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A robust and quantitative method for tracking liposome contents after intravenous administration

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ABSTRACT

We introduce a method for tracking the rate and extent of delivery of liposome contents *in vivo* based on encapsulation of 4-methylumbelliferyl phosphate (MU-P), a profluorophore of 4-methylumbelliferone (MU). MU-P is rapidly dephosphorylated by endogenous phosphatases *in vivo* to form MU after leakage from the liposome. The change in fluorescence spectra when MU-P is converted to MU allows for quantification of entrapped (MU-P) and released (MU) liposome contents by fluorescence or by a sensitive high performance liquid chromatography assay. We define the "cellular availability" of an agent encapsulated in a liposome as the ratio of the amount of released agent in the tissue to the total amount of agent in the tissue; this parameter quantifies the fraction of drug available for therapy. The advantage of this method over existing technologies is the ability to decouple the signals of entrapped and released liposome contents. We validate this method by tracking the circulation and tissue distribution of MU-P loaded liposomes after intravenous administration. We use this assay to compare the cellular availability of liposomes composed of engineered phosphocholine lipids with covalently attached cholesterol, sterol-modified lipids (SML), to liposomes composed of conventional phospholipids and cholesterol. The SML liposomes have similar pharmacokinetic and biodistribution patterns as conventional phospholipid–cholesterol liposomes but a slower rate of contents delivery into the tissue. Thus, MU-P enables the tracking of the rate and extent of liposome contents release in tissues and should facilitate a better understanding of the pharmacodynamics of liposome-encapsulated drugs in animals.

1. Introduction

A quantitative understanding of where and when liposome encapsulated agents are released *in vivo* is critical in the rational design of liposomes for drug therapy. While liposomes may accumulate at the target site, only drug released from the liposome, "cellular available" drug, has biological activity. Drug trapped in the liposome has little therapeutic consequence as illustrated by the poor performance of liposome-encapsulated cis-platinum in humans [1]. Liposomal therapeutics should be optimized to release their payload over a timescale defined by the pharmacology of the payload and the biology of the therapeutic target. While there are several methods to quantify liposome pharmacokinetics and assess accumulation of liposomes and payload in target tissues, few approaches can differentiate the signals of the encapsulated and released payload. This paucity of experimental approaches to measure cellular availability has limited the optimization of liposomes for drug delivery.

Microscopic, radioactive, magnetic resonance, and fluorescent tracers have been the principal tools for tracking liposomes *in vivo* [2]. Microscopy studies have demonstrated the cellular compartmentalization of liposomes and established the reticuloendothelial system (RES) as a mediator of liposome clearance [3]. Encapsulated radioactive tracers or iodinated lipid markers have confirmed that the liver, spleen, bone marrow, and tumor are the primary sites of liposome accumulation *in vivo* [4–8]. However, these studies have provided little insight into the release of liposomal payloads in tissues. Results that rely on bilayer embedded or encapsulated fluorescent tracers such as carbocyanine dyes [9] or fluorescence resonance energy transfer (FRET) pairs [10], can be confounded due to exchange of the probe into lipoproteins and cell membranes [11]. Encapsulation of self-quenching fluorescent compounds, such as carboxyfluorescein (CF) [12] and doxorubicin [13–15], or fluorophore-quencher pairs [16] is useful for measuring entrapped and released contents in plasma samples, but physical and chemical tissue homogenization steps that disrupt the lipid bilayer limit the ability of these probes to report on the cellular availability.

A small number of studies have focused on decoupling the signals of entrapped and released liposome contents in tissues [17–20]. Laginha and colleagues approximated the fraction of leaked doxorubicin by measuring doxorubicin in tumor nuclei and assuming that all released

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drug is bound to DNA [18]. However, this approach is specific for the *in vivo* disposition of doxorubicin crystallized in the liposome and reliant on the drug's interactions with DNA. The Baldeschwieler group used perturbed angular correlation spectroscopy to quantify entrapped and released ^{111}In [20]. While safe and broadly applicable, this method is limited by its sensitivity. Previously, our group quantified the cellular availability of liposomal contents using a dual radiolabeled reporter system: [^{51}Cr]EDTA and [^{22}Na] [21]. While [^{22}Na] is exported by the cell, [^{51}Cr] is not, and the ratio of the two components measures the liposome cellular availability. While promising, this method has proven to be too complicated for widespread use. Taken together, these studies show that there is a need for quantitative methods to distinguish between entrapped and released liposomal contents in tissues.

We developed a broadly applicable and sensitive method for tracking liposome cellular availability *in vivo* in which 4-methylumbelliferyl phosphate (MU-P), a water soluble profluorophore of 4-methylumbelliferone (MU) is encapsulated in liposomes (Fig. 1) [22]. Release of this compound from liposomes *in vivo* results in its rapid dephosphorylation to form MU (Fig. 2A); MU, MU metabolites and MU-P can then be quantified by fluorescence or by high-performance liquid chromatography (HPLC). This method allows researchers to obtain a new level of granularity when investigating liposome biodistribution.

We use this method to determine if restricting the transfer of cholesterol out of the liposome bilayer reduces the release of liposome contents in tissues. We used liposomes composed of sterol-modified lipids (SML), in which cholesterol is covalently attached to the phosphoglycerol backbone in place of an acyl chain [23,24]. We compare pharmacokinetics, biodistribution, and cellular availability of SML to that of liposomes composed of conventional phospholipids and cholesterol (CPL) and find that while SML and CPL have similar pharmacokinetic profiles; MU-P encapsulated in SML has greater accumulation and longer persistence in the liver and spleen. This indicates that at certain chain lengths SML release contents slower than CPL.

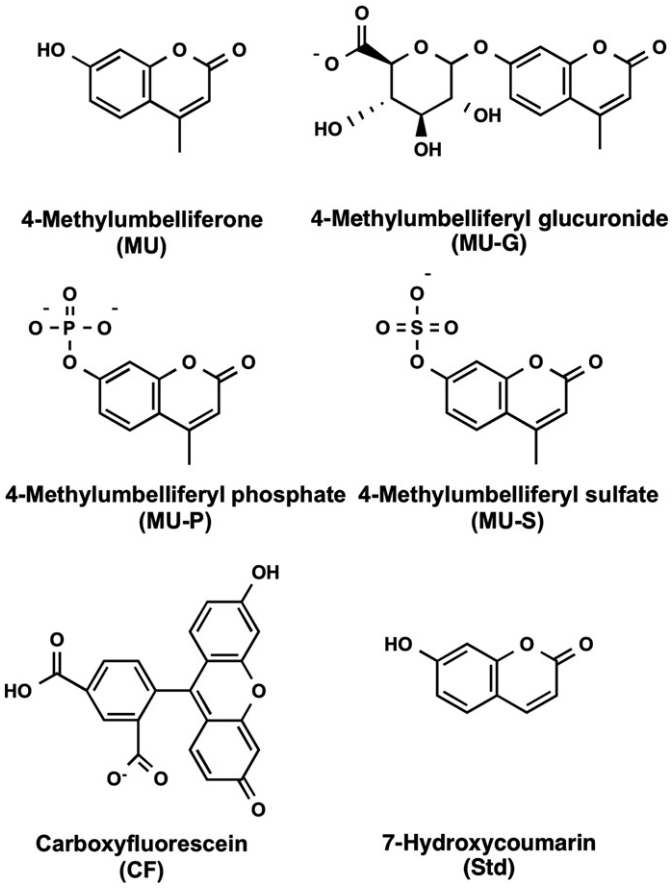

Fig. 1. *Relevant Structures.* Structures of 4-methylumbelliferone (MU), MU metabolite 4-methylumbelliferyl glucuronide (MU-G), MU prodrugs 4-methylumbelliferyl phosphate (MU-P) and 4-methylumbelliferyl sulfate (MU-S), carboxyfluorescein (CF), and HPLC standard 7-hydroxycoumarin (Std).

2. Materials and methods

2.1. Lipids

1,2-dilauroyl-*sn*-glycero-3-phosphocholine (C_{12} PC), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (C_{14} PC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (C_{16} PC), L-α-phosphatidylcholine hydrogenated (C_{18} PC), 1,2-diarachidoyl-*sn*-glycero-3-phosphocholine (C_{20} PC), 1,2-dibehenoyl-*sn*-glycero-3-phosphocholine (C_{22} PC) 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine ($C_{18:1}$ PC), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dicholesterylhemisuccinoyl-*sn*-glycero-3-phosphocholine (DCHEMSPC), 1-lauroyl-2-cholesterylhemisuccinoyl-*sn*-glycero-3-phosphocholine (C_{12} SML), 1-myristoyl-2-cholesterylhemisuccinoyl-*sn*-glycero-3-phosphocholine (C_{14} SML), 1-palmitoyl-2-cholesterylhemisuccinoyl-*sn*-glycero-3-phosphocholine (C_{16} SML), 1-stearoyl-2-cholesterylhemisuccinoyl-*sn*-glycero-3-phosphocholine (C_{18} SML), 1-rachidoyl-2-cholesterylhemisuccinoyl-*sn*-glycero-3-phosphocholine (C_{20} SML), 1-behenoyl-2-cholesterylhemisuccinoyl-*sn*-glycero-3-phosphocholine (C_{22} SML) 1-oleoyl-2-cholesterylhemisuccinoyl-*sn*-glycero-3-phosphocholine ($C_{18:1}$ SML), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG), and cholesterol were purchased from Avanti Polar Lipids, kindly donated by Dr. Zhaohua Huang, or synthesized as previously described [23,24].

2.2. Materials and instrumentation

MU, MU-P, MU-S, MU-G and 7-hydroxycoumarin were obtained from Sigma. Solvents were removed under reduced pressure using a rotary evaporator. Average liposome diameter and zeta potential measurements were determined using the Zetasizer Nano ZS (Malvern Instruments). Fluorescence spectroscopy was measured on a FluoroLog-3 spectrofluorimeter (Horiba Jobin Yvon) equipped with a temperature-controlled stage (LFI-3751) or using a Tecan Infinite 4300 (Tecan Group Ltd). Data acquisition was done through FluorEssence software (Horiba Scientific). High-pressure liquid chromatography (HPLC) was performed on an Agilent 1100 HPLC (Agilent).

2.3. Serum conversion of MU prodrugs

MU-P or MU-S were dissolved in phosphate buffered saline (2.7 mM KCl, 1.5 mM KH_2PO_4, 136.9 mM NaCl, 8.9 mM Na_2HPO_4, pH 7.4; PBS) and incubated with 50% mouse serum at 37 °C. Prodrug conversion to MU was monitored by measuring MU fluorescence every 5 min over 12 h. Data was fit to a Michaelis–Menten model using GraphPad Prism.

2.4. Liposomes for contents leakage

Lipids were dissolved in chloroform, dried to form a thin film, and placed under high vacuum overnight. Lipid mixtures included Diacyl:Chol:PEG (55:40:5), Diacyl:SML:PEG (55:40:5), SML:PEG (95:5), and DiChems:Diacyl:PEG (20:75:5) with acyl chain lengths of C_{12}–C_{22}. The films were re-hydrated with 1 mL CF (50 mM) or 1 mL MU-P (300 mM) in HEPES buffered saline (10 mM HEPES, 140 mM NaCl, pH 7.4; HBS) at 60 °C and vortexed to obtain a lipid concentration of 5 mM. The liposomes were sonicated at 60 °C until opalescent (~10 min) and extruded through 200 nm and 100 nm polycarbonate membranes at 60 °C. Liposomes were purified on a Sephadex G-25 size exclusion column. In order to quantify CF release from liposomes, CF fluorescence (excitation 492 nm, emission 517 nm) was measured