Published in final edited form as:

Adv Healthc Mater. 2014 February ; 3(2): 230–238. doi:10.1002/adhm.201300188.

Sustained Zero-Order Release of Intact Ultra-Stable Drug-Loaded Liposomes from an Implantable Nanochannel Delivery System

Dr. Christian Celia[#],

Department of Nanomedicine, The Methodist Hospital Research Institute, 6670 Bertner Ave. Houston, TX 77030 (USA)

Dr. Silvia Ferrati,

Department of Nanomedicine, The Methodist Hospital Research Institute, 6670 Bertner Ave. Houston, TX 77030 (USA)

Dr. Shyam Bansal[#],

Department of Nanomedicine, The Methodist Hospital Research Institute, 6670 Bertner Ave. Houston, TX 77030 (USA)

Dr. Anne L. van de Ven,

Department of Nanomedicine, The Methodist Hospital Research Institute, 6670 Bertner Ave. Houston, TX 77030 (USA)

Dr. Barbara Ruozzi,

Department of Life Sciences, University of Modena and Reggio Emilia, Via Campi 183, Modena, 41100 (Italy)

Ms. Erika Zabre,

Department of Nanomedicine, The Methodist Hospital Research Institute, 6670 Bertner Ave. Houston, TX 77030 (USA)

Dr. Sharath Hosali,

NanoMedical Systems, Inc., 2706 Montopolis Drive Austin, TX 78741, (USA)

Dr. Donatella Paolino,

Department of Health Sciences, University “Magna Graecia” of Catanzaro, V.le “S. Venuta” Germaneto – Catanzaro, 88100 (Italy)

Dr. Maria Grazia Sarpietro,

Department of Drug Sciences, University of Catania, V.le A. Doria 6, Catania, 95125 (Italy)

Dr. Daniel Fine,

Department of Nanomedicine, The Methodist Hospital Research Institute, 6670 Bertner Ave. Houston, TX 77030 (USA)

Dr. Massimo Fresta,

Department of Health Sciences, University “Magna Graecia” of Catanzaro, V.le “S. Venuta” Germaneto – Catanzaro, 88100 (Italy)

Dr. Mauro Ferrari, and

Correspondence to: Alessandro Grattoni, agrattoni@tmhs.org.

[#]Equal contribution

The authors S. H., D. F., M. F., and A. G. disclose a financial interest in NanoMedical Systems, Inc. All other authors declare no competing financial interest.

Department of Nanomedicine, The Methodist Hospital Research Institute, 6670 Bertner Ave. Houston, TX 77030 (USA); Department of Medicine, Weill Cornell Medical College, 1300 York Avenue, New York, NY 1006, (USA), Department of Bioengineering, Rice University, 6100 Main Street Houston, TX 77251, (USA), Alliance for NanoHealth, 6670 Bertner Ave., Houston, TX 77030, (USA)

Dr. Alessandro Grattoni

Alessandro Grattoni: agrattoni@tmhs.org

Abstract

Metronomic chemotherapy supports the idea that long-term, sustained, constant administration of chemotherapeutics, currently not achievable, could be effective against numerous cancers. Particularly appealing are liposomal formulations, used to solubilize hydrophobic therapeutics and minimize side effects, while extending drug circulation time and enabling passive targeting. As liposome alone cannot survive in circulation beyond 48 hrs, sustaining their constant plasma level for many days is a challenge. To address this, we developed, as a proof of concept, an implantable nanochannel delivery system and ultra-stable PEGylated lapatinib loaded-liposomes, and we demonstrate the release of intact vesicles for over 18 days. Further, we investigate intravasation kinetics of subcutaneously delivered liposomes and verify their biological activity post nanochannel release on BT474 breast cancer cells. The key innovation of this work is the combination of two nanotechnologies to exploit the synergistic effect of liposomes, demonstrated as passive-targeting vectors and nanofluidics to maintain therapeutic constant plasma levels. In principle, this approach could maximize efficacy of metronomic treatments.

Keywords

Liposomes; nanochannels; constant release; implantable device; subcutaneous delivery

1. Introduction

Liposomes have been investigated and clinically utilized as drug carriers for decades due to their ability to encapsulate and solubilize both hydrophilic and hydrophobic compounds avoiding the clinical use of organic solvents^[1]. Through the enhanced permeability and retention (EPR) effect, present in many types of cancer^[2], systemically circulated liposomes preferentially extravasate and accumulate at disease loci, leading to improved therapeutic efficacy, while minimizing side effects^[1, 3, 4]. In most cases, the liposomal surface is functionalized with polyethylene glycol (PEG) to decrease reticuloendothelial uptake^[5] thereby extending liposomes circulation time in the blood to greater than 24 hrs following intravenous (IV) administration^[1, 6]. Despite substantial increase in circulation time^[7], however, plasma proteins and systemic enzymes eventually erode the PEG-chains from the liposomal surface leading to their clearance by the spleen and liver in a matter of days^[7]. This process limits the use of liposomes as sustained, long-term delivery vehicles for applications in which drug administration is required for periods that range from weeks to months. A primary example is metronomic chemotherapy, a regimen involving small doses of drugs that are delivered at short time intervals for long treatment durations, attempting to mimic sustained continuous administration^[8]. To reach and maintain drug plasma concentrations within the therapeutic window from hours to days while avoiding multiple bolus injections, i.v. infusion has been used. However, this method subjects the patient to the inconvenience of long visits to the clinic, and the discomfort and increased risk of infection while carrying trans-cutaneous catheter infusion devices. In addition, i.v. infusion can lead to toxicity reactions known as infusion reactions, not predictable or explainable by the known toxicity profile of the drug^[9].

Due to their stability and tunable degradation kinetics, lipidic formulations have also been used as depots to deliver drugs subcutaneously and intramuscularly^[10], or, in combination with hydrogels, as coatings to release antiseptics from the surfaces of catheters both *in vitro*^[11] and *in vivo*^[12]. A variety of these depot formulations (DepoFoam®), based on highly stable, non-concentric multilamellar lipidic vesicles^[13], are already FDA-approved for the sustained delivery of several drugs, including bupivacaine (EXPAREL®)^[14], morphine (DepoDur®)^[15], and cytarabine (DepoCyt(e)®)^[16], for several days to weeks. These vesicles are employed exclusively to control the kinetics of drug release through polymer or lipid membrane degradation, not to mitigate local drug cytotoxicity or exploit the EPR effect as systemically circulated intact liposomal drug carriers.

To date, only a few attempts have been made to develop a sustained release approach where intact liposomes containing therapeutic agents are subcutaneously or intramuscularly released. Among these endeavors, biodegradable dextran microspheres have been shown *in vitro* to achieve a sustained release of intact liposomes up to 100 days^[17]. Alternatively, liposomes contained in a reservoir have been released *in vitro* from a microchannel membrane for a period of 30 hrs^[18]. Both approaches, however, suffer from a decaying diffusive release rate and therefore fail to achieve a long-term constant release profile^[17]. Additionally, the membrane and reservoir must be surgically implanted and may therefore only be valuable for treatment that exceed several weeks.

In this study, we have adapted our implantable nanochannel Delivery System (nDS), which has already demonstrated long-term sustained release of a broad range of molecular therapeutics^[19], to constantly and sustainably deliver intact chemotherapeutic-loaded liposomes *in vitro*. Ultra-stable liposomes, loaded with lapatinib (a small hydrophobic molecule tyrosine kinase inhibitor of HER1, HER2/ErbB2, and EGFR^[20]) were formulated and characterized in size, Z-potential (ζ), polydispersity index (PDI), and stability over 30 days at 23 and 37 °C. The size and morphology was verified by atomic force and transmission electron microscopy (AFM and TEM). The interaction between lipid membrane and lapatinib was investigated using differential scanning calorimetry (DSC). Loading efficiency, encapsulated drug, lapatinib release at 37 °C in both PBS (at pH 7.4 and 5.5) and fetal bovine serum (FBS) were quantified over 30 days using high-performance liquid chromatography (HPLC). Silicon membranes presenting 200 and 900 nm nanochannels, were microfabricated and employed for the sustained release of liposomes over 18 days. The integrity of the released vesicles was verified by DLS, whereas their bioactivity was measured through MTT assay with a breast cancer cell line (BT474). BT474 cells and confocal microscopy were also used to demonstrate the internalization of rhodamine-fluorescent liposomes. Finally, intravital microscopy (IVM) was adopted to study the intravasation of subcutaneously delivered liposomes in mice.

2. Results and Discussion

The aim of this study was to demonstrate that nanochannels can be exploited to passively control the sustained release of intact liposomes from subcutaneously implantable reservoirs, designed for systemic delivery. For such application, liposomes and delivery system must comply with the following requirements: 1) Liposomes must possess long-term physicochemical stability (weeks) in a reservoir at 37 °C in the potential presence of permeated biological molecules and 2) must retain encapsulated drug until reaching the disease site, 3) nanochannels must grant a constant and sustained release of intact vesicles, which 4) must intravasate into blood vessels, intact, to maintain extended circulation time and 5) biodistribution as conventional PEG-liposomes. 6) Finally, released liposomes must deliver a biologically active payload at the lesion site. The scope of this study is to analyze

requirements 1 through 4 (stability, drug retention, sustained release, and liposomal intravasation), as well as 6, in an *in vitro* setting.

2.1 Liposome Design and Characterization

To address the first of the above requirements, we engineered ultra-stable liposomes containing lipid/chol/PEG at 5.4/4.5/0.6 molar ratios. Large cholesterol content (39%) was employed to maximize liposome rigidity and compactness, and to stabilize the bilayer, while decreasing permeability to encapsulated drugs. Liposomes were also PEGylated for steric stabilization and to enhance plasma half-life^[7, 21, 22]. Lapatinib, a representative cytotoxic, small-molecule chemotherapeutic, which could potentially benefit from enhanced water solubility, reduction of side effects, and improvement of bioavailability, was used. A nominal vesicle size in the range 80-120 nm was selected to be optimal for EPR effect and low-clearance rates^[23-25], and to further promote stability while maintaining adequate diffusivity. Such a size was also chosen to ensure the ability of liposomes to intravasate intact into blood vessels^[26].

Optimized empty and lapatinib-liposomes (E-Lip and L-Lip, respectively), were compared in terms of stability. Covalently bonded rhodamine-labeled lipids (0.1% molar ratio) were used to obtain fluorescent empty vesicles (RE-Lip) for the *in vitro* analysis of internalization into BT474 breast cancer cells and *in vivo* intravasation study by confocal and IVM. RE-Lip were extruded in two sizes (~96 and ~117 nm) to investigate differences in intravasation kinetics. The DLS characterization (Figure 1A) shows nearly monodisperse liposomes with PDI values smaller than 0.07, except for 96 nm RE-Lip. Typically, negative ζ values in average equal to -10.4 mV were obtained, due to PEGylation. No significant differences in the physicochemical properties between RE-Lip and E-Lip were observed. Spherical vesicle morphology with an average size of 115 and 106 nm was verified for E-Lip and L-Lip, respectively, by DLS, in good agreement with AFM data performed in non-contact mode (Figure 1A, B, and Figure S2).

Spherical morphology and unilamellar structure were further confirmed by TEM (Figure 1C). Encapsulation efficiency (EE) and drug loading efficiency (DLE) were measured by HPLC equal to 62% and 58 $\mu\text{g}_{\text{drug}}/\text{mg}_{\text{lipids}}$, respectively. Such values of EE and DLE support the affinity between the hydrophobic drug and the phospholipid chains (Figure 1D), as later confirmed by DSC analysis.

2.2 Liposome Stability

Physicochemical stability of L-Lip and E-Lip in PBS at 37 and 23 °C, mimicking the implant environment and shelf-conditions respectively, was evaluated and compared by monitoring size, PDI, and ζ for 30 days. Results are shown in Figure 2.

L-Lip maintained a constant size, PDI, and ζ at both 37 °C and 23 °C for the duration of analysis, while the size and PDI of E-Lip rapidly increased at 10 (37 °C) and 20 days (23 °C) (Figure 2). This finding suggests that lapatinib stabilizes the liposomes. For E-Lip, at 25 and 30 days (37 °C), the PDI decreases suggesting that the vesicle population has become more homogeneous in regards to size distribution (Figure 2D). Broad variations in ζ were observed at 37 °C for both L-Lip and E-Lip. In contrast, a constant net negative charge or a small monotonic increase was recorded for L-Lip and E-Lip, respectively, at 23 °C. The effect of lapatinib on the lipid bilayer was further investigated using DSC analysis, by comparing thermograms of E-Lip and L-Lip. We used increasing lapatinib-to-lipid molar ratios (from 0.015 to 0.18) to demonstrate the effect of the drug on the lipid bilayer. The main transition peak (at 24.8 °C for E-Lip, from ripple to disordered liquid-crystalline phase) progressively broadens at increasing lapatinib content and shifts toward lower

temperature (Figure 2C, F). This, in conjunction with a decrease in the associated enthalpy variation, confirms a direct interaction of the loaded drug with lipids and the formation of a more compact but disorganized liquid-crystalline phase. This is in agreement with the strong retention of lapatinib inside the liposomes. Long-term stability of L-Lip at 37 and 23°C over a period of 21 days was supported by the low variation in backscattering (ΔBS always within 3%), measured through turbidimetric analysis (Figure S5).

2.3 Drug Retention

To demonstrate drug retention, lapatinib release from L-Lip was investigated at 37 °C in both PBS and 10% FBS/PBS (v/v, both at pH 7.4) and in PBS at pH 5.5 over 30 days. The first two conditions mimic the implanted reservoir environment, in the case of fresh liposomal solution and in the case of permeation of interstitial fluid, respectively, whereas lower pH mimics the acidic biological environments (e.g. tumor and lysosomal compartments) where liposomes are expected to accumulate. The amount of free lapatinib released from liposomes and diffused through a 25 kDa cutoff dialysis membrane into a sink reservoir was measured by HPLC. A 200 ml sink solution was employed to potentially allow the full solubilization of the total amount of lapatinib loaded in the used L-Lip (380 µg, lapatinib water solubility = 22.3 µg/ml). A high-sensitivity HPLC protocol (lower detection limit = 125 ng/ml) was used, presenting a detection limit corresponding to 0.65% of the total amount of encapsulated lapatinib. However, no lapatinib was detected in the sink solution for any of the three investigated conditions. DLS and ζ measurements on L-Lip retrieved from the dialysis membranes at day 30 confirmed that vesicles in PBS at pH 7.4 were intact, whereas L-Lip kept at pH 5.5 were significantly altered: average size, PDI, and ζ increased from 93.6 nm to 495.2 nm, from 0.028 to 0.208, and from -9.61 mV to -15.27 mV, respectively. Despite vesicle destabilization by acidic pH, lapatinib was not released into the sink fluid, which could be explained by its hydrophobic affinity for phospholipids^[27]. DLS analysis could not be performed on the PBS/FBS samples because of the interfering presence of biological aggregates from infiltrated FBS components in the dialysis membrane. However, absence of lapatinib in solution, despite its high affinity for albumin (99%), suggests liposome integrity, further supported by the neutral pH of the solution.

2.4 Release of Liposomes from Nanochannel Membranes

We have previously demonstrated that by exploiting the physical and electrostatic confinement on diffusing analytes, a constant and sustained release of therapeutics can be achieved through nanochannels^[19]. We also showed that the controlled release of highly hydrophobic drugs ($\text{LogD}_{\text{pH}7.4} > 3$) such as lapatinib ($\text{LogD}_{\text{pH}7.4} = 4.4$) can be achieved through nanochannels only with solubilizers^[28]. To confirm this, we attempted to release free lapatinib in water through 3.6 and 5 nm nanochannels nDS (channel-to-analyte size ratio (R_s) = 3.3 and 4.6, respectively), over 30 days. No drug release was detected, justifying the need for a liposomal formulation. Two hundred nanometer nanochannels nDS was used for the release of L-Lip and RE-Lip ($R_s = 2.1$) maintaining an optimal R_s value < 3.5 to achieve the constant release of negatively charged nanoparticles. Results were compared to larger channels (900 nm, $R_s = 10.7$), expected to produce an exponential release profile. Sustained release of intact L-Lip and RE-Lip was achieved for a period of 18 days (Figure 3) during which ~2.2 and 1.0 mg L-Lip, corresponding to ~77% and 35% of loaded amount respectively, were released from 900 and 200 nm nDS, respectively. As expected, zero-order kinetics was obtained for 200 nm nDS, while an exponential release profile was observed for 900 nm membranes. In view of therapeutic applications, these “*proof of concept*” delivery rates could be increased by orders of magnitude by adjusting nanochannel packing density and membrane surface area, while maintaining an implant size suitable for human use. It has to be noted, however, that because of the constant and sustained liposomal delivery, lower doses could ultimately be needed as compared to conventional chemotherapeutic treatments.

At intermediate times (days 7, 11, 14, and 18), at least one experiment replicate for each channel size and formulation was interrupted. DLS was performed on both source and sink solutions to assess vesicle integrity in terms of size, PDI, and ζ , both before and after release through nanochannels. Results (Figure 3E and F) show a slight increase in L-Lip size ($\sim 10\%$ over 18 days) in both source and sink solutions for both channel sizes, simultaneous to a significant increase in PDI for released L-Lip, especially from 200 nm channels (PDI = 0.4) (ζ data in Figure S8). The increase in PDI suggests an increase in size variability among liposomes released from the membrane. Similarly, RE-Lip remained stable in the source solution, but a more evident increase in size and PDI was observed after diffusion through 200 nm channels, with vesicle size ≈ 500 nm and PDI of 0.6 suggesting formation of a heterogeneous population of liposomal vesicles. Both L-Lip and RE-Lip data suggest that despite the nominal repulsive interactions between negatively charged channel walls ($\zeta = -26$ mV) and liposomes ($\zeta = -9$ mV), vesicles interact with the silica surface and are destabilized. It has been shown that low-density PEG (< 3 mol%) liposomes in aqueous solutions at pH 7.4 tend to adsorb to negatively charged silica surfaces forming a stable bilayer [29]. It is therefore likely that such a layer is formed within the channel during the first phase of diffusion, after which quasi-stable diffusive transport is established. The early decrease in slope of release profiles (from time 0 to day 2.5) seems to support such a hypothesis. However, while empty RE-Lip suffers major size changes (Figure 3H and 3I), L-Lip maintains a tighter population distribution (Figure 3E and 3F) probably due to the stabilizing effects of lapatinib on the bilayer. These results indicate feasibility of long-term constant delivery of intact L-Lip through nanochannel membranes.

2.5 Intravasation of Liposomes into Systemic Circulation

Intravital microscopy was used to verify that liposomes were able to intravasate into the systemic circulation from the subcutaneous environment. The nanochannel membrane was not suitable for this study because the fluorescence intensity associated to the slowly released liposomes would have been below the detectable limit. As such, high-resolution time-lapse images of the mice vasculature were taken every 30 sec over 55 minutes following a one-time subcutaneous injection of 80 μ l rhodamine or RE-Lip solution, containing equal amounts of rhodamine. Two RE-Lip formulations presenting different average sizes (96 and 117 nm) were used and compared. This was done to evaluate whether a 20% variation in average vesicle size could generate differences in intravasation kinetics, based on different diffusive properties. The mean fluorescence intensity of randomly selected ROI within vessels was then quantified over time and normalized with respect to the highest intensity value (Figure 4). Obtained standard deviation was 11.1% (data not shown for graphical clarity).

For all solutions used, an increase in the ROI fluorescence intensity was observed. In the case of 96 nm RE-Lip, a deviation from a monotonic increase in normalized intensity was observed with an early peak in the intensity profile. This increase was most likely due to an experimental artifact, linked to a temporary change in the imaging focal plane as a consequence of animal breathing. The kinetics of intravasation appeared to be size dependent, with the 96 and 117 nm RE-lip reaching equivalent intravascular fluorescence intensity 30 and 43 min later than free rhodamine. The increase in fluorescence could be due to the intravasation of fluorescent lipids rather than intact vesicles, and a faster increase in fluorescence for smaller vesicles could be due to a faster degradation rate. However, the stability tests performed over 30 days with both 96 and 117 nm RE-Lip (Figure S4 and S3, respectively) indicate long-term stability (beyond 7 days) at 37 °C and at neutral pH, which contrasts with the *in vivo* degradation hypothesis. Based on the above, we believe that RE-Lip was able to intravasate as intact vesicles and the difference in kinetics can be attributed to a difference in diffusivity, which is inversely proportional to vesicle size. Our conclusions

are in substantial agreement with previous studies, which showed that although a part of the subcutaneously injected PEGylated liposomal population (size 120 nm) were taken up by the lymphatic system, a significant fraction were able to reach the blood circulation within hours post-administration^[29]. Importantly, the same studies demonstrated that intravasated liposomes present similar biodistribution, pharmacokinetics, and half-life to intravenously injected vesicles^[29], supporting the feasibility of our approach.

2.6 Cellular Interaction and Biological Activity of Liposomes

Lapatinib-responsive BT474 breast cancer cells were employed to test liposome cellular interaction and biological activity, using confocal microscopy and proliferation assay. Based on the physicochemical properties, which are nearly identical to L-Lip, fluorescent RE-Lip represents a good model for imaging analysis. The 3D reconstructed z-stack image of 488-WGA-labeled BT474 incubated for 6 hrs with RE-Lip (Figure 5A) shows that our liposomal formulation is internalized by BT474. For the proliferation assay, BT474 were exposed to increasing concentrations of both fresh and nDS-released L-Lip (equivalent lapatinib concentration = 0.1, 0.05 and 0.01 μ M) for 24 and 72 hrs. MTT viability assay results, Figure 5B and C and Figure S9, show that L-Lip produces a characteristic dose- and time-dependent decrease in proliferation, comparable to equivalent concentrations of free lapatinib^[30]. In addition, long permanence in the reservoir and diffusion through nanochannels did not affect liposomes biological efficacy. DMSO, used as a vehicle for free lapatinib, and E-Lip were employed as negative controls.

4. Conclusions

In this study, the constant and sustained release of ultra-stable lapatinib-liposomes through the nanochannel Delivery System is demonstrated. This work provides a proof-of-concept for the potential use of nanochannels for time-controlled subcutaneous administration of liposomes commonly used for passive targeting. All requirements necessary to achieve our set objectives - long-term liposome stability and drug retention at 37 °C, constant and sustained release of intact vesicles from nDS, liposome intravasation into the systemic circulation from subcutaneous delivery and biological activity post release - were addressed step by step. PEGylated L-Lip formulated with high cholesterol content were shown to be stable and able to retain the encapsulated drug at 37 °C in simulated subcutaneous fluid for 30 days. Sustained zero-order release of intact liposomes was achieved for 18 days from 200 nm nanochannels nDS. The ability of liposome to intravasate into systemic circulation after subcutaneous administration was shown *in vivo* by means of IVM. Finally, the efficacy of released L-Lip in reducing BT474 cell proliferation was verified *in vitro* through an MTT assay.

Future directions of this work will involve the efficacy study of implanting nDS with L-Lip and targeting tumor tissue *in vivo*. The efficacy study will determine whether the L-Lip released from nDS will accumulate in sufficient concentration in tumor tissue to initiate cell death. The results presented in this paper provide the feasibility evidence necessary to initiate the investigation on the effectiveness of the L-Lip and nDS implant.

This approach, demonstrated with lapatinib, is in principle applicable to any hydrophobic therapeutic, that would benefit from increased solubility, bioavailability, and minimized toxicity offered by a liposomal formulation. This work supports the feasibility of combining nanofluidics and nanoparticles, to fully exploit the synergistic effects of both nanotechnologies in sustaining drug delivery and providing the means for passive targeting, while avoiding multiple bolus injections.

5. Experimental

5.1 Materials

The materials employed in the following experiments and their source are here listed: egg L- α -phosphatidylcholine (PCS100) (Lipoid LLC, NJ, USA); N-(carbonyl-methoxy polyethylene glycol-2000)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE-MPEG2000, purity 99%) (Avanti Polar Lipids, Inc. AL, USA); cholesterol (Chol) (Sigma-Aldrich Co., MO, USA); lissamineTM rhodamine B 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt (rhodamine DHPE) (Molecular Probes, Life Technologies, NY, USA); lapatinib (LC Laboratories, MA, USA); NaH₂PO₄•2H₂O and Na₂HPO₄ (Fluka, Sigma-Aldrich Co.); dimethyl sulfoxide (DMSO) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution (MTT reagent) (Sigma-Aldrich Co.); Spectra/Por cellulose membranes (Spectrum Laboratories, Inc CA, USA); centrifugal filters (Millipore, Billerica, MA, USA); human breast carcinoma cells (BT-474) (ATCC, VA, USA); Dulbecco's modified Eagle's (DMEM)/high glucose medium (Hyclone, UT, USA); pen/strep solution (10,000 IU penicillin and 10,000 μ g/ml streptomycin) and fetal bovine serum (FBS) (Gibco, Life Technologies); trypsin solution (Clonetics, NJ, USA); glass-bottomed culture slides (BD Falcon, New Jersey, USA); DiD lipophilic tracer, ProLong Gold mounting medium, and 488-wheat germ agglutinin (WGA) (Invitrogen, Life Technologies); paraformaldehyde solution (Electron Microscopy Science, PA, USA). Phosphate buffer (PB, at pH 7.4) (Na₂HPO₄/NaH₂PO₄•2H₂O) was prepared in Milli-Q water and filtered through 0.22 μ m Millipore membranes; Phosphate buffer saline (10x PBS) and all the remaining solvents (analytical grade) were from Fisher Scientific (PA, USA).

5.2 Liposome Preparation

Liposomes were prepared by thin-layer evaporation and small unilamellar vesicle extrusion^[31], using PCS100/Chol/DSPE-MPEG 2000 at a molar ratio of 5.4:4.5:0.6. After dissolving phospholipids (40 mg) and lapatinib (1 mg) in chloroform/methanol (3:1 v/v) and removing organic solvents by rotary evaporation (Heidolph Instruments, Germany), a lipid layer was obtained. Residual solvent was vacuumed (12 hr, 23 °C), and the lipid film was hydrated (1x PBS, 2 ml). Multilamellar liposomes were obtained with three heating/vortexing cycles (3 min each, 55 $\pm$ 0.5 °C, 700 rpm). PEGylated unilamellar lapatinib-liposomes were produced in the average size of 106 nm following a 10-cycle extrusion (at 60 $\pm$ 0.5 °C) per each filter size (400, 200, and 100 nm; Whatman Inc., NJ, USA) using a Lipex Biomembranes extruder (Northern Lipids Inc., BC, Canada). Each extrusion was performed through two stacked polycarbonate membranes. A similar protocol was used to prepare fluorescent and non-fluorescent empty liposomes (RE-Lip and E-Lip, respectively), for confocal and IVM experiments. For RE-Lip, rhodamine-labeled DHPE was co-dissolved with lipids at a 0.1% molar ratio. RE-Lip were produced in two sizes (96 and 117 nm) by an additional filtration cycle through the 80 nm membrane to generate the smaller vesicle population.

5.3 Liposome Dynamic Light Scattering (DLS) Characterization

Liposome size, polydispersity index (PDI) and Z-potential (ζ) were measured using a Zetasizer Nano ZS (Malvern Instruments Ltd., UK) through DLS and laser doppler electrophoresis. Details are given in supporting information. To avoid multi-scattering, samples were diluted in PB, and new cuvettes were used for each measurement. Three sets of 10 measurements were performed for each sample, and mean and standard deviation (s) were calculated.

5.4 Atomic Force and Transmission Electron Microscopy of Liposome

Size, morphology and structure of vesicles were investigated using AFM and TEM. Wet AFM analysis (Autoprobe AFM, Park Instruments, CA, USA) was performed on a 1:100 v/v diluted liposomal solution on a mica disc, using a triangular silicon soft tip in non-contact mode, (23 °C, 100 kPa), as previously reported^[32]. To avoid disrupting vesicles and to minimize noise, the analysis was performed in low-force mode, with a tip-to-sample distance ranging from 10 to 100 Å and a cantilever resonance frequency of 300 kHz. Topographical images were recorded, noise was filtered (ProScan software) and images were processed (Image J software) to obtain liposome size distribution. TEM was performed as previously reported^[32]. Briefly, a 1:100 v/v diluted sample of liposomal solution was placed on a 200-mesh formvar copper grid (TABB Laboratories Equipment, UK), stained with uranyl acetate solution (2% w/v, 5 min) and dried at 23 °C. Images were acquired using a JEM 2010 microscope (Jeol, MA, USA) at 200 kV.

5.5 Differential Scanning Calorimetry (DSC)

L-Lip with lipid-to-lapatinib molar ratios of 0.015, 0.03, 0.045, 0.06, 0.09, 0.12, and 0.18 were prepared as described above. Twenty microliters of L-Lip solutions, containing different lapatinib molar ratios, or E-Lip were loaded into 160-μl DSC aluminium pans in replicates of four and analyzed using a Mettler Toledo DSC-822®. Consecutive scans were performed by heating from 5 to 37 °C (2 °C/min) and cooling from 37 to 2 °C (4 °C/min). After DSC analysis, the phospholipid content was measured using a phosphorous assay. Enthalpy data were then normalized with respect to lipid content. (details in supporting information)

5.6 Drug Loading Efficiency (DLE) and Encapsulation Efficiency (EE)

DLE and EE were quantified by Chromaster HPLC (Hitachi High Technologies America, Inc., TX, USA). An analytical C₁₈ HyperSil column (Thermo-Scientific's, 4.6 × 150 mm, 5 μm) was used at 25 °C in series with a Zorbax Eclipse plus C₁₈ guard column (4.6 × 12.5 mm, 5 μm). Lapatinib was separated from liposomal components using a linear elution method (1 ml/min flow rate) with a 3:1 v/v methanol to ammonium formate (10 mM, pH 4.0) as the mobile phase, and an injection volume of 20 μl. Lapatinib absorbance was measured at 322 nm and a standard curve was generated (Figure S1). One hundred microliters of L-Lip were dried by vacuum-centrifugation (Vacufuge Plus, Eppendorf, NY, USA), and dry liposomal components were weighed (W_T) in a tared test tube. Residual liposomal structure was disrupted by dissolution in methanol (1 ml), and lapatinib content was measured by HPLC (W_{TL}). To evaluate the amount of non-encapsulated lapatinib (W_{FL}), the L-Lip solution was filtered through the 100 kDa cutoff centrifugal membrane and the membrane was rinsed with methanol to solubilize attached lapatinib. The recovered solution was further diluted 1:10 v/v in methanol and analyzed by HPLC. EE was determined as $EE = (W_{TL} - W_{FL}) \times W_{NL(100\mu l)}^{-1} \times 100$, where $W_{NL(100\mu l)}$ is the nominal amount of lapatinib in the formulation per 100 μl solution. DLE was calculated as $DLE = (W_{TL} - W_{FL}) \times W_T^{-1}$.

5.7 Liposome Stability

The stability of E-Lip and L-Lip in PBS (pH 7.4) at 37±0.1 °C and 23±0.5 °C was analyzed over 30 days using DLS, by monitoring vesicle size, PDI, and ζ. At time intervals not exceeding 4 days, 100 μl of the solutions were collected, diluted in 1 ml PB, and analyzed. Three measurements (4 runs each) were performed per sample and the mean and s of the data were calculated. Liposome stability in PBS (pH 7.4) at 37±0.1 °C and 23±0.5 °C was also evaluated by turbidimetric analysis (Turbiscan Lab® Expert, France) over 21 days at different times (days 1, 7, 11, 14, and 21). Variations in backscattering (ΔBS) profiles of the

liposomal solution^[33], which is directly correlated to particle size, were monitored at the different time points for 1 hr at 23.0±0.5 °C.

5.8 Lapatinib Release from Liposomes

Drug release from liposomes was evaluated over time by measuring in a sink reservoir the amount of free lapatinib diffusing from a cellulose dialysis membrane containing 1 ml L-Lip, by HPLC. Dialysis membranes (25 kDa cutoff) were first hydrated for 40 min in 1x PBS water to remove sodium azide, loaded with the L-Lip solution, and immersed in 200 ml sink solution. Three different sink solutions were used: 1) 1x PBS at pH 7.4; 2) 1x PBS at pH 5.5; and 3) 10% FBS/PBS (v/v, pH 7.4); the experiment was performed over 30 days at 37 °C. Samples of the sink solutions (1 ml) were collected at regular intervals (1, 2, 3, 4, 5, 6, 8, 12, and 24 hrs and every day until day 30), replaced with the same amount of fresh buffer solution, dried under vacuum (10 hrs), reconstituted in methanol (100 µl), and analyzed by HPLC. In the case of the PBS/FBS samples, lapatinib was extracted with methanol before drying. With the exception of the PBS/FBS samples, DLS and ζ analysis were performed on the liposomal suspension contained in the dialysis membranes at the end of the experiments.

5.9 Liposomes Release from nDS

Nanochannel Delivery System membranes (nDS) with 3.6, 5, 200 and 900 nm nanochannels were microfabricated employing a sacrificial layer technique using silicon-on-insulator substrates by NanoMedical Systems, Inc. (NMS, TX, USA) as described elsewhere. All membranes were cleaned in 30% H₂O₂:70H₂SO₄ and gas tested to assess their quality as described elsewhere^[23]. Release of L-Lip and 96 nm RE-Lip through the 200 and 900 nm nDS was tested *in vitro* at 23±0.5 °C over 18 days with at least 5 replicates for each channel size. A validated custom UV-diffusion device^[19] was used, which hosted 150 µl liposomal solution (source) and a 2.5 ml UV-cuvette loaded with Milli-Q water (sink), which were separated and sealed by a pre-wetted nDS (Figure S6A). Liposome release of the sink solution was measured (~ every 3 hrs) (λ =270 and 280 nm for L-Lip and RE-Lip, respectively) by means of a DU730 UV-spectrophotometer (Beckman Coulter, Inc., CA, USA). Data were normalized with respect to absorbance at t=0 and liposome concentrations were obtained using the standard curve (Figure S7). On days 7, 11, 14, and 18, one or more experimental replicates for each channel size was interrupted, and DLS was performed on both source and sink solutions to assess liposomes integrity (size, PDI, and ζ). Meanwhile, the release of free lapatinib was tested over 30 days from 3.6 and 5 nm nDS (5 replicates each). nDS were assembled with 350 µl titanium capsules loaded with 3 mg lapatinib powder, sealed, and filled with 250 µl Milli-Q water through a septum (Figure S6B). Capsules were then incubated at 37° C in borosilicate bottles containing 10 ml Milli-Q water. Sink solution was continuously homogenized through magnetic stirring, samples were collected daily, and lapatinib release was measured by HPLC.

5.10 Intravital Microscopy (IVM)

Transgenic Tie-2 mice possessing GFP-labeled endothelial cells were employed. Twenty-four hours before imaging, mice were injected with autologous red blood cells (RBCs, ~3% hematocrit) labeled with DiD for blood flow visualization^[24]. Animals received a single subcutaneous injection of 80 µl RE-Lip (96 and 117 nm) or free rhodamine, and were immediately imaged alive under an upright Nikon A1R scanning confocal microscope customized for IVM. The subcutaneous areas of interest were exposed for IVM via an aseptic skin-flap procedure under isofluorane anesthesia. High-resolution images of the mammary vasculature and surrounding tissues (~10 mm from the injection site) were acquired for 1 hr at 30 sec intervals across randomly selected fields-of-views (FOVs).

Simultaneous excitation with 488, 541, and 640 nm lasers, was performed while collecting emissions centered at 525, 579, and 670 nm for GFP, rhodamine and RBCs, respectively, using band-pass filters of approximately 30 nm. Animals were handled according to the IACUC protocol AUP-0611-0032 at The Methodist Hospital Research Institute. Multiple, circular regions-of-interest (ROI) were randomly placed inside vessel segments in each collected frame, maintaining at least 1 pixel separation between the ROI and vessel margins. The mean fluorescence intensity of each ROI was obtained for each video frame using Nikon NIS Elements v4.0 software, and plotted as a function of time, after background correction, and normalization with respect to the maximum intensity value.

5.11 Biological Activity of Liposomes: MTT Assay

BT474 cells were grown in DMEM/ high glucose medium with 10% FBS and 1% Pen/Strep (5 ml stock). Cells were maintained as monolayers that were 80% confluent at 37 °C in 5% CO₂ and detached with a 0.25% trypsin solution. BT474 cells were seeded into 96-well plates (20,000 cells/well in 200 µl medium) and left to adhere overnight. Cells were treated with fresh L-Lip or L-Lip released from the nDS (day-7 sample) or lapatinib in DMSO at concentrations of 0.1, 0.05, and 0.01 µM. E-Lip, DMSO, and untreated cells were used as controls (corresponding amounts to those of treated groups). Cell proliferation was checked at 24 and 72 hrs. MTT reagent (150 µl, 0.5 mg/ml) was added to each well, incubated for 1 hr (37 °C), replaced by DMSO (150 µl), and incubated for 15 mins (23 °C). The UV-absorbance of the supernatant was then measured at 590 nm (Synergy H4 plate reader, BioTek, USA). Each treatment group was performed in 6 replicates for which the mean and s of the data were calculated.

5.12 Confocal Microscopy of Liposome Internalization

BT474 cells (10⁵) were left to adhere overnight on a glass-bottomed chamber and then incubated with diluted 96 nm RE-Lip (50 µg/ml) for 6 hrs at 37 °C. After staining cellular membranes with 488-WGA (10 min, 37 °C), cells were washed with PBS, fixed with 4% paraformaldehyde solution and mounted with ProLong Gold reagent containing DAPI for nuclear staining. Zeta (z)-stack images were collected with a Nikon Eclipse confocal microscope and a 60x objective, and were analyzed with Nikon NIS Elements v4.0 software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thanks Thomas Geninatti, Eugenia Nicolov, Juliana Sih, and Fatema Dalal for support in the *in vitro* testing, and Rohan Bhavane and Fazle Hussain for the support in manuscript preparation. Membranes were provided by NanoMedical Systems (NMS). This work was supported with funds from the Methodist Hospital Research Institute, The Cancer, Prevention, and Research Institute of Texas (CPRIT) for the Innovative Thinking Fellowship, the Department of Defense (DODW81XWH-09-1-0212), NIH/NCI PS-OC grant U54CA143837, and the Research Fellowship Ministero Universita e Ricerca Scientifica (MIUR), Italy.

References

1. Torchilin VP. Nat Rev Drug Discov. 2005; 4:145. [PubMed: 15688077]
2. Maeda H, Wu J, Sawa T, Matsumura Y, Hori K. J Control Release. 2000; 65:271. [PubMed: 10699287]
3. Drummond DC, Meyer O, Hong K, Kirpotin DB, Papahadjopoulos D. Pharmacol Rev. 1999; 51:691. [PubMed: 10581328]
4. Barenholz Y. Nat Nanotechnol. 2012; 7:483. [PubMed: 22864165]

5. Papahadjopoulos D, Gabizon A. *Prog Clin Biol Res.* 1990; 343:85. [PubMed: 2198586]
6. Crommelin DJ, Storm G. *J Liposome Res.* 2003; 13:33. [PubMed: 12725726]
7. Pasut G, Veronese FM. *J Control Release.* 2012; 161:461. [PubMed: 22094104]
8. Scharovsky OG, Mainetti LE, Rozados VR. *Curr Oncol.* 2009; 16:7. [PubMed: 19370174]
9. Song X, Long SR, Barber B, Kassed CA, Healey M, Jones C, Zhao Z. *Curr Clin Pharmacol.* 2012; 7:56. [PubMed: 22299770]
10. Mantripragada S. *Prog Lipid Res.* 2002; 41:392. [PubMed: 12121719]
11. DiTizio V, Karlgard C, Lilge L, Khoury AE, Mittelman MW, DiCosmo F. *J Biomed Mater Res.* 2000; 51:96. [PubMed: 10813750]
12. Pugach JL, DiTizio V, Mittelman MW, Bruce AW, DiCosmo F, Khoury AE. *J Urol.* 1999; 162:883. [PubMed: 10458402]
13. New, RRC. *Liposomes as a tool in basic research and industry.* CRC press; 1995. p. 3
14. Golf M, Daniels SE, Onel E. *Adv Ther.* 2011; 28:776. [PubMed: 21842428]
15. Carvalho B, Riley E, Cohen SE, Gambling D, Palmer C, Huffnagle HJ, Polley L, Muir H, Segal S, Lihou C, Manvelian G. *G. DepoSur Study. Anesth Analg.* 2005; 100:1150. [PubMed: 15781537]
16. Jaeckle KA, Batchelor T, O'Day SJ, Phuphanich S, New P, Lesser G, Cohn A, Gilbert M, Aiken R, Heros D, Rogers L, Wong E, Fulton D, Gutheil JC, Baidas S, Kennedy JM, Mason W, Moots P, Russell C, Swinnen LJ, Howell SB. *Journal of Neuro-Oncology.* 2002; 57:231. [PubMed: 12125986]
17. Stenekes RJ, Franssen O, van Bommel EM, Crommelin DJ, Hennink WE. *Pharm Res.* 1998; 15:557. [PubMed: 9587951]
18. Farrell S, Sirkar KK. *Journal of Membrane Science.* 1997; 127:223.
19. Grattoni A, Shen H, Fine D, Ziemys A, Gill JS, Hudson L, Hosali S, Goodall R, Liu X, Ferrari M. *Pharm Res.* 2011; 28:292. [PubMed: 20593302]
20. Baselga J, Bradbury I, Eidtmann H, Di Cosimo S, de Azambuja E, Aura C, Gomez H, Dinh P, Fauria K, Van Dooren V, Aktan G, Goldhirsch A, Chang TW, Horvath Z, Coccia-Portugal M, Domont J, Tseng LM, Kunz G, Sohn JH, Semiglazov V, Lerzo G, Palacova M, Probachai V, Pusztai L, Untch M, Gelber RD, Piccart-Gebhart M, Neo AST. *Lancet.* 2012; 379:633. [PubMed: 22257673]
21. Rameez S, Palmer AF. *Langmuir.* 2011; 27:8829. [PubMed: 21678920]
22. Redondo-Morata L, Giannotti MI, Sanz F. *Langmuir.* 2012; 28:12851. [PubMed: 22873775]
23. Sih J, Bansal SS, Filipini S, Ferrati S, Raghuwansi K, Zabre E, Nicolov E, Fine D, Ferrari M, Palapattu G, Grattoni A. *Anal Bioanal Chem.* 2012
24. van de Ven AL, Kim P, Haley O, Fakhoury JR, Adriani G, Schmulen J, Moloney P, Hussain F, Ferrari M, Liu X, Yun SH, Decuzzi P. *Journal of Controlled Release.* 2012; 158:148. [PubMed: 22062689]
25. Mayer LD, Tai LC, Ko DS, Masin D, Ginsberg RS, Cullis PR, Bally MB. *Cancer Res.* 1989; 49:5922. [PubMed: 2790807]
26. Allen TM, Hansen CB, Guo LS. *Biochim Biophys Acta.* 1993; 1150:9. [PubMed: 8334142]
27. Shabbits JA, Chiu GNC, Mayer LD. *Journal of Controlled Release.* 2002; 84:161. [PubMed: 12468219]
28. Ferrati* S, Fine* D, You J, De Rosa E, Hudson L, Zabre E, Hosali S, Zhang L, Hickman C, Bansal S, Cordero-Reyes AM, Genanatti T, Sih J, Goodall R, Palapattu G, Kloc M, Ghobrial RM, Ferrari M, Grattoni A. *Nature Medicine (In Submission).* 2012
29. Kaufmann S, Papastavrou G, Kumar K, Textor M, Reimhult E. *Soft Matter.* 2009; 5:2804.
30. Rusnak DW, Lackey K, Affleck K, Wood ER, Alligood KJ, Rhodes N, Keith BR, Murray DM, Knight WB, Mullin RJ, Gilmer TM. *Mol Cancer Ther.* 2001; 1:85. [PubMed: 12467226]
31. Calvagno MG, Celia C, Paolino D, Cosco D, Iannone M, Castelli F, Doldo P, Frest M. *Curr Drug Deliv.* 2007; 4:89. [PubMed: 17269921]
32. Ruozzi B, Belletti D, Tombesi A, Tosi G, Bondioli L, Forni F, Vandelli MA. *Int J Nanomedicine.* 2011; 6:557. [PubMed: 21468358]

33. Celia C, Trapasso E, Cosco D, Paolino D, Fresta M. *Colloids Surf B Biointerfaces*. 2009; 72:155. [PubMed: 19376689]

Figure 1.

Tabulated physicochemical properties of liposomes, including size, polydispersity index (PDI), Z-potential (ζ), encapsulation efficiency (EE) and drug loading efficiency (DLE) in terms of μ g of loaded drug per mg of liposomal formulation (A); Wet AFM micrograph and (B); TEM micrograph of E-Lip vesicles (C); schematic of the L-Lip structure (D).

Figure 2.

Results of stability analysis performed at 37 and 23 °C in PBS on L-Lip compared to E-Lip, over a period of 30 days. DLS data for vesicle size (A and B), PDI (D and E), and ζ (G and H) are shown at 37 and 23 °C, respectively. Differential scanning calorimetric thermograms (C), ripple-to-liquid crystal transition temperature T_m (F), and enthalpy variation ΔH (I), for L-Lip at increasing molar ratios of lapatinib are shown.

Figure 3.

Picture of a silicon nanochannel membrane employed in this analysis (A); SEM micrograph of a cross-section (CS) of the internal architecture of the nDS; magnified SEM micrograph of a 200 nm nanochannel cross section displaying, by means of size comparison, a schematic of a 100 nm liposome (C); cumulative and percentage release profiles of L-Lip and RE-Lip through 200 and 900 nm channels over a period of 18 days (D and G, respectively); DLS results of size (E, H) and PDI (F, I) of L-Lip and RE-Lip contained in source and sink reservoirs of diffusion devices for 900 nm (top-graph) channels and 200 nm channels (bottom-graph). Correlation coefficient values R^2 are also shown for linear trend for L-Lip and Re-Lip released through 200 nm membranes.

Figure 4. Mice vessels fluorescence intensity profiles over time after subcutaneous injection of free rhodamine and RE-Lip (96 and 117 nm).

Figure 5.

Cellular interaction and biological activity of liposomes. Z-stack confocal image of RE-Lip internalized in 488-WGA-labeled BT474 breast cancer cells, top and 3D side views (A); MTT proliferation assay of BT474 cells treated with fresh or nDS-released L-Lip for 72 hrs at the concentrations of 0.1, 0.05, and 0.01 μM (B); proliferation rate profiles for L-Lip at 0.1 μM lapatinib (C).