Research Article

Dynamic Probing of Nanoparticle Stability In Vivo: A Liposomal Model Assessed Using In Situ Microdialysis and Optical Imaging

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Nanoparticle-mediated drug delivery and controlled release has been a vigorous research area in contemporary nanomedicine. The in vivo stability of nanoparticle delivered on site is a prerequisite for the design of drug-controlled release by any means. In this study, the first methodology comprised of microdialysis and optical imaging to assess the liposome stability in vivo is reported. Macroscopically, we demonstrated the DPPG liposomes with negative surface charge fast accumulated in the rat liver upon their i.v. administration using optical imaging. Microscopically, the concurrent analysis of fluorescent molecules leaching from the liposomes, in situ sampled using microdialysis probe, provides the dynamic information of stability of DPPG liposomes locus in quo. The current combination of in situ microdialysis and optical imaging possesses a great potential for use as a platform technology to evaluate the nanoparticle stability and the bioavailability of drug payload released on targeted site in vivo.

1. Introduction

Nanoparticle-mediated drug delivery has been emerging as a vigorous research area. The nanoformulation of chemotherapeutics can improve the pharmacokinetics; the controlled release of drug can be further exploited with either inherent pathophysiological conditions like acidic pH in tumor, or external physical means such as photoirradiation and alternating magnetic fields [1–4]. The high stability of nanoparticle delivered on-site is a critical prerequisite for the design of drug controlled release by any means. The otherwise spontaneous release of drug will result in unwanted side effects and collateral tissue damages, to compromise the advantages of using nanoparticles as carriers. Thus, to define a methodology that can dynamically monitor the stability of targeted nanoparticles and bioavailability of drug payload locus in quo is important in validation of the activatable drug release strategy.

Nanomedicine approaches to drug delivery center on developing nanoscale particles to improve the bioavailability of a drug. The biodistribution of nanoparticles including blood circulation, immunosystem interaction, clearance, and metabolism are synergically determined by many factors such as size, shape, surface chemistry, and charge. Among nanoparticles, liposomes have played an important role in formulation for potential drugs to improve their therapeutic index. Due to their biocompatibility and biodegradability, liposomes are by far the most studied colloidal particles applied in medicine, particularly in antitumor therapy. The use of liposomes as drug carriers requires the liposomal preparations with various clearance rates and biodistribution patterns to better fit the specifics of each particular application. Liposome charge and liposome coating with different polymers, such as PEG, are among the parameters known to strongly affect biological properties of liposomes [5]. During the delivery process, after extravasation into
tumor tissue, liposomes remain within tumor stroma as a drug-loaded depot and eventually are subject to enzymatic degradation and/or phagocytic attack, leading to release of drug for subsequent diffusion to tumor cells. Therefore, the temporal profiles of biodistribution and stability of nanoformulated contrast agent/drug carrier in vivo are critical in determining the imaging/therapeutic efficacy and the necessity for advanced design of activatable controlled release.

There are a number of imaging modalities that have been reported to trace the biodistribution of nanoparticles in vivo, including positron emission tomography (PET) [6], magnetic resonance imaging (MRI) [7, 8], and optical image system [9]. However, these techniques, though noninvasive, are not sensitive enough to reflect the dynamic changes of nanoparticle stability incurred by variations of pathophysiological microenvironments or activations of external means for drug-controlled release. The indiscernibility of image signals contributed from the contrast agent of intact nanoparticles and that leaching from the disintegrated nanoparticles can seriously impede the interpretation of nanoparticle biodistribution. Current maneuvers to validate the nanoparticle biodistribution measured using imaging modalities are to acquire ex vivo tissue dissections for inductively coupled plasma-mass spectroscopy (ICP-MS) measurement and/or TEM imaging with energy dispersed X-ray spectroscopy (EDX) for element analysis [10, 11]. By verifying the physical residence of nanoparticles in tissues that colocalized with the imaging contrast, it can assure an appropriate interpretation of nanoparticle biodistribution using the aforementioned non-invasive imaging modalities. Nevertheless, these processes are time consuming and difficult to acquire dynamic profiles of nanoparticle biodistribution and stability locus in quo.

Alternatively, to assess the stability of nanoparticle in response to local variations of microenvironments as residing in tissue, we exploited an in vivo microdialysis system. Such a technique used for intracerebral sampling, for example, can provide continuous monitoring of levels of compounds of a single animal and has been widely used for pharmacological and physiological studies to assay endogenous neurotransmitters or exogenous compounds [12, 13]. In the previous report [14], we applied fluorescent polystyrene nanospheres (20 nm) to study the blood-brain barrier (BBB) permeability and used microdialysis probe which was implanted in the cerebral cortex of an anesthetized rat to in situ monitor the extravasation of administered fluorescent nanospheres into the brain across the blood-brain barrier. As illustrated in Scheme 1, since the microdialysis probe contains membrane with a specific molecular weight cutoff, it possesses a selective permeability for molecules with different sizes at locus of tissue, in the close vicinity of where the probe is positioned. If the contrast agent- or drug-encapsulating nanoparticles on site can maintain their morphological stability, their size will be too large to be selectively collected through the microdialysis membrane. On the contrary, the contrast agents or drug molecules leaching from the disintegrated nanoparticles, due to their small size, can easily diffuse through the probe membrane and be collected in microdialysates for further analysis. Thus, the microdialysis system has the potential of usage as a platform for dynamic monitoring of the nanoparticle stability in vivo, locus in quo.
Figure 2: (a) The time-lapse optical images showed that liposome containing 10 mM CF, with the size of 200–240 nm, fast accumulated in rat liver as soon as 5 min after i.v. administration. The fluorescence intensity observed from liver gradually increased and reached its plateau in 100 min. To evaluate the stability of these on-site DPPG-liposomes in liver, the microdialysis probe with the molecular size cutoff at 100 kDa was implanted in situ to simultaneously analyze the fluorescence signals of extracellular fluid in liver, as indicated with a red-dotted circle. (b) The profile of fluorescence intensity in microdialysate showed no significant variation throughout the concurrent optical imaging course after the i.v. injection of CF-encapsulating liposomes.

In the present study, we synthesized carboxyfluorescein-(CF-)encapsulating liposomes with different surface charges as models of drug carrier. An analytical method was implemented that combined the optical image system as well as in vivo microdialysis sampling technique to simultaneously monitor the biodistribution of liposomes in vivo and to assess their on-site stability at the extracellular space of liver tissue, where the liposomes accumulated the most after the intravenous (i.v.) administration. The current instrument configuration comprised of the optical image system and the microdialysis platform can provide dynamic observations of nanoparticle stability in vivo, from the macroscopic to microscopic scales.

2. Experimental Section

2.1. Preparation of Referenced CF-Encapsulating Liposomes (Dipalmitoylphosphatidyl Choline-Liposome: DPPC-Liposome). Liposomes were prepared using the film hydration method [14]. The lipid mixture consisted of a 10 : 10 : 1 molar ratio of DPPC, cholesterol, and DPPG. The total lipid mixture was dissolved in 4 mL of a solvent mixture consisting of chloroform and methanol (8 : 2), followed by a 1-min sonication at 45°C. The organic solvent was then removed under vacuum on a rotary evaporator, leaving a milky white, gel-like suspension of proliposomes. It was followed by addition of 1 milliliter of CF solution (10 mM or 100 mM) to the lipid mixture. After sonication for 3 more min, the liposome preparation was ready to pass through a 0.2-µm polycarbonate filter 20 times for the production of a homogeneous suspension of uniform size. Any unencapsulated dye or trace organic solvent was removed from the liposome preparation by gel filtration on Sephadex G-50 column at room temperature, followed by dialysis (MWCO, 12–14 kDa) against 0.01 M potassium phosphate buffer (pH 7.0) at 4°C in the dark.

2.2. Preparation of Relatively Cationic CF-Encapsulating Liposomes (Dipalmitoylphosphatidyl Ethanolamine-Liposome: DPPE-Liposome). The procedure was similar to that for DPPC liposome, except the lipid mixture used for DPPE liposome consisted of a 10 : 10 : 3 molar ratio of DPPC, cholesterol and DPPE.
2.3. Preparation of Relatively Anionic CF-Encapsulating Liposomes (Dipalmitoylphosphatidyl Glycerol-Liposome: DPPG-Liposome). The procedure was similar to that for DPPC liposome, except the lipid mixture used for DPPG liposome consisted of a 10 : 10 : 3 molar ratio of DPPC, cholesterol, and DPPG.

2.4. The HepG2 Cell Line Experiments. Human hepatoma cells (HepG2) were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco; Carlsbad, CA) with 10% fetal bovine serum (Hyclone; Logan, Utah). Cells were seeded onto glass bottom dishes (35 mm OD × 10 mm High, glass area: 22 mm dia., and glass thickness: 0.17 mm) (WillCo Wells BV; Amsterdam, Netherlands) at density of 1 × 10^5 cells /dish, and cultured overnight in culture medium. Cell nucleus were labeled by 10 mM Hoechst33342 (Molecular probe, Carlsbad, CA) for 5 min and rinsed twice with PBS buffer. DPPC liposome or DPPE liposome, encapsulating 100 mM carboxyfluorescein, were added to cells at concentration 5 × 10^5 liposome/ml in serum-free medium and cells were incubated for different time periods from 1 h to 4 h. Unfused liposome were removed by washing twice with PBS and replaced with DMEM medium without phenol red. Live cells were observed with Leica AS MDW system (Leica Microsystems; Wetzlar, Germany) at high magnification (63X). Carboxyfluorescein was imaged using 490 nm excitation wavelengths and 530/30 nm BP emission filter. Hoechst 33342 was excited by 380 nm and its emission was collected by 465/20 nm BP emission filter.

2.5. In Vivo Microdialysis System. The microdialysis system was perfused with 50 mM phosphate buffer solution (pH 7.4) at a flow rate of 2 µL/min. Microdialysis probes, made of polyethylene sulfonate with a molecular weight cutoff at 100,000, were purchased from CMA (Carnegie Medicine Association, Solna, Sweden). The microdialysates were allowed to flow through a polyimide-coated fused-silica capillary (360-µm o.d., 250-µm i.d.). A 2-cm segment of the capillary was stripped of the polyimide coating to serve as the detection window and placed inside a fluorescence detector (Argos 250 FL detector, Flux Instruments, Basel, Switzerland). Polyethylene catheters were inserted into the liver of the rats.

2.6. Optical Imaging System. The light from a 100 W mercury lamp (color temperature is 5700 K) was passed with
The average diameters of the liposomes were measured using the Coulter LS particle analyzer (Coulter Corp., Miami, FL). The spectral analysis displayed the maximal absorption and fluorescence emission of CF-encapsulated liposomes at 495 nm and 520 nm, respectively, which resembled those of CF solution (Figure 1).

Since the biodistributions of liposome and of other nanoparticles were reported to greatly depend on the surface charge [15–17], the zeta potentials of DPPG- and DPPE-liposomes were measured and listed in Table 2. As described in Experimental Section, to vary the molar ratios of DPPG and DPPE to DPPC and cholesterol in lipid mixtures, the constructed liposomes can possess different surface charges. The DPPG-liposomes containing 10 mM and 100 mM CF exhibited negative zeta surface potentials of $-43.30$ mV and $-38.16$ mV at pH 7.4, respectively. In contrast, the DPPE-liposomes encapsulating 10 mM and 100 mM displayed the relatively positive surface potentials of $-10.99$ mV and $-15.00$ mV at pH 7.4, respectively (Table 2).

### Table 1: Characteristics of fluorescent DPPG-liposome.

<table>
<thead>
<tr>
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<th>10 mM CF-liposome</th>
<th>100 mM CF-liposome</th>
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<tbody>
<tr>
<td>Diameter</td>
<td>202.33 nm$^a$</td>
<td>250.3 nm$^a$</td>
</tr>
<tr>
<td>Volume of liposome</td>
<td>$4.34 \times 10^{-12}$ $^b$</td>
<td>$8.21 \times 10^{-12}$ $^b$</td>
</tr>
<tr>
<td>Number of Liposome</td>
<td>$1.44 \times 10^{13}$ $^c$</td>
<td>$8.95 \times 10^{12}$ $^c$</td>
</tr>
<tr>
<td>Dye molecule/liposome</td>
<td>23132</td>
<td>448557</td>
</tr>
<tr>
<td>Concentration of CF</td>
<td>10 mM</td>
<td>100 mM</td>
</tr>
</tbody>
</table>

$^a$The particle size is average.
$^b$The unit is µL.
$^c$The unit is No. per mL.

3.2. Dequenching of Fluorescent Liposomes upon Lysis by Nonionic Surfactant. The 100 mM fluorescent CF encapsulated within DPPG liposomes with the sizes around 200 nm indicated apparent self-quenching of fluorescence, due to its relatively high concentration confined within a relatively small volume (Figure 1). The quenching effect could be evaluated by disintegrating liposomes with the nonionic surfactant, Triton X-100, to compare the fluorescence intensity before and after lysis. The lysis buffer containing surfactant Triton X-100 of 0.1% in PBS buffer (pH 7.4) was added and mixed gently to 3 mL of the liposome solution for 10 minutes at room temperature. As demonstrated in Figure 1, the liposome-encapsulated CF at 10 mM showed comparable levels of fluorescence intensity before and after lysis, while the 100 mM specimens indicating ninefold increase of that after the liposomes being lyzed. Thus, this dramatic change of fluorescence intensity before and after liposome disintegration could be further applied to sense the stability of liposomal structure as delivered on site in tissue. On the other hand, for tracking biodistribution of liposome in vivo, the 10 mM specimen was selected, due to its traceable, bright fluorescence intensity.

3.3. In Vivo Biodistribution versus In Situ Stability of Fluorescence Liposomes. The biodistribution of CF-encapsulating DPPG liposome was monitored dynamically by homemade small animal optical imaging system, and its on-site stability was simultaneously assessed using in situ microdialysis probe. Prior to imaging, male Sprague Dawley rats (weight ca. 250 g) were placed on Teklad 2916 diets for 5 days and fasted for 12 h, to minimize rodent chow autofluorescence within the GI-tract and anesthetized with urethane (1.5 g/kg) i.p. injection. DPPG-liposomes containing 10 mM and 100 mM CF, at a dosage of 16 mg/kg, were injected i.v. via the tail vein of animals, respectively. The time-lapse optical images showed that liposome containing 10 mM CF, with the size of 200–240 nm, fast accumulated in rat liver as soon as 5 min after i.v. administration (Figure 2(a)). The fluorescence intensity observed from liver gradually increased and reached its plateau in 100 min. To evaluate...
Figure 5: The HepG2 cells treated with DPPG liposomes containing 100 mM CF (surface charge ca. $-40$ mV) showed no CF fluorescence in the cytoplasm, implicating that no cell uptake of liposomes took place during the 4h imaging course. The scale bar is 10 µm.

Figure 6: The HepG2 cells treated with DPPE-liposomes containing 100 mM CF (surface charge ca. $-10$ mV) showed pronounced increase of CF fluorescence in the cytoplasm, implicating that avid cell uptake of liposomes took place after the treatment. The scale bar is 10 µm.

Table 2: The zeta potentials of fluorescent DPPG- and DPPE-liposomes.

<table>
<thead>
<tr>
<th></th>
<th>DPPG-liposome (10 mM CF)</th>
<th>DPPG-liposome (100 mM CF)</th>
<th>DPPE-liposome (10 mM CF)</th>
<th>DPPE-liposome (100 mM CF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zeta potential (mV)</td>
<td>-43.30</td>
<td>-38.16</td>
<td>-10.99</td>
<td>-15.00</td>
</tr>
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</table>
the stability of these on-site DPPG liposomes in liver, the microdialysis probe with the molecular size cutoff at 100 kDa was implanted in situ to simultaneously analyze the fluorescence signals of extracellular fluid in liver (Scheme 1). As depicted in Figure 2(b), the profile of fluorescence intensity in microdialysates showed no significant variation throughout the concurrent optical imaging course after the i.v. injection of CF-encapsulating liposomes. It implicated that liposomes in liver were able to maintain their structural stability, preventing the encapsulated CF from leaching into the extracellular fluid and being sampled by the microdialysis probe. On the other hand, since the liposomes were not disintegrated, with the intact size of 200 nm, they were simply too large to permeate through the membrane of microdialysis probe.

For DPPG liposomes containing high concentration of 100 mM CF, due to the self-quenching phenomenon, there was no observable increase of fluorescence signal in liver of anesthetized rat during the optical imaging course, following the i.v. administration (Figure 3(a)). If the liposomes with 100 mM CF disintegrate on site, it would result in CF molecules permeating into the interstitial space of liver, consequently to compromise the self-quenching of fluorescence. Therefore, the dark time-lapse images of liver could be accounted for the macroscopic evidence that implicating the integrity of liposomes was remained intact at their loci of liver. To microscopic probing of the stability of liposomes encapsulating 100 mM CF, the positioned microdialysis in liver also revealed no increase of CF fluorescence in microdialysates over the 100-min concomitant optical imaging session (Figure 3(b)). The upshift of fluorescence baseline (from less than 0.01 to ca. 0.03 V) upon the i.v. injection of liposomes was due to the high concentration of encapsulated CF molecules. At the equilibrium, with 100 mM liposome-encapsulated CF, the concentration of free CF molecule in the prepared ready-to-use solution was much higher than that with 10 mM liposome-encapsulated CF. Consequently, it incurred the increase of baseline following the injection of liposome solution, as delineated in Figure 3(b). At later phase of imaging course, the lysis buffer of Triton X-100 was locally infused at 2 μl/min via microdialysis probe. It caused the rupture of liposomal lipid integrity, resulting in an immediate increase of CF fluorescence intensity measured in the microdialysate (Figure 3(b)).

Since the lysis buffer diffused to the tissue only in the vicinity of microdialysis probe, its effective volume on tissue was very small; only those liposomes situated around the tip of microdialysis probe were subject to the lysis. Thus, this increase of fluorescence intensity, resulted from the leaching CF, could only be sensed by the microdialysis in situ, but not by the optical imaging measurement. As such, to deliver Triton-X for liposome disintegration was not an efficient means of controlled release, albeit it provided indirect evidence to substantiate the intact of liposomal structure in situ of liver. The release of CF molecules during the Triton-X lysis was a process of concentration-dependent diffusion, therefore, to probe the integrity of liposome via this means was better to use liposomes encapsulating 100 mM, rather than 10 mM.

To further validate the CF sensing of in situ microdialysis probe, 10 μM free CF molecules were i.v. administered. As shown in Figure 4, with this CF concentration before the plasma dilution—less than a thousandth of that previously used with the liposome encapsulation, a prominent fluorescence signal was still able to be measured via the microdialysis sampling of extracellular fluid in liver. It implicated that the in situ microdialysis was suitable for monitoring of the drug controlled release in tissue locus in quo. In summary, all the evidence, from macroscopic optical imaging to microscopic microdialysis measurement, indicates that, following i.v. administration, the DPPG-liposomes would fast accumulate in the liver and reside in the extracellular space with the high stability of morphological integrity over the period of 2 h.

3.4. The Hepatoma Cell Uptakes of Liposomes versus Different Surface Charges. There are a number of reports demonstrating that the biodistribution of nanoparticles is surface charge-dependent [15–17]. In general, the nanoparticles with greater surface charge tend to be more avid for cell uptake via process like endocytosis. In the current study, the DPPG-liposomes, with the surface zeta potential of ca. −40 mV, were demonstrated to reside in the extracellular space of liver tissue. How the surface charge of liposomes leads the propensity of their interactions with cells was investigated with the in vitro model of human hepatoma cell line (HepG2). The HepG2 cells (1 × 10^5 cell number) were seeded onto a glass bottom dish and cultured overnight in culture medium. The cell nuclei were labeled by 10 mM Hoechst 33342 for 5 min and then rinsed twice with PBS buffer. Afterwards, the DPPG-liposomes (ca. −40 mV) or DPPE-liposomes (ca. −10 mV), both encapsulating 100 mM carboxyfluorescein, were respectively added to HepG2 cells at concentration of 5 × 10^5 lipidosome/ml in serum-free medium, incubated for various time periods, from 1 h to 4 h. If the treated liposomes are engulfed into cells, the encapsulating 100 mM CF can be released from the disrupted liposomal structure via two possible pathways: direct lipid-cell membrane fusion or cell endocytosis. In the latter pathway, the lipase in endosomes is responsible to digest the liposome to release the CF molecules [5]. Both cases will result in the increase of intracellular fluorescence intensity, due to the compromise of CF fluorescence self-quenching by its dilution in cytoplasm. In our study, the time-lapse fluorescence microscopy illustrated that, with the DPPG-liposomes, no CF fluorescence was observed in the cytoplasm of HepG2 cells, implicating no cell uptake of liposomes occurred in 4 h (Figure 5). However, with the DPPE-liposomes possessing relatively positive surface charge as compared to the DPPG liposomes, the pronounced intracellular fluorescence intensity was observed throughout the 4 h imaging course (Figure 6). These results of liposome-cell interaction are consistent to that for other reported nanoparticles [15–17] and are supportive to the in vivo observation of extracellular residence of DPPG liposomes in liver.
4. Conclusions

In this study, we successfully implement the first methodology comprised of microdialysis and optical imaging to dynamically assess the stability of liposome in vivo. Macroscopically, with the time-lapse optical imaging, we demonstrated that the DPPG-liposomes with surface charge around −40 mV would accumulate in the liver of anesthetized rat upon their i.v. administration in 5 min. Microscopically, the concurrent analysis of fluorescent molecules in extravascular fluid of liver tissue, in situ-sampled using microdialysis probe, provided the dynamic information of stability of DPPG-liposomes in vivo. The macroscopic results, all the evidence implicated that the DPPG-liposomes could reside in the extracellular space of liver, with stable and intact morphological integrity, over a period of 2 h at least. The microdialysis probing the lipid integrity of liposome, by analyzing the increase of fluorescence intensity in microdialysates that attributed to the leaching of CF probe, provided itself as a platform technology used for evaluation of nanoparticle stability (not only for liposome) and the bioavailability of drug payload released on targeted site in vivo. As such, the current combination of in situ microdialysis and optical imaging possesses a great potential to be used vigorously in the research of nanoparticle-mediated drug targeted delivery and controlled release.

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