Investigating the Stability of eLiposomes at Elevated Temperatures

Ghaleb A. Husseini, William G. Pitt and Marjan Javadi

Technol Cancer Res Treat published online 26 September 2014
DOI: 10.1177/1533034614551480

Technology in Cancer Research & Treatment
1–4

DOI: 10.1177/1533034614551480
tct.sagepub.com

Investigating the Stability of eLiposomes at Elevated Temperatures

Ghaleb A. Husseini, PhD[1], William G. Pitt, PhD[2], and Marjan Javadi, PhD[2]

Abstract

eLiposomes encapsulate a perfluorocarbon nanoemulsion droplet inside a liposome. Ultrasound is then used as a trigger mechanism to vaporize the perfluorocarbon, break the liposome, and release the desired drug to the tumor tissue. The purpose of this research is to show that eLiposomes synthesized using perfluoropentane are stable above the normal boiling point of the perfluoropentane and at body temperature and thus has potential for use in vivo. Experiments involving the release of fluorescent calcein molecules were performed on eLiposomes to measure the release of calcein at various temperatures in the absence of ultrasound. Results showed that eLiposomes are stable at body temperatures and that as the temperature increases above 40°C, calcein release from these novel nanocarriers increases.

Keywords

nanodrug delivery, calcein, emulsion liposome, eLiposome, temperature stability, perfluoropentane (PFC_5)

Introduction

A novel delivery vehicle for cancer treatment is an eLiposome.[1] Inside a liposome, these carriers encapsulate a small perfluorocarbon (PFC) nanoemulsion droplet that can be changed to gas using acoustic droplet vaporization.[2-4] Ultrasonic pressure waves produce negative pressure fluctuations in the nanoemulsion droplet, causing it to vaporize, burst the liposome open,[2] and release its contents only at the site where the ultrasound (US) is focused (ie, the tumor tissue).[5]

Liposomes and eLiposomes have many advantages as drug delivery systems. (1) They can carry hydrophilic therapeutics in their aqueous interior and hydrophobic drugs in their membranes or in the emulsion droplets of eLiposomes.[1,5,6] (2) Liposomes retain their structural integrity in physiological conditions. (3) Their size can be controlled to be small enough to extravasate at the tumor site and large enough to escape renal excretion. (4) Their surface can be modified to reduce opsonization and increase their circulation time in blood (by reducing their clearance by cells of the reticuloendothelial system). (5) Since they are composed of phospholipid bilayers similar to cell membranes, they are both biocompatible and biodegradable. A noninvasive triggering stimulus, such as US, can be used to release liposomal drug contents only at a focused spot, that is, malignant tissue,[7] while reducing the agent's interaction with healthy cells.

The eLiposomes were designed to be activated by acoustic droplet vaporization (ADV), a process involving the phase change of a nanoemulsion droplet (encapsulated inside a liposome), from a small liquid droplet to a gas bubble about 125-fold larger in volume (the expansion for submicron-sized droplets may be smaller in size). Acoustic droplet vaporization occurs when US is focused on these emulsion-containing drug delivery vehicles, and the vaporization of the PFC droplet bursts the liposome carrier and releases the encapsulated drug. Perfluorocarbons (especially perfluoropentane [PFC_5]) can be used to form eLiposomes because they are nontoxic,[8] have a low-boiling point (that will require a minimal amount of subpressurization to produce the change phase),[9] and have low solubility in blood.[10]

Lattin et al[11] synthesized eLiposomes encapsulating both calcein and different size droplets of perfluorohexane (PFC_6) or PFC_5 emulsions. The group studied the effect of applying 20-kHz US to these carriers. Results were promising as it was shown that eLiposomes released 3- to 5-fold more calcein than conventional liposomes (liposomes that did not have emulsion

[1] Department of Chemical Engineering, American University of Sharjah, Sharjah, United Arab Emirates
[2] Department of Chemical Engineering, Brigham Young University, Provo, UT, USA

Received: May 3, 2014; Revised: June 22, 2014; Accepted: July 29, 2014.

Corresponding Author:
Ghaleb A. Husseini, PhD, Department of Chemical Engineering, American University of Sharjah, EB1-230, AUS, Sharjah, United Arab Emirates.
Email: ghusseini@aus.edu

droplets). As with micelles and liposomes, the released amount increased with the power intensity of US. The calcein release was higher when using large (400 nm) emulsion droplets than when using the small (100 nm) ones, and when PFC_5 was used, compared to PCF_6, indicating that vapor pressure and Laplace pressure played a strong role in ADV.

Javadi et al also studied eLiposomes as drug and gene delivery vehicles.[1,5] In a recent article, they synthesized eLiposomes with PFC_5 emulsions and then chemically conjugated a folate moiety to the carrier's surface, which induced binding and endocytosis. Then they studied the effect of several US parameters on the delivery of calcein and DNA plasmids into HeLa cells.[5] Their study supports the hypothesis that exposure to low frequency US vaporizes the liquid emulsion droplets, forcing the rupture of the eLiposome and thus the release of calcein. The folate targeting strategy also paid off, as eLiposomes were observed inside the HeLa cells, and calcein and plasmids were delivered to the cell cytosol.

But the question remains unanswered as to whether these eLiposomes would be stable in the blood stream of the patient at physiological temperatures, given that the boiling point of PFC_5 is 29°C. We suspected that the Laplace pressure would prevent the droplets from boiling at 29°C, but we did not know how stable the droplets would be at physiological temperatures. Would there be a spontaneous generation of gas that would prematurely permeabilize the eLiposomes before US was applied at the target site?

The purpose of this note is to show that PFC_5 nanoemlusions encapsulated inside eLiposomes are stable above the PFC boiling point (up to 37°C) and may have potential for in vivo applications. We performed experiments to measure the release of a fluorescent molecule (namely calcein) from our eLiposomes at various temperatures up to 59°C.

Materials and Methods

Materials

Lipids dipalmitoylphosphatidylcholine (DPPC), cholesterol, and 1,2-distearoyl-sn-glycero-3 phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG(2000)-amine) were purchased from Avanti Polar Lipids, Inc (Alabaster, Alabama). Calcein was purchased from Sigma-Aldrich (St Louis, Missouri). Perfluoropentane was purchased from SynQuest Labs, Inc (Alachua, *Florida*). Sucrose was obtained from Avantor Performance Materials (Phillipsburg, New Jersey) and United Biochemical Corp. (Cleveland, Ohio). Other chemicals were reagent grade from Sigma-Aldrich.

Nanoemulsion Formation

Nanoemlusions of PFCs were made by placing 0.1 mL of ice-cold 1,1,1,2,2,3,3,4,4,5,5,5-perfluoropentane (with a normal boiling point of 29°C) in a cold flask previously coated with a layer of DPPC and sonicating on ice with a 20-kHz US probe at 1 W/cm^2 for 1 minute. The resulting droplets have a size range between 100 and 200 nm. They were then extruded through a 100-nm filter to decrease their size distribution.

Liposome Formation

The liposomes were made as follows. Lipids (DPPC, cholesterol, and DSPE-PEG (2000)-amine in a 3:1:1:0.004 molar ratio) were dried onto a glass flask and then hydrated with buffer, forming liposomes of various sizes. The mixture was extruded through a 200-nm filter to produce a uniform size distribution of unilamellar liposomes.

eLiposome Formation

The eLiposomes were made by mixing 1 mL of 100-nm PFC_5 emulsions with 1 mL 200-nm of liposomes and added these to 2 mL of 30 mmol/L calcein in phosphate-buffered saline (PBS) at the same osmolarity as the liposomes and emulsions. Then, the mixture was sonicated on ice 3 times for 30 seconds to give a calcein concentration of 15 mmol/L. The sonication transiently breaks open a liposome and the emulsion droplet enters inside the drug delivery vehicle before the liposome membrane reseals. External emulsions (not captured inside the eLiposome) are removed by centrifugation on a sucrose/glucose/NaCl density column with a resultant eLiposome having an average diameter of 200 nm.[5] The size distribution of the eLiposomes was measured by dynamic light scattering on a *Brookhaven 90Plus* Particle Sizer (Brookhaven Instruments, New York). Inside the resultant eLiposomes, a 100-nm PFC_5 droplet was imaged using cryo transmission electron microscopy.[12] The calcein concentration of 15 mmol/L was in the self-quenched region and thus has minimal fluorescence inside the carrier. The fluorescence then increases upon the rupture of this drug delivery vehicle during the application of US when the emulsion vaporizes causing the liposome bilayer to break open and the drug concentration to decrease, which in turn increases its emitted fluorescence.

To measure the amount of calcein released from these eLiposomes, 30 μL of the calcein-loaded nanocarriers were mixed in 2 mL of PBS (pH = 7.4) and placed in a cuvette in a fluorometer. Excitation and emission wavelengths were set at 488 and 520 nm, respectively, and the fluorescence level continuously recorded.

Results and Discussion

Our goal was to show that PFC_5 eLiposomes are stable above the normal boiling point of PFC_5 and even stable at 37°C. Therefore, we performed fluorescence experiments on our freshly synthesized eLiposomes at various temperatures. We have previously shown low calcein (encapsulated in eLiposomes) uptake by cancer cells after 2 hours of incubation.[5] Here we examine the release of the model drug at various temperatures to determine whether any nonacoustically activated release occurs using these novel drug delivery vehicles.

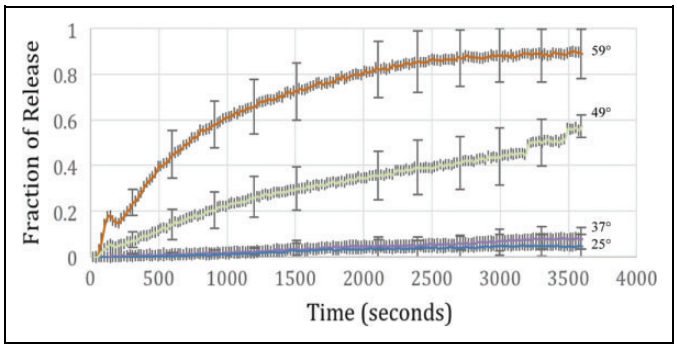

Figure 1. Fraction of calcein release from eLiposomes incubated, not insonated, at various temperatures (n = 3 for experiments at 25°C and 37°C and n = 5 at 49°C and 59°C). Error bars are 95% confidence intervals.

Figure 1 shows the release of calcein over a period of 1 hour. Rapid release was observed at 49°C and 59°C. At 37°C and 25°C, there was very little release. There was no statistically significant difference at temperatures above and below 29°C (PFC_5 boiling point; P value = 0.44). However, the release was statistically significant at 49°C and 59°C when compared to release at physiological temperatures with P values of .0029 and .00001, respectively. (P values were calculated using a student t test on the maximum release percent reached in each experiment).

These results suggest that eLiposomes could be stable in the blood stream of a patient at normal physiological temperatures. The release observed at higher temperatures could be due to either thermal vaporization of the droplets or increased lipid permeability due to a thermal phase transition of the lipids used in liposomal shell (the transition temperature [T_m] of DPPC = 41°C).

Cancer drug delivery is concentrated on treating patients with cancer and improving their lives. There are 3 principle categories of drug delivery techniques involved with nanocarriers, namely passive, ligand, and triggered targeting. Our eLiposomes appear to be an important chemotherapeutic vehicle and once labeled with a targeting moiety and used in conjunction with US, eLiposomes can employ all 3 targeting mechanism.

Passive targeting occurs naturally if the particle size is small enough; the ideal drug delivery vehicle should be below 0.5 μm in diameter to escape from the circulatory system and collect in tissues of tumors with malformed capillaries. Ligand targeting is also heavily researched in drug delivery. The essential aspect of this type of targeting is the proper selection of the moiety/ligands that have the ability to deliver the carrier to the target site and then induce uptake via endocytosis. Once inside the lysosome/endosome, the drug needs to be released into the cytosol which can be achieved using fusogenic peptides/lipids and proton sponges. However, the use of these drug release activators into the cytosol is limited due to their toxicity which prompts researchers to find other ways to burst the carrier and the endosome when needed. An external trigger mechanism can be useful here.

Ultrasound appears to be an ideal trigger for this type of drug delivery system because it can be easily focused on the cancerous site. In order to make these drug delivery vehicles echogenic, several strategies can be utilized, including the use of microbubbles that are decorated with the drug-encapsulated carriers or by inducing a phase change inside the carrier. The latter can be achieved by encapsulating a liquid nanoemulsion inside the delivery vehicle. The phase change can be induced by a small acoustic nudge using US.[13,14] Here, PFC_5 appears to be an ideal compound to form these nanoemulsions because it has a low boiling point and it is nontoxic.

Once at the desired tissue, physicians will have the ability to control the temporal release of the drug from these nanocarriers. If drug delivery is not adequately controlled, the drug may be either quickly released upon arrival at the desired location or we may observe a sustained slow release over time. The latter is problematic because the drug may never reach the needed concentrations to achieve the desired therapy which in turn may expedite the development of multidrug resistance in the tumor. Therefore, in most cases, it is highly desirable to release the drug instantly and simultaneously to achieve a rapid high and lethal concentration. Here, US appears to be a viable trigger mechanism.

Conclusion

The results reported in this technical note validate the use of PFC_5 as the main ingredient in making nanoemulsions and encapsulating them inside liposomes to form ideal chemotherapeutic nanocarriers. The eLiposome will not rupture prematurely at body temperature and hence can be activated using US once it reaches the desired malignant location.

Authors' Note

Authors certify that this article has not been published in whole or in part nor is it being considered for publication by another journal. There is no conflict of interest to declare.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: The authors acknowledge the financial support of the Faculty Research Grant Type 1 (FRG1-2012) from the American University of Sharjah.

References

1. Javadi M, Pitt WG, Belnap DM, Tsosie NH, Hartley JM. Encapsulating emulsions inside liposomes for drug delivery. *Langmuir*. 2012;28(41):14720-14729. doi:10.1021/la303464v.
2. Sheeran PS, Luois S, Dayton PA, Matsunaga TO. Formulation and acoustic studies of a new phase-shift agent for diagnostic and therapeutic ultrasound. *Langmuir*. 2011;27(17):10412-10420. doi:10.1021/la2013705.

3. Fabiilli ML, Haworth KJ, Fakhri NH, Kripfgans OD, Carson PL, Fowlkes JB. The role of inertial cavitation in acoustic droplet vaporization. *IEEE Trans Ultrason Ferroelectr Freq Control*. 2009;56(5):1006-1017. doi:10.1109/Tuffc.2009.1132.
4. Kripfgans OD, Fowlkes JB, Miller DL, Eldevik OP, Carson PL. Acoustic droplet vaporization for therapeutic and diagnostic applications. *Ultrasound Med Biol*. 2000;26(7):1177-1189.
5. Javadi M, Pitt WG, Tracy CM, et al. Ultrasonic gene and drug delivery using eLiposomes. *J Control Release*. 2013;167(1): 92-100. doi:10.1016/j.jconrel.2013.01.009.
6. Pitt WG, Husseini GA, Roeder BL, et al. Preliminary results of combining low frequency low intensity ultrasound and liposomal drug delivery to treat tumors in rats. *J Nanosci Nanotechnol*. 2011;11(3):1866-1870.
7. Martins S, Sarmento B, Ferreira DC, Souto EB. Lipid-based colloidal carriers for peptide and protein delivery–liposomes versus lipid nanoparticles. *Int J Nanomedicine*. 2007;2(4):595-607.
8. Spahn DR. Blood substitutes. Artificial oxygen carriers: perfluorocarbon emulsions. *Crit Care*. 1999;3(5):R93-R97. doi:10.1186/cc364.
9. Diaz-Lopez R, Tsapis N, Fattal E. Liquid perfluorocarbons as contrast agents for ultrasonography and (19)F-MRI. *Pharm Res*. 2010;27(1):1-16. doi:10.1007/s11095-009-0001-5.
10. Spiess BD. Perfluorocarbon emulsions as a promising technology: a review of tissue and vascular gas dynamics. *J Appl Physiol (1985)*. 2009;106(4):1444-1452. doi:10.1152/japplphysiol.90995.2008.
11. Lattin JR, Pitt WG, Belnap DM, Husseini GA. Ultrasound induced calcein release from eLiposomes. *Ultrasound Med Biol*. 2012;38(12):2163-2173.
12. Lattin JR, Belnap DM, Pitt WG. Formation of eLiposomes as a drug delivery vehicle. *Colloids Surf B Biointerfaces*. 2012;89: 93-100. doi:10.1016/j.colsurfb.2011.08.030.
13. Singh R, Husseini GA, Pitt WG. Phase transitions of nanoemulsions using ultrasound: experimental observations. *Ultrason Sonochem*. 2012;19(5):1120-1125. doi:10.1016/j.ultsonch.2012.02.005.
14. Pitt WG, Singh RN, Perez KX, Husseini GA, Jack DR. Phase transitions of perfluorocarbon nanoemulsion induced with ultrasound: a mathematical model. *Ultrason Sonochem*. 2014;21(2): 879-891. doi:10.1016/j.ultsonch.2013.08.005.