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Lipid–calcium phosphate nanoparticles for delivery to the lymphatic system and SPECT/CT imaging of lymph node metastases

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ABSTRACT

A lipid/calcium/phosphate (LCP) nanoparticle (NP) formulation (particle diameter ~25 nm) with superior siRNA delivery efficiency was developed and reported previously. Here, we describe the successful formulation of ^{111}In into LCP for SPECT/CT imaging. Imaging and biodistribution studies showed that, polyethylene glycol grafted ^{111}In-LCP preferentially accumulated in the lymph nodes at ~70% ID/g in both C57BL/6 and nude mice when the improved surface coating method was used. Both the liver and spleen accumulated only ~25% ID/g. Larger LCP (diameter ~67 nm) was less lymphotropic. These results indicate that 25 nm LCP was able to penetrate into tissues, enter the lymphatic system, and accumulate in the lymph nodes *via* lymphatic drainage due to 1) small size, 2) a well-PEGylated lipid surface, and 3) a slightly negative surface charge. The capability of intravenously injected ^{111}In-LCP to visualize an enlarged, tumor-loaded sentinel lymph node was demonstrated using a 4T1 breast cancer lymph node metastasis model. Systemic gene delivery to the lymph nodes after IV injection was demonstrated by the expression of red fluorescent protein cDNA. The potential of using LCP for lymphatic drug delivery is discussed.

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1. Introduction

The lymphatic system is a central component of the immune system and serves as the secondary circulation system responsible for the drainage of fluid, proteins, and waste products from tissues into the blood. Lymph nodes also play an important role in infectious disease, inflammation, and cancer [1]. Primary tumors commonly invade draining lymph nodes, which then serve as a reservoir for further metastatic spread of cancer cells [2–4].

The delivery of genes and drugs to both local, draining lymph nodes and the lymphatic system as a whole is a challenging task. Certain lipophilic compounds such as long-chain fatty acids, cholesterol esters, triglycerides, and lipid-soluble vitamins can be transported through the lymphatic channels [1,5]. However, most chemotherapy agents do not gain access to the lymphatic system and lymph node metastases after conventional intravenous (IV) infusion [1,5]. Consequently, the development of clinical chemotherapy for lymph node metastasis has remained elusive.

Many different types of nanoparticle (NP), including liposomes, silica NPs, and other polymer-based drug delivery systems, have exhibited improved efficiency in regionally delivering drugs to the lymphatic system [6–10]. For example, intraperitoneally (IP) injected liposomes containing doxorubicin result in an 8- to 14-fold (4 h post injection) and a 3- to 6-fold (24 h post injection) increase in doxorubicin concentration in the draining lymph nodes in rats compared to free doxorubicin [6]. However, no significant difference was observed after IV administration. Thus, methods for effective delivery of IV administered NP to the lymphatic system are still needed for the detection of lymph node metastasis.

Non-invasive imaging techniques using nuclear medicine, such as single photon emission computer tomography (SPECT) and positron emission tomography (PET) have been developed [11,12]. Among the radionuclides used in clinical practice, ^{111}In is the second most widely used radionuclide in clinical practice, surpassed only by ^{99m}Tc. ^{111}In displays major decay at photon energy levels of 171.3 and 245.4 keV, within the ideal range of the detector device. The half-life of ^{111}In (2.83 days) is also advantageous because prolonged exposure to the radionuclide may cause undesired toxicity and should be prevented. Many studies have demonstrated *in vivo* imaging of tumors with various types of NPs using SPECT/CT or PET/CT technique [12–14].

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Lipid/calcium/phosphate (LCP) NPs were first developed for siRNA delivery [15–17] and can also successfully deliver plasmid DNA to hepatocytes [18]. Based on the mechanism of formation of the CaP core, we hypothesized that any drug or radionuclide that can form co-precipitates with CaP has the potential to be formulated into LCP. Since indium (In) can form precipitates with phosphate very efficiently (solubility product constant, Ksp, of $InPO_4 = 2.3 \times 10^{-22}$) in a manner similar to that of calcium (Ksp of $Ca_3(PO_4)_2 = 1.0 \times 10^{-25}$), we hypothesize that ^{111}In will be a good candidate to add to LCP formulations to provide *in vivo* imaging capability.

Systemic accumulation of NPs in the lymph nodes after IV administration has rarely been reported. A 25 nm, dextran-coated, ultrasmall super-paramagnetic iron oxide (USPIO, Feridex®) NP has shown systemic lymphotropism after IV administration and has been evaluated for MRI of clinical lymph node metastasis [19–22]. However, larger iron oxide NPs coated with the same dextran coating were found to preferentially accumulate in the liver and spleen [19]. Another self-luminescing, near-infrared semiconductor polymer NP (30–40 nm) capable of accumulating in the lymph node at a certain level has been described recently for lymph node mapping and tumor imaging [23]. However, quantitative data showing the level of accumulation is missing and liver showed the highest accumulation according to near-infrared fluorescent imaging.

Other NPs of similar size (25–30 nm) or smaller may exhibit prolonged circulation time in the blood. No preferential accumulation in lymph nodes has been reported, however. For example, one recent publication reported that PET imaging demonstrated a long-circulating, 15 nm, micellar NP had minimal accumulation in the liver and spleen, with ~6% ID/g tumor accumulation. However, this micellar NP did not show lymphotropism [24]. Another recent publication reported 30 nm Au nanocages for use in tumor PET imaging also exhibit minimal accumulation in the liver and spleen but no lymphotropism [25]. Thus, NP lymphotropism may be correlated not only with NP size but also other surface properties.

In this paper, we demonstrate that LCP were able to achieve both systemic delivery of genes to the lymphatic system and imaging of lymph node metastasis by a mechanism similar to the aforementioned Feridex®. The systemic lymphotropism of LCP and Feridex® was achieved by tissue penetration and an atypical lymphatic drainage mechanism that is different from phagocytic uptake mechanisms used by locally injected NPs to accumulate at regional draining lymph nodes. Coating of the NP surface with a lipid bilayer or dextran may contribute to the unusual lymphotropism.

2. Materials and methods

2.1. Materials

Cholesterol, 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), dioleoylphosphatydic acid (DOPA), 1,2-Dioleoyl-3-trimethylammonium-propane chloride salt (DOTAP), 1,2-distearoryl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethyleneglycol-2000) ammonium salt (DSPE-PEG2000), and 1-oleoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino] dodecanoyl]-*sn*-glycero-3-phosphocholine (NBD-PC) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). ^{111}InCl in 0.05 N HCl was purchased from PerkinElmer (Waltham, MA). Double-strand oligo DNA (sense sequence, 5′-CAAGGGACTGGAAGGCTGGG-3′) and Texas-Red labeled sense-strand oligo DNA (sequence: 5′-[TxRd]CAAGGGACTGGAAGGCTGGG-3′) were both synthesized by Sigma–Aldrich (St. Louis, MO). ^{3}H labeling of oligonucleotides was prepared by hydrogen exchange with $^{3}H_2O$ at the C8 positions of the purines [26]. 4T1-luc2-GFP Bioware® Ultra Green) cell line was purchased from Caliper (Hopkinton, MA). CR8C peptide was synthesized by Peptide 2.0 Inc. (Chantilly, VA). Other materials were obtained from Sigma–Aldrich (St. Louis, MO).

2.2. The preparation of ^{111}In-LCP core

^{111}In-LCP core was prepared using a previously described method [15–17] with some modifications (Supplementary Fig. 1). Two water-in-oil microemulsions were prepared: 1) a calcium emulsion: $^{111}InCl_3$ was premixed with $CaCl_2$ to make a final 50 μL of 500 mM $CaCl_2$ in 4 mL of cyclohexane oil phase (cyclohexane/Igepal CO-520 = 71/29, v/v), and 2) a phosphate emulsion: a sufficient amount of 0.05 N NaOH was added to pH 9.0 Na_2HPO_4 (to neutralize the extra HCl in the calcium emulsion) to make final 50 μL of 100 mM Na_2HPO_4 also in 4 mL of cyclohexane oil phase. DOPA (92.5 μL 34.6 mM in chloroform) as the inner leaflet lipid was also added in the phosphate emulsion.

After mixing the two microemulsions for 40 min, 8 mL of absolute ethanol was added to the micro-emulsion to break the microemulsion system. The mixture was stirred for another 30 min. Then, the mixture was centrifuged at 12,500× *g* for 15 min to collect the ^{111}In-LCP cores. The cores were washed once with 10 mL absolute ethanol to remove residual surfactants. Then, the cores were washed with 1.2 mL cyclohexane and mixed with 1.4 mL absolute ethanol to remove any residual DOPA. Finally, the cores were washed with 2 mL of absolute ethanol to ensure the removal of cyclohexane. After all washes, the pellets were dispersed in 250 μL chloroform. The product was centrifuged at 10,000× *g* for 5 min. Precipitate containing excess salts and aggregates was discarded and the supernatant containing LCP cores was collected and stored in a glass vial at −20 °C.

2.3. Outer leaflet coating

For outer leaflet coating, 100 μL of 20 mM cholesterol, 100 μL of 20 mM DOPC, and 50 μL of 20 mM of DSPE-PEG_{2000} (all in $CHCl_3$) were mixed with the LCP cores in a glass vial. After complete removal of the $CHCl_3$ by using a stream of nitrogen and vacuum desiccation for 1 h, the cores were first suspended in 100 μL of pre-warmed absolute alcohol (55 °C) and then dispersed in total 1 mL pre-warmed aqueous solution containing 5% dextrose.

2.4. Sucrose gradient centrifugation

Sucrose gradient centrifugation assays were performed using NBD-PC, which had a structure similar to that of DOPC, to mix with DOPC at 1% of the total DOPC to label the outer leaflet of LCP with fluorescence. The outer leaflet coated LCP was loaded into a tube containing a sucrose density gradient ranging from 0% to 60% (w/w). After ultra-centrifugation at 337,000× *g* for 4 h, excess lipids that were not associated with the LCP floated to the upper part of the gradient and were separated from the dense LCP which formed a sharp band above the 60% layer in the gradient.

2.5. Tuning ^{111}In-LCP NP size by adjusting surfactant system

To tune the ^{111}In-LCP core size, a Triton surfactant system (cyclohexane/hexanol/Triton X-100 = 75/10/15, v/v/v) was mixed with the original Igepal surfactant system (cyclohexane/Igepal CO-520 = 71/29, v/v). As the portion of the Triton surfactant system increased, the size of ^{111}In-LCP cores could be enlarged to ~50 nm, making the final larger-LCP (L-LCP) around 65 nm in diameter.

2.6. Particle size and zeta potential analysis

Particle size and zeta potential of LCP NPs were determined using a Malvern ZetaSizer Nano series (Westborough, MA). Before measurement, LCP NPs were purified using sucrose gradient centrifuge method to remove excess lipids. Collected NPs were measured in water using protocols suggested by the manufacturer.

2.7. TEM microscopy and sample preparation

LCP cores suspended in chloroform was carefully applied on the formvar/carbon coated copper 200 mesh grid and allowed to dry for 10 min at room temperature. The volume of chloroform used should be as small as possible (~1 μL) to prevent dissolution of the formvar/carbon film. For outer leaflet coated LCP in 5% dextrose solution, 10 μL of the final LCP solution was applied on the formvar/carbon coated copper 200 mesh grid. The LCP was allowed to settle on the grid for 10 min. Then the remaining LCP solution was carefully removed by using a Kimwipes wiper. Another 10 μL of water was used to rinse the grid to prevent formation of dextrose crystals. After the 10 μL of water was removed carefully by using Kimwipes wiper, the grid was placed in a dust-free area to allow complete dryness for at least 30 min before observation using a TEM microscopy (JEOL 100 CS II).

2.8. Determining siRNA entrapment efficiency

To ensure modifications would not compromise the ability to encapsulate siRNA, 35 μg total double-stranded oligo DNA (as surrogate to siRNA) including 10 μg of ^{3}H-labeled DNA oligo was added to the calcium emulsion during ^{111}In-LCP preparation. After the LCP core preparation, oligo entrapment efficiency was determined using liquid scintillation counting for ^{3}H.

2.9. In vivo SPECT/CT imaging and biodistribution study

All animal work was performed in accordance with and approved by the University of North Carolina Institutional Animal Care and Use Committee guidelines. Athymic nude (nu/nu) mice and wild type C57BL/6 mice purchased from National Cancer Institute were used. SPECT/CT experiments were performed using a GE eXplore speCZT system. A mouse 7-pin-hole collimator was used for high resolution SPECT imaging. Each mouse was injected through the tail vein with 200 μL of the final ^{111}In-LCP containing ~0.5 mCi of ^{111}In. Mice were anesthetized with isoflurane