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Ultrasonic gene and drug delivery using eLiposomes

Marjan Javadi [a], William G. Pitt [a,*], Christopher M. Tracy [b], Jeffery R. Barrow [c], Barry M. Willardson [b], Jonathan M. Hartley [a], Naakaii H. Tsosie [a]

[a] Department of Chemical Engineering, Brigham Young University, Provo, UT, 84602, USA
[b] Department of Chemistry and Biochemistry, Brigham Young University, Provo, UT, 84602, USA
[c] Department of Physiology and Developmental Biology, Brigham Young University, Provo, UT, 84602, USA

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ABSTRACT

eLiposomes are liposomes encapsulating emulsions and therapeutics for targeted delivery. By applying ultrasound to eLiposomes, emulsion droplets can transform from liquid to gas and rupture the lipid bilayer of the eLiposome to release a drug or plasmid. In this study, perfluoropentane (PFC5) emulsions were encapsulated inside folated eLiposomes carrying a model drug (calcein) or a model GFP plasmid to examine the effects of a folate ligand, PFC5 emulsion and various ultrasonic acoustic parameters in drug delivery and gene transfection into HeLa cells.

Confocal microscopy was used to quantify drug delivery and the level of plasmid transfection into HeLa cells. The results showed that drug delivery or transfection was minimal without incorporation of internal PFC5 emulsions and folate ligand on the eLiposome surface. It was also shown that application of ultrasound greatly enhanced the drug delivery and plasmid transfection. Delivery of these therapeutics appears to be to the cytosol, indicating that the expansion of the emulsion droplets disrupted both the eLiposomes and the endosomes.

1. Introduction

The rapid advancement of nanotechnology has greatly benefited drug and gene delivery, since nano-sized carriers can be designed to deliver both small molecules and macromolecules. One of the main goals of nanotechnology in drug delivery is to encapsulate drugs or genes into a biocompatible and biodegradable carrier, and then deliver the contents to targeted locations in the body with active, passive and triggered targeting [1–4]. These carriers have included micelles, polymer conjugates, nanocapsules and nanoparticles [5–9].

Drugs and/or genes can be delivered actively or passively. In passive delivery, carriers accumulate within tumors that have leaky vasculature [10]. This phenomenon is generally called the enhanced permeation and retention (EPR) effect. Active targeting occurs through specific binding interactions between the carrier and the cell. Ligands and receptors play a key role in active delivery to the cells. Furthermore the sub-micron size of the nanovehicles can allow them to be taken into the cells *via* endocytosis, particularly if they have ligands that induce endocytosis [11]. In an ideal scenario, nanocarriers passively enter tumor tissue *via* the EPR effect, bind to selected tumor cells *via* specific ligands, and are endocytosed into tumor cell. All that remains is to actuate the release of the drug from the carrier once it has been endocytosed.

One method to assist release from a carrier and to promote uptake by cells involves ultrasonically induced cavitation of bubbles [5]. Microbubbles are used in both diagnostic and therapeutic applications because of their response to ultrasound. Some current therapeutic applications of bubbles are in drug delivery, gene delivery and thrombolysis [12]. Although ultrasound has been used to actuate drug delivery [13,14], the disadvantage of bubbles is that they are too large to extravasate into tumors *via* the EPR effect.

An alternative for pre-existing microbubbles is to use acoustic droplet vaporization (ADV), a phenomenon in which a small liquid emulsion droplet is converted to a much larger gas bubble by a pulse of ultrasound [15,16]. Perfluorocarbons (PFCs) have been proposed for medical ADV because PFCs are non-toxic and have low solubility in blood [17]. PFCs in the form of emulsions have been found useful as oxygen carriers in artificial blood [18] and as contrast agents in medical sonography [19]. When formulated properly and with sufficiently small size, PFC emulsions can remain stable as a liquid phase even at temperatures above their normal boiling points [15,20,21]. Such meta-stability is possible due to the droplet's highly curved interface that raises the internal pressure within the PFC emulsion droplet. Thus PFCs with low boiling points – such as perfluoropentane (PFC5) – could be used as a relatively stable emulsion in the human body even though its boiling point (29 °C) is lower than body temperature (37 °C).

* Corresponding author at: 350 Clyde Bldg., Brigham Young University, Provo, UT, 84602, USA. Tel.: +1 801 422 2589; fax: +1 801 422 0151.
E-mail address: pitt@byu.edu (W.G. Pitt).

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Upon insonation the emulsion droplets can change phase from liquid to gas, and thus can be used to mechanically trigger drug delivery. Many medical applications could take advantage of this phase change. For example, Fabiilli et al. reported that by using ultrasound frequencies above 1 MHz and applying microsecond pulse lengths, small PFC emulsion droplets were changed from liquid to gas [22].

Ultrasound itself has been shown to improve the efficiency of gene and drug delivery to cells [13]. Ultrasonic cavitation perturbs the cell membrane, making transient pores in the membrane that facilitates drug and gene delivery [23]. Microbubbles in the form of ultrasound contrast agents can enhance the efficacy of gene and drug delivery without damaging the cells [24]. However, to avoid cell damage, careful application of the optimum intensity and exposure duration is critical [25,26].

To actively direct a drug carrier to a tumor cell, folate is sometimes attached [27–29]. Folate receptors are expressed in limited amounts on normal cells but are present in large numbers on many cancer cells. Also, there is a correlation between the folate receptor expression and the stage of tumor development [30]. Drugs [28,31], gene therapy products [32], and radiopharmaceuticals [33] have better uptake when the delivery vehicle is decorated with folate. When folate is attached to a polyethylene glycol (PEG) tether, it can interact better with receptors in the cell membrane to enhance uptake of the therapeutic. For example, KB (human carcinoma of the nasopharynx) cells and HeLa (cervical cancer) cells, which both overexpress folate receptors, showed more uptake of doxorubicin compared to WI-38 (lung cancer) cells that do not overexpress folate receptors [31].

Calcein has often been employed as a model drug in drug delivery studies. Calcein is a fluorescent, hydrophilic molecule with low molecular weight that can be easily detected by fluorescence and confocal microscopy. Another advantage is that calcein is self-quenching at high concentrations. Thus vesicles carrying high local concentrations of calcein are not detectable by fluorescence microscopy; however, upon release from the carrier and subsequent dilution, the green fluorescence of calcein can be observed.

Gene therapy offers a promising alternative for the treatment of severe diseases and genetic disorders including cancer. Research on the delivery of plasmids has resulted in a variety of gene delivery vehicles. Recent studies involve the delivery of plasmid DNA for expressing therapeutic agents to block harmful genes and to restrict the activity of defective genes [34]. Ultrasound has been used as an effective tool for delivering plasmid DNA into cells [35].

Recently we have formulated an emulsion-containing liposome, which we call an eLiposome [36–38]. This novel nano-carrier can be used as both a gene and a drug delivery vehicle. It meets all requirements for our delivery scenario: it is small enough to be passively extravasated; it can be modified with folate or other ligands for active delivery and endocytosis; and it can release a therapeutic when triggered by ultrasound.

Fig. 1 illustrates an ideal eLiposome for targeted drug and gene delivery, which consists of a phospholipid bilayer surrounding a solution of drug or plasmid and emulsion droplet(s), and having active targeting ligands and stealth polymers outside [39]. We have shown that these eLiposomes can encapsulate and deliver calcein to the cells, and we will show herein that we can also deliver plasmids. When the eLiposomes bind to cancer cells with a targeting ligand and are endocytosed, application of ultrasound changes the phase of emulsion droplets from liquid to gas and causes the liposome bilayer to break and the endosome to rupture, thus releasing the plasmid or drug to the cell cytosol.

2. Materials and methods

Calcein was obtained from MP Biomedicals (Aurora, OH). Phosphate buffered saline (PBS) was purchased from Fisher Scientific (Fair Lawn, NJ). Sodium chloride and dimethyl sulfoxide (DMSO) were purchased from Mallinckrodt (Paris, Kentucky). Sucrose was purchased from Avantor Performance Materials (Phillipsburg, NJ) and glucose from United Biochemical Corp. (Cleveland, OH).

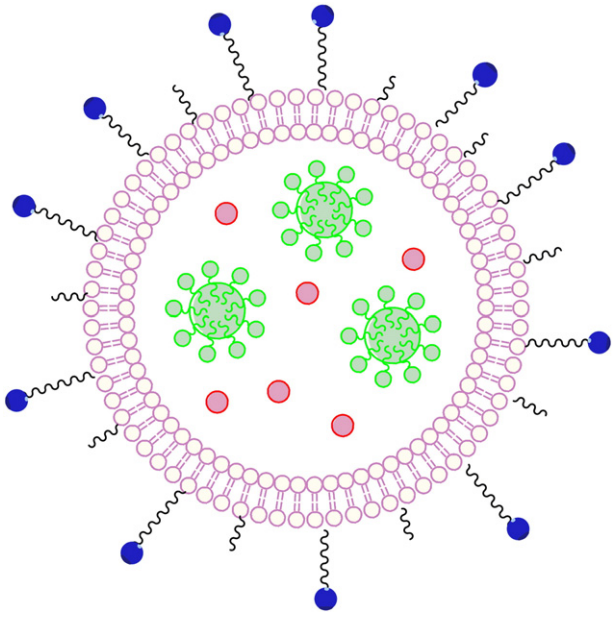

Fig. 1. Schematic of the ideal eLiposome. The lipid bilayer (pink) encapsulates emulsion droplets (green spheres) along with therapeutic molecules (red spheres). The outside of the bilayer contains active targeting ligands (blue circles) and stealth polymers. Sizes are not to scale.

Phospholipids 1, 2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG2000-amine) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). 1, 2-Dipalmitoyl-sn-glycero-3-phosphate (DPPA) was purchased from Echelon Biosciences (Salt Lake City, UT). Folic acid and N,N′-dicyclohexylcarbodiimide (DCC) were purchased from Sigma-Aldrich (St. Louis, MO). Perfluoropentane (PFC5) was obtained from SynQuest Labs, Inc. (Alachua, FL).

2.1. PFC5 emulsion droplet formation

PFC5 emulsion droplets were prepared as described previously [38]. Briefly, 1.0 mL of DPPA solution (10 mg/mL in chloroform) was dried onto a flask under vacuum and then hydrated by rotation in 2 mL of PBS. Following hydration the flask was placed in an ice-water bath. Then 1.0 g of PFC5 was added to the flask. Emulsions were made by sonicating with a 20 kHz probe (Sonics and Materials, CVX400, Newtown, CT) 1.25 W/cm^2 for 90 s, (3 times, 30 s each time with 1 min between sonications). Then the emulsion was extruded through a 100-nm filter (Hamilton Co, Reno, NV).

2.2. eLiposome formation

eLiposomes were formed using the “ultra method” described previously [38]. Briefly, liposomes were prepared by hydrating a DMPC film (50 mg) on a round-bottomed flask with PBS (1.0 mL) at 23 °C. The suspension was sonicated at 20 kHz, 1.5 W/cm^2 for 10 min to reduce the size of liposomes. The suspension was extruded through a 200-nm-filter 10 times. Then 1.0 mL of 100-nm-emulsion was added to 1.0 mL of the 200-nm-liposome suspension, and the mixture was sonicated at 20 kHz, 1.5 W/cm^2 on ice for 90 s (3 times, 30 s each time with 1 min between each sonication). The resulting eLiposomes were extruded through a 200-nm filter. Non-encapsulated emulsions