pKa value of the primary amine on ADA was determined to be 8.56 ± 0.04 (n = 4) by acid-base titration and LogD values of -0.6 (pH 5) and 0.6 (pH 7.4) were estimated using the ChemAxon software package (see Supporting Information Fig. S1 and Fig. S2). The pKa and corresponding change in hydrophilicity upon protonation of the amine allows ADA to traverse lipid membranes at neutral pH but not at lower pH values. This makes ADA suitable for remote-loading into the liposomes.
Figure 2. Remote loading of non-radioactive ADA into liposomes via transmembrane ammonium sulfate or citrate pH gradients. (A) UV-VIS spectra of ADA loaded liposomes before and after removal of free ADA by MicroSpin column separation. Inset: Normalized UV-VIS spectra where the absorbance of Rho-DPPE at 560 nm has been set to unity. The normalized spectrum enables direct comparison of the ADA to lipid ratio before and after removal of non-loaded ADA by spin column separation. (B) The loading efficiency of ADA into ammonium sulfate gradient liposomes is shown as function of time and external pH. The loading experiments (B) were conducted at a 0.1 ADA-to-lipid molar ratio and at 55 °C. (C) The loading efficiency of ADA into citrate gradient liposomes is shown as function of the ADA-to-lipid ratio and time. The loading experiments (C) were conducted at 55 °C with an external pH of 7.0. (D) Cryo-TEM images of ADA-loaded ammonium sulfate liposomes with an ADA-to-lipid ratio of 0.2. (E) Cryo-TEM images of ADA-loaded sodium citrate liposomes with an ADA-to-lipid ratio of 0.2. The scale bars are 100 nm.

Optimizing the remote loading of ADA into liposomes
Remote loading of ADA into stealth liposomes was conducted using either an ammonium sulfate or a citrate based pH gradient method. A Rho-DPPE lipid fluorophore was included to enable quantification of the lipid fraction. The loading efficiency could therefore be determined by UV-VIS spectrophotometry (Fig. 2A), via the absorbance of ADA at 243 nm and Rho-DPPE at 560 nm. By use of Eq. 1, the loading efficiency, %LE, was determined via the ADA to lipid ratio before and after removal of non-loaded ADA by spin column separation (see Supporting Information...
Fig. S3 and Fig. S4 for UV-VIS spectra of ADA and liposomes used for quantification of the loading efficiencies). Iodine and phosphor concentrations of several UV-VIS samples were in addition quantified by ICP-MS for verification of the method (see Supporting Information Fig. S5). For both sets of liposomes, dry ADA solids were stirred directly with the liposomes, and all reported loading efficiencies describe the combined process of initial dissolution and subsequent loading of ADA into the liposomes. Both solubility and membrane permeation are highly dependent on the overall charge of ADA and thereby dependent of the pH outside the liposomes. An optimal pH of 7.0 was identified for the ammonium sulfate gradient liposomes (at 55 °C and 0.1 ADA-to-lipid ratio), yielding a maximal loading efficiency of 86 ± 2% after 6 h of stirring, followed by a 12% decline in the loading efficiency at 24 h (Fig. 2B). Liposomes with an external pH of 6.0 exhibited the lowest loading efficiencies reaching a maximal loading of 27 ± 1% after 24 h of stirring (Fig. 2B). The reduced loading efficiency at pH 6.0 is likely caused by poor membrane permeation of the protonated ADA species leading to slow loading kinetics. At external pH 8.0 and 9.0, the maximal loading efficiency was reached at 2 h, yielding 56 ± 1% and 78 ± 3% respectively (Fig. 2B). The reduction in loading efficiency obtained at pH 8.0 and 9.0 compared to pH 7, may be explained by lower solubility and/or slow dissolution kinetics of the dry ADA at higher external pH values due to limited protonation. Further studies are required to determine the exact cause of the reduced loading efficiency found at pH 6, 8 and 9 compared to pH 7.

Having identified an optimal external pH of 7.0, citrate gradient based liposomes were investigated. These liposomes were prepared in citrate buffers with an internal lumen pH of 4.0, and an external pH of 7.0. This procedure utilizes the polyprotic nature of citrate, and provides an instant pH gradient across the liposome membranes in a quick and simple manner. Using this method, concentrated liposome samples (50 mM lipid) were prepared with membrane gradients of ΔpH = 3.0, without any purification steps and with limited sample dilution (~20% upon addition of sodium carbonate). Such liposomes, of high lipid concentration and simple gradient formation, are highly warranted when large compound payloads are desired. In order to determine the maximal payload of ADA into the citrate based liposomes, the loading efficiency was determined for the ADA-to-lipid molar ratios 0.1, 0.25, 0.5 and 1.0. For all ADA-to-lipid molar ratios, the loading efficiency remained approximately constant at the 1, 2, 3 and 6 h time-point, beyond which a slight decrease in loading efficiency was observed for the 0.1 ADA-to-lipid ratio (Fig. 2C). This decrease in loading efficiency after 12-24 h incubation may be governed by slow dissipation of the citrate pH gradient resulting in unloading of the ADA compound. Similar observations have been reported by Forster et al. 43 Loading efficiency levels of 98 ± 1%, 90 ± 2%, 73 ± 3% and 38 ± 2% were obtained at ADA-to-lipid ratios of 0.1, 0.25, 0.5 and 1.0 respectively demonstrating that the citrate pH gradient is gradually exhausted as more ADA compound is loaded into the core of the liposomes. This observation is in accordance with previous reports. 44 The effectively entrapped payload of ADA per liposome, given as the product of the loading efficiency and the ADA-to-lipid molar ratio, increases

12
from [ADA]/[Lipid] = 0.098, 0.23, 0.37 to 0.38 for ADA-to-lipid molar ratio 0.1, 0.25, 0.5 and 1.0 respectively. The effective entrapped payload thus converges towards [ADA]/[Lipid] = 0.38, which corresponds to 14.5 mg iodine/mL for a 100 mM lipid solution. Hence, production of long circulating high CT contrast liposomes by remote loading using the current approach seems to not be feasible, as 50-200 mg/mL iodine is often required to obtain significant CT contrast levels. Improved payloads of iodine in liposomes may still be obtained by entrapping higher citrate concentrations and/or utilizing liposomes with increased diameter. However, such changes impact the osmotic transmembrane gradient and systemic circulation properties of the liposome. Cryo-TEM images of citrate and ammonium sulfate gradient liposomes loaded with ADA are shown in figure 2D-E displaying populations of spherical monodisperse liposomes in both cases. Neither the citrate nor ammonium sulfate based liposomes were found to entrap visible precipitates of ADA at a ADA-to-lipid ratio of 0.2, such as is found in Doxil where crystals of doxorubicin are present after remote loading.

**Radiolabeling of ADA with $^{124}$I and $^{125}$I**

$^{124}$I substitution of iodine on ADA was conducted using a modified Cu(I)-catalyzed nucleophilic isotopic exchange reaction (Scheme 1). The radiochemical purity of the purified [$^{124}$I]ADA was > 90%, with a radiochemical yield of 34-55% and a specific activity of 83 MBq/µmol. ADA was in addition radiolabeled with $^{125}$I through a 30 minute reaction, which resulted in a radiochemical purity of 94% and a higher purified radiochemical yield of 64%. Since $^{124}$I could only be acquired in lower concentration than $^{125}$I, it was necessary to conduct the radiolabeling with this radionuclide at higher water concentrations. This was observed to drastically lower the reaction rate, which made it necessary to extend the reaction time. This led to an increased presence of impurities and lowered radiochemical yields for the $^{124}$I radiolabeling. Radiochemical conversion (not purified) for similar reaction conditions on an ortho-iodinated benzyl group has previously been reported to be around 75-82% after 30 minutes of reaction, with a slight drop to around 70% after 45 minutes. Accordingly, our RCYs and conversion for $^{125}$I are on par with reported values and the extended reaction time may compromise yields, likely due to harsh reaction conditions. The reaction used is theoretically applicable to all isotopes of iodine, but as was observed, the concentration and manner in which an isotope can be acquired may affect practical procedures.
Figure 3. Characterization of $^{124}\text{I}}$ADA loaded liposomes by size exclusion column separation and radio-TLC. (A) Elution profile of $^{124}\text{I}}$ADA loaded liposomes by PD-10 column separation. The liposomes were eluted with 25 mM HEPES buffer (150 mM NaCl, pH 7.4, 310 mOsm/kg), with a loading efficiency of 77.2%. (B) Radio-TLC peak of $^{124}\text{I}}$ADA after loading into liposomes.

Remote loading of $^{124}\text{I}}$ADA and $^{125}\text{I}}$ADA into liposomes

For remote loading of the radiolabeled analogue of ADA, liposomes entrapping citrate were chosen due to their higher loading efficiency and faster loading kinetics (Fig. 2B-C) when compared to their ammonium sulfate counterparts. The longer lived $^{125}\text{I}}$ADA analogue was used for the initial optimization of the radio-TLC and PD-10 size exclusion column analyses, as well as for the test loading of liposomes. Subsequently, the $^{124}\text{I}}$ADA analogue was synthesized for evaluation of the liposome biodistribution in mice by PET/CT imaging and organ well counting. The average radiochemical purity of $^{125}\text{I}}$ADA and $^{124}\text{I}}$ADA was 93.0 ± 0.6% ($n = 4$) when assessed by radio-TLC (see Supporting Information Fig. S6). However, remote loading of both $^{125}\text{I}}$ADA and $^{124}\text{I}}$ADA, with subsequent size exclusion purification, resulted in radiochemical purities of more than 99% (Fig. 3B) in all cases. We believe this to be a result of only the target compound (ADA) undergoing remote-loading, whereas other small-molecular radioactive impurities were removed by the PD-10 purification. The average loading efficiency was 77 ± 1% ($n = 4$) when assessed by PD-10 column separation (Fig. 3A). Overall, this correspond to 84% of the loadable activity being entrapped in the liposomes, which is a 30% improvement compared to the Bolton-Hunter reagent method reported by Mougin-Degraef et al.32

For the in vivo studies, the liposome sample was purified on a PD-10 column, with concomitant exchange of the medium from hypertonic citrate (380 mOsm/kg) to isotonic HEPES buffer. After column separation, citrate gradient liposomes were collected with lipid and radioactivity concentrations of 10.2 mM and 20.5 MBq/mL respectively at the time of injection. The loading efficiency after column purification was 98%. The stability of the encapsulation of the loaded radiotracer was assessed by incubating the $^{124}\text{I}}$ADA liposomes at 25 °C for 168 hours in HEPES buffer. This
resulted in an encapsulation efficiency of 98 ± 1% (n = 3), testifying to the excellent stability of this loading method.

![MIP and axial PET/CT images of systemically administered free $^{[124]}$IADA and $^{[124]}$IADA loaded liposomes in CT26 tumor-bearing mice at 15 min, 90 min 20 h, 48 h and 70 h pi.. The position of the axial image projections is illustrated for the 70 h timepoint using yellow dotted lines.](image)

**Figure 4.** MIP and axial PET/CT images of systemically administered free $^{[124]}$IADA and $^{[124]}$IADA loaded liposomes in CT26 tumor-bearing mice at 15 min, 90 min 20 h, 48 h and 70 h pi. The position of the axial image projections is illustrated for the 70 h timepoint using yellow dotted lines.

**In vivo evaluation of $^{[124]}$IADA loaded liposomes**

$^{[124]}$IADA and $^{[124]}$IADA liposomes were well tolerated by all mice and no reactions to the injections or during the observation period were seen. Representative MIP and axial PET/CT images are shown in Fig. 4 (See supporting information S8 for Sagittal and Coronal PET/CT images). Biodistributions of free and liposome loaded $^{[124]}$IADA in CT26 tumor–bearing mice at each time-point were determined from the PET scans (Fig. 5A-E). The biodistributions were further verified by gamma counting organs including the thyroid regions after a distribution period of 72 hours (Fig. 5F,G).

For free $^{[124]}$IADA, rapid blood clearance (Fig. 5A) and urinary excretion during the first 15 minutes were observed (Fig. 5E). Rapid clearance of free $^{[124]}$IADA from the spleen, liver and kidney, and low tumor activity was also observed (Fig. 5B-C), reflecting the rapid clearance of free $^{[124]}$IADA from the blood pool within these organs. $^{[124]}$IADA thus exhibits the typical renal clearance of small, polar substances. After 72 h, a total retention of 0.015 %ID was determined in the thyroid by well counting (Fig. 5F). Assuming a thyroid weight of 11 mg, this corresponds to 1.4%ID/g accumulation in the thyroid of the non-liposomal $^{[124]}$IADA. This is likely to be a result of the bulk of the radioactivity clearing from the organism before appreciable
deiodination of $^{[124]}$IADA has occurred. The limited thyroid accumulation indicates that the compound is relatively stable in vivo. In comparison, administration of free $^{131}$I in rats resulted in 270 %ID/g accumulation in the thyroid after 6 h.\(^9\)

Contrary to the free compound, $^{[124]}$IADA loaded liposomes circulated with a half-life of 19.7 h, when estimated by a mono-exponential fit of data collected during 0-72 h ($R^2 = 0.98$). At the first PET scan (t = 15 min), 29.3 %ID/g, 12.0 %ID/g and 10.8 %ID/g were found in the blood, liver and spleen respectively, accounting for approximately 74% of the total activity when assuming organ/blood weight percentages (%w/w) of 8.5%, 4.9% and 0.54% for blood, liver and spleen\(^{50}\) and a 23 g average body weight of the Balb/c mice. The circulating half-life of the $^{[124]}$IADA-loaded liposomes is comparable to earlier reports for liposomal CT contrast agents\(^{27}\) and for liposomes carrying $^{64}$Cu- and $^{52}$Mn-radiolabels that are known to be stable in vivo\(^8,13,15,19,51\). This, as well as the minimal radioactivity in the urinary bladder, which could be either in the form of $^{[124]}$IADA or breakdown products, (Fig. 5E) substantiate that the stealth liposomes entrapping $^{[124]}$IADA are stable during circulation in the blood. Liver, spleen and kidneys also displayed the expected activity of liposomal

Figure 5. PET-derived biodistribution of free $^{[124]}$IADA and $^{[124]}$IADA loaded liposomes. (A-E) The accumulation of free $^{[124]}$IADA (n = 3) and $^{[124]}$IADA loaded liposomes (n = 5) in (A) blood, (B) liver, kidney and spleen, (C) tumor, and (D) tumor to blood ratio are shown as a function of time post injection (pi.). The radioactivity in the urinary bladder (E) was measured 15 min post injection. The results are presented as mean ± SEM for all organs and blood, whereas the tumor is given as both mean ± SEM and max ± SEM. (F-G) Ex vivo biodistribution data, 72 h post injection, of free $^{[124]}$IADA (n = 3) and $^{[124]}$IADA loaded liposomes (n = 5), based on organ well counting. The data are presented as mean ± SEM. The thyroid uptake is expressed as %ID ± SEM per thyroid.
[\textsuperscript{124}I]ADA, which is in correspondence with previous reports on comparable nanoparticles. The highest activity was observed at 1.5 h post injection (pi.) for these organs, which contradicts earlier reports on long circulating liposomes where the maximal accumulation typically occurred after 12-40 h.\textsuperscript{15,18} This indicates that radioactivity was able to leave these organs during the imaging period, which is not expected to occur if [\textsuperscript{124}I]ADA is maintained within the stealth liposome. The tumor to blood ratio, shown in figure 5D, increases as a function of time for the liposomal administered [\textsuperscript{124}I]ADA further indicating the extravascular activity in this region increases with respect to the intravascular as a result of EPR dependent liposome accumulation. Previous biodistribution studies of stealth liposomes have proven these to be very stable in circulation\textsuperscript{5,15,51}, which suggests that free [\textsuperscript{124}I]ADA or breakdown products were released from the liposomes after accumulation. The absence of a precipitate in the cryo-TEM images (Fig. 2D-E) lends support to this hypothesis, as formation of crystalized [\textsuperscript{124}I]ADA inside the liposomes would have been likely to slow down the release kinetics.\textsuperscript{52} This release may be a result of uptake and digestion of the liposomes by resident immune cells, or loss of the trans-membrane pH-gradient. The latter possibility was previously suggested as a reason for unloading of liposomal doxorubicin.\textsuperscript{53} In vitro studies by Forster et al. and Mayer et al. showed that the pH gradient of liposomes composed of EPC or DPPC may dissipate over time when incubated at 37 °C in buffer or serum, which supports our hypothesis that [\textsuperscript{124}I]ADA may have leaked from the liposomes after they had accumulated.\textsuperscript{43,54} Since higher thyroid accumulation was observed for the liposomal [\textsuperscript{124}I]ADA (1.06 %ID/thyroid, 96%ID/g assuming a thyroid weight of 11 mg,\textsuperscript{48} Fig. 5F) compared to freely administered [\textsuperscript{124}I]ADA, the compound is probably released from the organs of uptake, with subsequent renal excretion and only limited deiodination, as thyroid entrapment of free iodine would have been more pronounced. In comparison, Ke and coworkers reported a 12-fold higher thyroid accumulation (12.9%ID/thyroid) for euthyroid mice upon administration of \textsuperscript{131}I.\textsuperscript{55}

The stability of [\textsuperscript{\textit{\textasteriskceil}}I]ADA was further investigated in vitro to shed light on the possible deiodination of the compound (see supporting Information, S7). In this test assay, the radiolabeled compound was incubated at 37 °C with mouse serum (50%), mouse liver homogenate in PBS and neat PBS as control, and the mixtures were monitored by radio-TLC for 3 days. Liver homogenate was included as a test for deiodination, due to the high presence of deiodinases in this organ. No breakdown was observed in PBS, whereas about 5% [\textsuperscript{\textit{\textasteriskceil}}I]ADA was degraded after 3 days in serum and about 10% in the liver homogenate. Multiple metabolites were observed, suggesting that other pathways of degradation besides deiodination took place. That degradation was slightly more pronounced in the liver homogenate may be a result of deiodination occurring. In general, however, the compound exhibited good stability in both biological samples, which is also supported by the relatively limited thyroid accumulation of radioiodine observed in vivo.

The low short term degradation and deiodination of the [\textsuperscript{\textit{\textasteriskceil}}I]ADA compound observed in vitro further supports the low thyroid accumulation observed for [\textsuperscript{124}I]ADA administered in HEPES. The longer blood circulation, higher liver accumulation, and
the more pronounced *in vitro* degradation of $^{[124]}$IADA incubated in liver homogenate, 
also supports the 70-fold higher thyroid accumulation for the liposomal $^{[124]}$IADA compared to free $^{[124]}$IADA (Fig. 5F). Partially lipophilic drugs, like $^{[124]}$IADA, are expected to be taken up in hepatocytes and possible undergo enzymatic modifications, which may explain the higher thyroid accumulation for the liposomal administered $^{[124]}$IADA. Liposomes are on the other hand expected to be taken up in the liver by resident Kupffer cells. Further studies are needed to decipher the histological distribution of $^{[124]}$IADA and its consequences for release and deiodination. The mean tumor accumulation of 1.5%ID/g (Fig. 5C) is likewise lower than the 4-6%ID/g normally reported for CT26 tumor-bearing mice after accumulation for 24h,\(^{15}\) which suggests that $^{124}$I leaves the tumor in an undetermined form. The potential indication of a continuous release of $^{124}$I in some chemical form, possibly $^{[124]}$IADA, could bias our calculated half-life for $^{[124]}$IADA-liposomes in circulation. Considering the high blood radioactivity observed, this is only of minor concern following the observed rapid urinary excretion of $^{[124]}$IADA when administered in its free form.

The procedure for radiolabeling liposomes with radioiodine presented here involves an early radiolabeling step, giving a radiolabeled small molecule that is subsequently purified and loaded into liposomes. This mirrors previous procedures, such as the use of the Bolton-Hunter reagent\(^ {32}\) as well as the use of radiolabeled prosthetic groups. For a clinically used radiopharmaceutical this is a disadvantage, due to the increased handling of radioactive material. This could potentially be limited by automation. In the case at hand this could involve the initial radiolabeling happening in a vessel containing CuSO\(_4\) and the substrate, to which AcOH and the radioiodine could be added, followed by heating. Automated Sep-Pak purification would benefit from a fixed aq. NH\(_4\)OH concentration in the eluting medium, which is likely to be possible. To the dried compound, the liposome dispersion would simply be added and heated to effectuate loading. To avoid size exclusion chromatography, an improved Sep-Pak purification step would be beneficial, to reduce radioactive impurities to below 5%. As the loading itself is quantitative, this would likely give a sufficiently radiochemically pure product.

Despite the potential concern of using the $^{[124]}$IADA-liposomes as imaging agents to determine the maximum accumulation levels of liposomes in tumors and the biodistribution over extended periods, the observations in the present study are highly encouraging towards developing liposomes for therapeutic use. The prolonged retention in and slow release of drugs from liposomes are a potential Achilles heel for liposomal delivery of anti-cancer drugs. The $^{[124]}$IADA-liposomes display a continuous relatively stable activity level in tumors and this indicates that their content of $^{[124]}$IADA is continuously released at a rate high enough to equilibrate their EPR dependent accumulation. New classes of anti-cancer therapeutics or pro-drugs etc. constructed with chemical characteristics comparable to $^{[124]}$IADA may therefore prove beneficial to avoid slow release kinetics and become directly available to cells within the tumors.
Conclusion

We introduced a versatile technique to formulate radioiodine in liposomes in quantitative encapsulation yields. This was achieved by radioiodination of a membrane-crossing compound, \([^{124}\text{I}]\text{ADA}\), that was remote-loaded by an ion gradient. The loaded radioiodinated specie was found to be stably encapsulated in circulation, as well as in vitro. In vivo, however, evidence of release of the radioiodine was observed, after uptake in organs such as liver and spleen as well as tumor. This observation is highly encouraging for the future development and construction of liposomal anti-cancer drugs, as drugs with compatible chemical characteristics as ADA may provide means to overcome the problematic entrapment stability of liposomes and subsequent slow release kinetics of chemotherapeutics.

Acknowledgement

We acknowledge the Core Facility for Integrated Microscopy, Faculty of Health and Medical Sciences, University of Copenhagen

References


(33) Madden TD, Harrigan PR, Tai LCL, Bally MB, Mayer LD, Redelmeier TE, Loughrey HC, Tilcock CPS, Reinish LW, Cullis PR. The Accumulation of


