Rigid-Liposomes with Engineered 'Raft-Switches' for controlled release of Therapeutics

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1. Introduction

In cancer therapies, advances in liposome research show strong potential in vitro, but, in the clinic, disseminated metastatic cancer is still incurable. This is largely due to low tumor absorbed doses, and low drug bioavailability within the cancer cells that constitute the tumor. In drug delivery to metastatic tumors with developed vasculature, the preferential tumor accumulation and retention of liposomes is primarily dependent on their size (EPR effect), and results in adequate tumor absorbed doses [1]. One major issue of concern, however, is the inadequate release of drug inside the tumors' cancer cells in vivo. pH-sensitive liposomes that release their 'cargo' after their endocytosis by cancer cells have been extensively studied for this purpose [2-4], but have limited in vivo application due to either loss of the liposome pH-sensitive character in the biological milieu [5] or due to their structural instability in vivo that results in low tumor accumulation (low tumor absorbed doses) and toxicity in vital organs [6]. We propose a novel type of liposomes that can combine efficient release of contents in cancer cells in vivo without loss of their intact structure, exhibiting, therefore, adequate tumor accumulation.

Increased phospholipid-membrane rigidity prevents liposome clearance, thus increasing liposome accumulation in tumors, and also enhances drug retention in liposomes during blood circulation [7]. But, after endocytosis, conventional rigid-membrane liposomes have limited capacity to release their contents during the acidification of the endosomal lumen, resulting in low drug bioavailability in cancer cells. On the other hand, conventional pH-sensitive liposomes have significantly shorter blood-circulation times which translate into decreased tumor uptake,and higher toxicity in normal organs [2]. In conventional pH-sensitive liposomes, addition of PEGylated lipids that increase the circulation times, aborts their pH-sensitive character [5].

We developed pH-sensitive liposomes with rigid membranes that combine complete release of contents in the acidic endosome, and, potentially, long blood circulation times. To achieve this, we designed and engineered liposomes containing raft-forming rigid lipids that are triggered to assemble into rafts as a response to the endosomal pH. At physiological pH (during circulation) the liposome membrane is 'homogeneous' and the contents cannot leak. At the endosomal pH, raft-formation takes place, and the encapsulated contents are released due to membrane discontinuities along the raft boundaries. The liposomal membrane is composed of rigid lipids, and is covered with PEG-chains that reportedly increase blood circulation times. PEGylation does not interfere with the pH-sensitive properties of the developed liposomes. Serum proteins accelerate the release of contents at the endosomal pH. These liposomes will potentially combine high tumor absorbed doses and high drug bioavailability in vivo.

2. Materials and Methods

2.1 Materials

The lipids 1,2-Dipalmitoyl-sn-Glycero-3-Phosphocholine (DPPC), 1,2-Distearoyl-sn-Glycero-3-Phosphate (Monosodium Salt) (DSPA), 1,2-Dipalmitoyl-sn-Glycero-3-Phosphoethanolamine-N-[Methoxy(Polyethylene glycol)-2000] (Ammonium Salt),1,2-Distearoyl-sn-Glycero-3-Phosphoethanolamine-N-[Methoxy(Polyethylene glycol)-2000] (Ammonium Salt), 1,2-Dipalmitoyl-sn-Glycero-3-Phosphoethanolamine-N-(Lissamine Rhodamine B Sulfonyl) (Ammonium Salt), 1,2-Dioleoyl-sn-Glycero-3-Phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (Ammonium Salt) (purity > 99%) were purchased from Avanti polar lipids (Alabaster, AL). Fetal bovine serum was purchased from Omega scientific (Tarzana, CA). RPMI 1640 was purchased from Cellgro Mediatech Inc. (Herndon, VA). The polycarbonate membranes for liposome extrusion were purchased from Avestin Inc., (Ottawa, Canada). Sodium azide, Calcein, cholesterol and Triton X-100 were purchased from Sigma Aldrich Chemical Company (Milwaukee, WI).

2.2 Preparation of liposomes

Lipids (in chloroform) were combined in a 25ml round bottom flask. Chloroform was evaporated in a Buchi rotavapor R-200 (Buchi, Flawil, Switzerland) for 10 minutes at 55^{o}C followed by evaporation under N_2 stream for 5 minutes. The dried lipid film was then hydrated in 1 ml PBS (1mM EDTA, pH = 7.4) for 2 hours at 50 ^{o}C. The lipid suspension was then extruded 21 times through two stacked polycarbonate filters of 100 nm pore diameter (Avestin Inc., Ottawa, Canada). Extrusion was carried at 80^{o}C in a water bath. For calcein quenching efficiency experiments, the lipid film was hydrated in 1 ml of 55mM calcein solution (self quenching concentration) iso molar to PBS. After extrusion the unentrapped calcein was removed by size exclusion chromatography (SEC) using a G-50 column (11 cm in length) eluted by PBS (1mM EDTA, pH = 7.4).

2.3 Calcein leakage

Liposomes containing self quenching concentrations of calcein were incubated in PBS and serum supplemented media (10% FBS) at pH 7.4, 5.5, 4.0 at 37^{o}C. Leakage of calcein from liposomes and its dilution in the surrounding solution results in increase in fluorescence. Calcein leakage was measured at different time points for the different conditions by adding fixed quantities of incubated liposomes into cuvettes (1 cm path length) containing PBS (1mM EDTA, pH 7.4). Calcein fluorescence was measured using a Fluoromax -2 (Horiba Jobin Yvon, NJ) before and after addition of Triton-X 100 (excitation: 495 nm and emission: 515 nm). Calcein quenching efficiency was calculated by taking the ratio of fluorescence intensity before and after addition of Triton-X 100. Percentage decrease in quenching efficiency was calculated from the following expression:

$$\%\ \text{decrease in quenching efficiency} = \left(1 - \frac{Ratio_i - 1}{Ratio_{\max} - 1}\right) \times 100$$

where,

Ratio $_i$ is the calcein quenching efficiency in the corresponding buffer,

Ratio $_{max}$ is the calcein quenching efficiency in PBS 7.4 immediately after separation of liposomes by SEC. Ratio of 1 indicates complete leakage.

2.4 Interliposome fluorescence resonance energy transfer (FRET)

Interliposome FRET was used to determine whether liposome fusion occurs at the various conditions studied. Two populations of liposomes were prepared. One population contained 1,2-Dipalmitoyl-sn-Glycero-3-Phosphoethanolamine-N-(Lissamine Rhodamine B Sulfonyl) (Ammonium Salt) and 1,2-Dioleoyl-sn-Glycero-3-Phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (Ammonium Salt) (0.5 mole% each). The other population did not contain any fluorophores. Samples from both populations of liposomes were mixed and compared to samples containing only liposomes from the fluorescent liposome population. Fusion increases the effective distances between fluorophores, resulting in lower interliposomal FRET intensities. Fluorescence intensities were measured at excitation: 496 nm and emission: 590 nm for all samples. The fluorescent intensities of samples were monitored over time and the ratios of the intensities of samples containing both liposome populations divided by the intensities of samples containing only the fluorescent liposomes were calculated. Ratios close to unity would indicate no fusion.

2.5 Dynamic Light scattering

Dynamic light scattering (DLS) of liposome suspension was studied with an N4 plus autocorrelator (Beckman-Coulter, Fullerton, CA), equipped with a 632.8 nm He-Ne laser light source. Scattering was detected at 23.0, 30.2, 62.6 and 90^o. Particle size distributions at each angle were calculated from autocorrelation data analysis by CONTIN [8]. The average liposome size was calculated to be the y-intercept at zero angle of the measured average particle size values versus $\sin^2(\theta)$ [9]. All buffer solutions used were filtered with 0.22 μm filters just before liposome preparation. The collection times for the autocorrelation data were 1 - 5 minutes.

3 Results and discussion

3.1 Calcein Quenching efficiency

The calcein quenching efficiency of DPPC: DSPA ‘raft-forming’ liposomes at different pH values at 37^{o}C was evaluated in PBS and serum supplemented media (10 % FBS). Figure 1 shows increase in content release with decreasing pH. pH sensitivity was not altered by the addition of PEG.

Leakage of encapsulated contents from liposomes should be fast while liposomes are in the endosomal or early lysosomal stage (30-60 minutes upon endocytosis within pH range of 6.5 - 5) to prevent enzymatic degradation at the lysosome (pH = 4) [10]. In the case of serum supplemented media, the kinetics of content release was observed to be faster than in PBS.

Figure 1 (A) indicates instantaneous and almost complete leakage at pH 4 and significant leakage at pH 5.5 within the first 100 minutes.

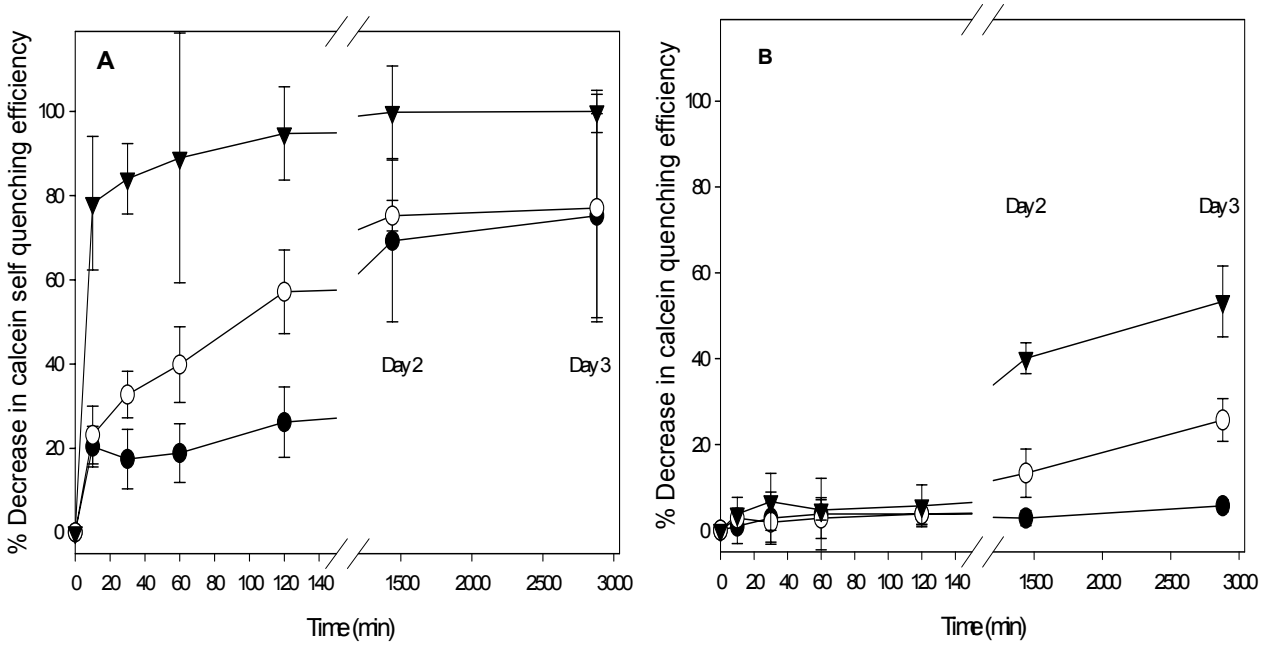

Figure 1. (A) Percent decrease in calcein self quenching in serum supplemented media at 37°C for different pH values. ***(B)*** Percent decrease in calcein self quenching in PBS at 37°C for different pH values. ● :pH 7.4,○:pH 5.5, ▼:pH 4.0.Error bars correspond to standard deviations of repeated measurements (n=3).

3.2 Interliposome Fluoroscence resonance energy transfer (FRET)

Liposome fusion was studied by interliposome FRET. Tables 1 and 2 show that the ratio of intensities of suspensions containing both labeled and non labeled liposomes are close to unity for all pH values and do not change over time, indicating no fusion.

Table 1. *Ratios of intensities for interliposome FRET in PBS at different pH values measured over time*

	pH 7.4	pH 5.5	pH 4
Day 1	1.0 ± 0.1	1.0 ± 0.1	1.0 ±0.0
Day 2	1.0 ± 0.1	1.0 ± 0.1	1.1 ± 0.1
Day 3	1.0 ± 0.0	1.0 ± 0.1	1.0 ± 0.1

Table 2. *Ratios of intensities for interliposome FRET in serum supplemented media at different pH values measured over time*

	pH 7.4	pH 5.5	pH 4
Day 1	1.1 ± 0.0	1.1 ± 0.1	1.0 ± 0.0
Day 2	0.9 ± 0.0	1.1 ± 0.0	1.0 ± 0.0
Day 3	0.9 ± 0.0	1.1 ± 0.0	1.1 ± 0.0

3.3 Dynamic Light scattering

Measurements of the size distribution of liposome suspensions in different solutions and at different pH values over time, showed no significant change in liposome size that suggests no fusion occurs between the liposomes and agrees with the hypothesis that content leakage occurs due to formation of phase-separated lipid domains at lower pH values.

4. Conclusion

This study shows that non-fusing, pH sensitive, rigid PEGylated liposomes containing 'raft switches' hold great potential for enhancing drug bioavailability within the endosomal pathway of cancer cells. The liposomal membrane rigidity combined with the PEGylated surface should result in long blood circulation times.

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