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^{64}Cu loaded liposomes as positron emission tomography imaging agents

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ABSTRACT

We have developed a highly efficient method for utilizing liposomes as imaging agents for positron emission tomography (PET) giving high resolution images and allowing direct quantification of tissue distribution and blood clearance. Our approach is based on remote loading of a copper-radionuclide (^{64}Cu) using a new ionophore, 2-hydroxyquinoline, to carry ^{64}Cu(II) across the membrane of preformed liposomes and deliver it to an encapsulated copper-chelator. Using this ionophore we achieved very efficient loading (95.5 ± 1.6%) and retention stability (>99%), which makes the ^{64}Cu-liposomes highly applicable as PET imaging agents. We show the utility of the ^{64}Cu-liposomes for quantitative *in vivo* imaging of healthy and tumor-bearing mice using PET. This remote loading method is a powerful tool for characterizing the *in vivo* performance of liposome based nanomedicine, and has great potential in diagnostic and therapeutic applications.

1. Introduction

Lipid nanoparticles (liposomes) are increasingly being investigated as diagnostic imaging agents [1] due to their potential as scintigraphic radiotracers for fast visualization of tumors and metastases. Positron emission tomography (PET) [2] imaging has emerged as a clinical cornerstone in cancer staging and restaging for a number of malignancies, and is one of the leading molecular imaging technologies approved by FDA [3]. PET/computed tomography (CT) imaging using 2-[^{18}F]fluoro-2-deoxy-D-glucose (FDG) has revolutionized the imaging of oncology patients by adding 3D functional and anatomic imaging data [4–6]. However, FDG is not specific for malignant transformation [5], and alternative specific tumor biomarkers would be highly valuable. In the current study, we have developed a highly versatile method for loading liposomes with copper-radionuclides applicable for PET imaging. Liposomes have only recently been investigated as diagnostic probes for disease imaging and as radiotherapeutic agents for tumor and metastases targeting, even though liposome based chemotherapeutics reached the market 15 years ago [7] with considerable success. Copper-based radionuclides are currently being evaluated as ideal radioisotopes for PET imaging (^{64}Cu and ^{61}Cu) [8,9] and radiotherapy (^{67}Cu and ^{64}Cu) [8,10,11], and the well-established coordination chemistry of copper allows for utilization of a variety of chelator systems that can be linked to biologically relevant molecules [8]. PET imaging is now routinely used in the clinic, but it will not reach its full potential in oncology before significant advances are made with longer-lived PET isotopes, such as ^{64}Cu [12]. ^{64}Cu is one of the few useful metallic positron-emitting radionuclides with a relatively long half-life (12.7 h), permitting studies to be performed for as long as 48 h after administration [9]. Moreover, because ^{64}Cu has fairly low maximum positron energy (0.66 MeV) and short positron range, similar to ^{18}F, the resulting PET images are of high quality [9]. Compared to single photon emission computed tomography (SPECT), PET has higher detection sensitivity measuring biomarker concentrations as low as 1×10^{-12} mol per litre, and has higher spatial resolution [2,5,13]. Dynamic imaging and mathematically constructed time-activity data are also possible with PET [2,5] using the annihilation coincidence detection (ACD) technique. Furthermore, the sensitivity of PET is approximately 10^6 times higher than magnetic resonance imaging (MRI). Another strong advantage of PET is the ability to calculate the amount of radioactivity specifically sequestered in a region of interest (ROI), allowing direct quantitative determinations based on differences in signal intensity [2,14,15].

When liposomes are utilized for *in vivo* imaging, the radionuclide can be entrapped in the aqueous core of the liposomes, using

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a method called “remote loading” or “after loading”. This method can provide high loading efficiency (>90%) and high *in vivo* stability, and is superior to other labelling approaches [16–19] due to the protected location of the radionuclide inside the liposome and the higher concentration of radionuclide per liposome. To date, remote loading approaches have been developed with different lipophilic transporters (ionophores), e.g. oxine [20], A23187 (calcimycin) [21] and hexamethylpropylene amine oxime (HMPAO) [22] to load preformed liposomes with ^{111}In, ^{177}Lu, ^{99m}Tc and ^{67}Ga, radioisotopes for SPECT applications. However, no remote loading of positron-emitters into liposomes has been reported even though the value of such imaging agents has been described in multiple papers [23,24].

The goal of this study was to develop an efficient remote loading of the PET radionuclide ^{64}Cu into liposomes. The efficiency of the method was measured as loading efficiency and retention stability of ^{64}Cu in liposomes, and the remote loading efficiency of 2-hydroxyquinoline was compared to other ionophores. A comparison of different chelators, temperatures and pH conditions was conducted to optimize the remote loading method. These studies were performed using isothermal titration calorimetry (ITC). In addition, the *in vivo* performance of ^{64}Cu-liposomes as a PET tracer was evaluated. ^{64}Cu-liposomes were administered to healthy and tumor-bearing mice, and were analyzed where biodistribution and tumor accumulation was quantified directly by PET/CT imaging.

2. Materials and methods

2.1. Preparation of liposomes

PEGylated liposomes consisting of 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), cholesterol (Chol) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy (polyethylene glycol)-2000] (DSPE-PEG$_{2000}$) in the molar ratio 55:40:5, liposomes consisting of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), liposomes consisting of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and liposomes consisting of 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) were prepared using the standard thin-film hydration followed by repeated extrusions through polycarbonate membranes with pore sizes of 100 nm to generate small unilamellar liposomes. The chelating agent, 1,4,7,10-tetra-azacyclododecane-1,4,7,10-tetraacetic acid (DOTA) was dissolved in HEPES buffer (10 mM pH 7.4, 150 mM NaCl) adjusted to pH 4.0 with a final DOTA concentration of 10 mM. DOTA was trapped within the liposomes during the thin-film hydration and residual DOTA was removed by size exclusion chromatography (SEC) (Sephadex G-25) eluted with HEPES buffer (10 mM pH 7.4, 150 mM NaCl). All lipids were purchased for Avanti Polar Lipids, DOTA was purchased from Macrocyclics, and all other chemicals from Sigma Aldrich.

2.2. ^{64}Cu production

Copper-64 was produced on a PETtrace cyclotron (GE Healthcare) equipped with a beamline. The production of ^{64}Cu was carried out via the ^{64}Ni(p,n)^{64}Cu nuclear reaction using a solid target system consisting of a water cooled target mounted on the beamline. The target consisted of ^{64}Ni metal (enriched to >99%) electroplated on a silver disc backing. A proton beam of 16 MeV and a beam current of 20 μA were used. After irradiation, the target was transferred to the laboratory for further chemical processing where ^{64}Cu was isolated using ion exchange chromatography. Final evaporation from aqueous HCl yielded 2–6 GBq ^{64}Cu as ^{64}CuCl$_2$.

2.3. Remote loading of ^{64}Cu into liposomes

A total of 10 μL 2-hydroxyquinoline (0.314 mM) (Sigma Aldrich) in HEPES buffer (10 mM pH 7.4, 150 mM NaCl) was added to a dried vial containing radioactive ^{64}CuCl$_2$ (~150 MBq). Immediately hereafter, 500 μL DOTA-containing liposomes were added followed by incubation. The loading was successful using incubation times varying from 30 to 60 min at 20–50 °C. The loaded ^{64}Cu-liposomes were assayed by separating the un-entrapped ^{64}Cu from ^{64}Cu-liposomes by size exclusion chromatography (SEC) on a Sephadex G-25 packed 1 × 25 cm column, eluted with HEPES buffer (10 mM pH 7.4, 150 mM NaCl). For the purpose of calculating the loading efficiency, the ^{64}Cu-liposomal fraction collected between 20 and 70 min was expressed as a percentage of the total radioactivity collected between 20 and 100 min. The elution profile was monitored on an in line radioactivity detector. The remote loading was also possible with higher radioactivity (100–400 MBq), with different liposome concentrations (1–50 mM) and different liposomal compositions (PEGylated liposomes, POPC, DPPC and DSPC liposomes).

2.4. Isothermal titration calorimetry

In the search of the optimal ionophore and chelator for the remote loading of Cu(II) into liposomes, isothermal titration calorimetry (ITC) was employed. The exchange of Cu(II) ions, between the ionophore and the chelator, was measured at two different temperatures (25 °C and 50 °C) and two different pH (pH 4.0 and pH 5.9) in MES buffer (10 mM pH 5.9, 150 mM $NaNO_3$) and acetate buffer (10 mM pH 4.0, 150 mM $NaNO_3$) using an isothermal titration calorimeter (iTC200, MicroCal, GE). The experiments were carried out by injecting buffered solutions of the chelator into a buffered solution of Cu (2-hydroxyquinoline)$_2$ using a 40 μL syringe for the titrant. Mixing was effected by stirring the syringe at 1000 rpm during equilibration and experiment. The following chelators were tested; DOTA, 1,4,7,10-tetra-azacyclododecane-1,4,7,10-tetra (methylene phosphonic acid) (DOTP), 1,4,7,10-tetra-azacyclododecane (cyclen), 1,4,8,11-tetraazacyclotetradecane (cyclam) and 1,4,8,11-tetraazacyclo-dodecane-1,4,8,11-tetraacetic acid (TETA). For experiments, 19 injections of 2 μL each were performed with a 150 s interval between injections. All solutions were degassed prior to use. The heats of ligand exchange (Q_i) were determined by integration of observed peaks. The heat of dilution was measured and subtracted during the data fitting procedure. Modelling the heat of the *i*'th injection was done by:

$$Q_i = \Delta H \cdot V_{cell} \cdot C_{Cu(2HQ)_2} \cdot (\xi_i - \xi_{i-1}) + q_{dil} \quad (1)$$

where ΔH is the molar enthalpy of the ligand exchange reaction, equation (2), $V_{cell} = 204$ μL is the volume of the ITC cell, $\xi \in [0;1]$ is the degree of conversion of the ligand exchange reaction, equation (2), and q_{dil} is the heat of dilution. In the following equations (2–4) DOTA serves as an example of a chelator. The ligand exchange reaction, equation (2), is defined by the equilibrium:

$$Cu(2HQ)_2 + DOTA \rightleftarrows CuDOTA + 2(2HQ) \quad (2)$$

The ligand exchange constant (K) is defined as:

$$K = \frac{C_{CuDOTA} \cdot C_{2HQ}^2}{C_{Cu(2HQ)_2} \cdot C_{DOTA} \cdot C_w} \quad (3)$$

where 2HQ is 2-hydroxyquinoline and C_{CuDOTA}, C_{2HQ}, $C_{Cu(2HQ)_2}$, C_{DOTA} and C_w are the molar concentrations of CuDOTA, 2HQ, Cu(2HQ)$_2$, DOTA and water respectively.

The ligand exchange constant (K), equation (3), was obtained by fitting equation (1) to the experimentally acquired heats of reaction by least-square minimization (utilizing methods from Numerical Recipes), and ξ was calculated by solving:

$$\left(\frac{4 \cdot C_{Cu(2HQ)_2}^2}{K \cdot C_w}\right) \cdot \xi^3 - C_{Cu(2HQ)_2} \cdot \xi^2 + \left(C_{Cu(2HQ)_2} + C_{DOTA}\right) \cdot \xi - C_{DOTA} = 0 \quad (4)$$

2.5. Characterization and stability of liposomes

Liposome preparations (before and after ^{64}Cu-loading) were analyzed on size and zeta-potential (ZetaPALS, Brookhaven, SE). Purified ^{64}Cu-liposome solution was tested for radionuclide retention stability by incubating for 24 h at 37 °C. The retention stability was assayed by separating free ^{64}Cu from ^{64}Cu-liposome by SEC. Additionally stability in human serum 37 °C for 24 h was tested by mixing human serum and ^{64}Cu-liposome (1:1).

2.6. Administered doses in the in vivo studies

Administered dose levels of ^{64}Cu-liposomes were 0.24 ± 0.04 μmol lipid animal^{-1} with a specific activity of 36.0 ± 2.7 MBq μmol lipid^{-1} (11.2 ± 2.6 MBq animal^{-1}), and 22.5 nmol DOTA mouse^{-1} (12.3 ± 2.6 MBq animal^{-1}) for ^{64}Cu-DOTA.

2.7. Micro-PET/CT imaging

Positron emission tomography (PET) data were acquired on a MicroPET® Focus 120 (Siemens Medical Solutions, Malvern, PA, USA). The voxel size was 0.866 × 0.866 × 0.796 mm^3 and in the centre field of view the resolution was 1.4 mm. Data were reconstructed with the maximum a posterior (MAP) reconstruction algorithm. For anatomical localization of activity, computer tomography (CT) images were acquired with a MicroCAT® II system (Siemens Medical solutions, Malvern, PA, USA) with X-ray tube settings of 60 kVp and 500 μA. CT images were acquired in a 7-min scan with 360 rotation steps, a 310 ms exposure time and voxel size of 0.095 × 0.095 × 0.095 mm^3. After data reconstruction PET- and CT images were fused with Inveon Software (Siemens), and PET data were analyzed by defining regions of interest (ROIs) on selected tissues. PET values are expressed as mean Becquerel ml^{-1} (Bq ml^{-1}). Multiplanar- and 3D reconstructions were obtained with Inveon Software (Siemens).

2.8. Animal models

Human colon adenocarcinoma (HT29) tumor cells (~2 × 10^6 cells) were inoculated in the left and right flank of female NMRI (Naval Medical Research Institute) nude mice ($n = 8$) and allowed to grow 4 weeks in the mice. ^{64}Cu-liposome and