Remote loading of doxorubicin into liposomes by transmembrane pH gradient to reduce toxicity toward H9c2 cells

Mohamed Alyane a,b,*, Gillian Barratt b, Mesbah Lahouel a

a Laboratoire de Toxicologie Moléculaire, Université de Jijel, BP 98, Ouled Aissa, 18000 Jijel, Algeria
b Université Paris sud-11, UMR 8612, 5 rue JB Clément, 92296 Châtenay-Malabry, France

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Abstract The use of doxorubicin (DOX) is limited by its dose-dependent cardiotoxicity. Entrapped DOX in liposome has been shown to reduce cardiotoxicity. Results showed that about 92% of the total drug was encapsulated in liposome. The release experiments showed a weak DOX leakage in both culture medium and in PBS, more than 98% and 90% of the encapsulated DOX respectively was still retained in liposomes after 24 h of incubation. When the release experiments were carried out in phosphate buffer pH5.3, the leakage of DOX from liposomes reached 37% after 24 h of incubation. Evaluation of cellular uptake of the liposomal DOX indicated the possible endocytosis of liposomes because the majority of visible fluorescence of DOX was mainly in the cytoplasm, whereas the nuclear compartment showed a weak intensity. When using unloaded fluorescent-liposomes, the fluorescence was absent in nuclei suggests that liposomes cannot cross the nuclear membrane. MTT assay and measurement of LDH release suggest that necrosis is the form of cellular death predominates in H9c2 cells exposed to high doses of DOX, while for weak doses apoptosis could be the predominate form. Entrapped DOX reduced significantly DOX toxicity after 3 and 6 h of incubation, but after 20 h entrapped DOX is more toxic than free one.

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1. Introduction

DOX is one of the most efficient anticancer drugs used against solid tumors and hematological malignancies (Hrelia et al., 2002); however, its side effects, in particular its cardiotoxicity, limit its use (Rahman et al., 2007). The mechanisms implicated in cardiotoxicity and cytotoxicity are different. The antineoplastic mechanism of DOX has been attributed to intercalation of the planar anthracycline ring into the DNA helix and/or covalent binding to proteins involved in DNA replication...
and transcription (Sardão et al., 2009). On the other hand, generation of reactive oxygen species has been proposed to be responsible for the cardiotoxicity of DOX (Alyane et al., 2008). This difference in mechanisms suggests that the acute and chronic toxicities associated with DOX could be reduced and higher doses could be used if the drug could be more efficiently targeted to cancer cells, for example, by encapsulation in liposomes.

Liposomes, unilamellar or multilamellar lipid vesicles (Santra and Mukherjee, 2009), are well established as drug delivery systems for low molecular weight molecules; when administrated intravenously, may improve the therapeutic index of some drugs due to the EPR (enhanced permeability and retention) effect (Zuker et al., 2009). The accumulation of liposomes in tumors is favored not only by a leaky microvasculature but also by an insufficient lymphatic drainage. The combination of these two factors is referred to as the “EPR phenomenon” (Minotti et al., 2004).

Liposomal formulations of DOX have been studied for a number of years as a means of reducing the cardiac toxicity of the drug because liposomal encapsulation would be expected to shift DOX away from myocardial tissue (with a normal endothelial barrier), while maintaining tumor exposure (Rahman et al., 2007). Furthermore, encapsulation of DOX within long-circulating liposomes decorated with poly(ethylene glycol) (PEG liposomes), reduces its uptake by the reticuloendothelial system and prolongs its serum half-life to around 50 h compared with 10 min for the free drug (Berry et al., 1998). As a result, DOX in PEG liposomal has been shown to have an increased therapeutic efficacy and reduced cardiotoxicity compared to free DOX (Gabizon et al., 2003) and a commercial formulation is marketed as Doxil or Caelyx (Janssen Pharmaceuticals).

In order to achieve a high yield of DOX encapsulation in liposomes a remote loading approach is used (Ogawara et al., 2009). The method involves the addition of drug to preformed liposomes through a pH gradient or an ion gradient capable of generating a pH gradient (Mayer et al., 1990). Due to their high payload of drug, liposomal systems are the best method to passively target anthracyclines to tumors, compared with microspheres or nanoparticles (Minotti et al., 2004).

Although the distribution of liposomal DOX in the body has been extensively studied, less work has been done on its interaction with cardiac cells. The objective of this work was to encapsulate DOX in PEG liposomes, investigate the mechanism of interaction between these liposomes and H9c2 cells using flow cytometry and confocal microscopy and assess the consequences for cell viability using well established tests.

2. Material and methods

2.1. Preparation of PEG liposomes

PEGylated liposomes, composed with HEPC (Hydrogenated Egg Yolk Phosphatidylcholine), CHOL (Cholesterol) and DSPE-PEG2000 (Methoxy polyethylene glycol (Mw 2000)-distearylphosphatidylethanolamine) (185:100:015, molar ratio), were prepared as previously described by Zucker et al. (2009). Briefly, the lipids were dissolved in chloroform, and after evaporation of the organic solvent with rotary evaporator (Büchi Rotavapor R-215, Switzerland) under reduced pressure at room temperature. The resulting lipid film was hydrated at 70 °C in 250 mM ammonium sulfate (pH 5.4) to form unilamellar vesicles (MLV). The lipid size was reduced by stepwise extrusion in two steps, firstly through 0.2 μm and then through 0.1 μm pore-diameter polycarbonate filters (Whatman, Maidstone, Kent, UK). Each extrusion step was performed 5–10 times at 70 °C using a high-pressure extruder (Northern Lipids, Inc. Burnaby, BC, Canada).

The mean diameters of the resulting liposomes were determined by photon correlation spectroscopy (PCS) on a Malvern Zetasizer (Zetasizer Nano ZS Zen 3600, Malvern instruments, UK) at 25 °C, using water as the dispersant. Three measurements were taken on each sample.

In the aim to study interaction between liposomes and H9c2 cells, fluorescent liposomes were prepared following the same steps but with the addition of 1% of Rhodamine B-DHPE (Sigma) to the lipid mixture.

2.2. DOX loading

DOX (Chemos GmbH, Germany) was loaded into preformed liposomes via a transmembrane pH gradient method and the free DOX was removed by gel filtration. Briefly, the liposomal suspension was dispersed within a vial containing a solution of DOX (10 mg/mL) was added at pH 7.4 to create a transmembrane PH gradient (drug to lipid ratio 1/20 w/w). This suspension was stirred for 1 h at 60 °C. Subsequently, unloaded DOX was removed by Sephadex G25 column pre-equilibrated with PBS pH7.4.

The amount of encapsulated DOX in PEG liposomes (DOXEnc) was determined by measuring absorption at 483 nm using UV–visible spectrophotometer (UVikon 930, Kontron Instruments, Germany) after lysis of the liposomes with 10% SDS (w/v) (Mayer et al., 1990).

Drug encapsulation efficiency (EE%) was calculated as follows:

\[
\text{EE\%} = \left( \frac{\text{DOX}_{\text{Enc}}}{\text{DOX}_{\text{Tot}}} \right) \times 100
\]

2.3. DOX release

The in vitro DOX release from liposomes was studied in PBS pH7.4, in complete Dulbecco’s modified Eagle’s medium (DMEM) (supplemented with 10% of fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin) and in phosphate buffer at pH5.3. 200 μL of the DOX-loaded liposome suspension was dispersed within a vial containing 2.0 mL of PBS, phosphate buffer and DMEM, protected from light and stirred gently at 37 °C. At predetermined time intervals, the entire release medium was subjected to ultracentrifugation at 300,000 g for 12 h at 4 °C (Beckman Coulter Optima L.90 K Ultracentrifuge). The concentration of released and retained DOX was evaluated in the supernatant and in the pellet respectively using UV spectrophotometry at 483 nm. A calibration curve was constructed using different concentrations of free DOX in PBS, DMEM and phosphate buffer.
The chemical stability of the entrapped drug was investigated by recording the UV–visible absorbance spectra of free and encapsulated DOX in PBS, phosphate buffer, and in DMEM.

2.4. Absorption spectra

To verify the influence of different solvents on the stability of DOX structure, absorption spectra were recorded using a Spectroscopy System (cell path length = 1.00 cm). DOX was added to buffers at pH 7.4 (PBS), pH 7.4 (complete DMEM medium) and pH 5.39 (phosphate buffer).

2.5. Cell culture

H9c2 cells, CRL 1446 (the rat embryonic ventricular myocardiad cell line) were obtained from the American Type Culture Collection. The cells were cultured and maintained in complete DMEM at 37 °C in a humidified incubator with 5% CO2. Cells were subcultured at a 1:4 ratio every 3 days after detachment with trypsin. For MTT and LDH assays, the cells were plated in 96-well plates (TPP Tissue culture dishes, Switzerland) at a density of 10000 cells per well and incubated overnight to allow cell attachment.

2.6. MTT assay

The MTT assay (Mosmann, 1983) was used to estimate the cytotoxicity of free DOX, encapsulated DOX and empty liposomes toward H9c2 cells. Cells were incubated with increasing concentration of each compound at concentration ranging between 1 and 80 μM, in triplicate. Untreated cells were used as controls. The cells were exposed for 48 h in a 5% CO2 incubator at 37 °C. After incubation, 20 μl MTT (Sigma) (5 mg/mL) solution was added in all wells, except in one well that served as blank, and incubated at 37 °C for 4 h. Finally, the medium was removed and formazan salt crystals were dissolved by addition of 200 μl of dimethylsulfoxide (Biobasic INC) to all wells. Plates were analyzed in an ELISA plate reader (Labsystems multiskan RS-232C, Finland) at 570 nm. Cell viability was defined relative to untreated control cells as follows:

\[
\text{Cell viability} = \frac{\text{absorbance of treated sample}}{\text{absorbance of control}}
\]

2.7. LDH assay

The LDH cytotoxicity detection kit (Roche Diagnostics GmbH, Mannheim, Germany) was used according to the

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![Figure 1](image-url)

**Figure 1** UV/visible spectra of free and entrapped DOX and empty liposomes. (a) UV/visible absorbance of DOX in different solvent with different pH. No noticeable differences in the obtained absorbance spectra. (b) UV–visible spectra of empty liposomes clearly indicate the absence of absorbance of lipid at 483 nm. The resemblance of spectra between free DOX and entrapped DOX confirms the presence of drug in the loaded liposomes.
manufacturer’s protocols. The test is based on the determination of LDH activity released from the cytosol of damaged cells into the medium, thus indicating cell membrane damage. Cells were incubated under the same conditions as for the MTT assay described above. LDH released into the extracellular medium of cells treated with different concentrations of free DOX, DOX-loaded liposomes and empty liposomes was expressed as a percentage of the total LDH activity in the cells [% of LDH released = extracellular LDH/(extracellular LDH + intracellular LDH)].

2.8. Analysis of cellular uptake of DOX by flow cytometry

Flow cytometry studies were conducted to compare free DOX, lipo-DOX, and empty fluorescent liposome uptake by H9c2 cells. H9c2 cell plated in two 6-well plates (TPP Tissue culture dishes, Switzerland) at a density of 200,000 cells per well and incubated for 24 h. Then cells were exposed to free or encapsulated DOX (20 μM, final concentration) or to the empty fluorescent liposomes. Untreated cells were used as negative control. After 3 or 20 h, the medium of each well was removed and the cells were then washed three times with 2 mL of PBS (pH7.4), harvested using 0.5% EDTA/trypsin and diluted in small volume of PBS. Finally, cells were examined by flow cytometry using a FACSCalibur (Becton Dickinson, USA). Cell-associated DOX or rhodamine was excited with an argon laser (488 nm) and fluorescence was detected at 585 nm (FL2 filter). 10,000 gated events were collected and analyzed with the CellQuest software.
Figure 3  Flow cytometry measurements of DOX uptake by H9c2 cells after 3 h (left panel) and 20 h (right panel) of incubation with entrapped DOX (A2, B2) empty fluorescent liposomes (A3, B3). Untreated cells served as negative control (A1, A2), free DOX was used as positive control (A4, B4). These graphs show the fluorescence intensity on the x-axis and the number of cells with this level of fluorescence on the y-axis.
2.9. Confocal microscopy

Confocal fluorescent microscopy was also used to compare the uptake of free and encapsulated DOX and the uptake of empty fluorescent liposomes and investigate their cellular distribution. Cells were grown in 6 well plates on glass coverslips (40,000 cells per mL). After 24 h, cells were treated free and encapsulated DOX (10 μM final concentration) and with empty fluorescent liposome’s, then incubated for 3 or 20 h at 37 °C. After incubation, cells were washed two times with cold PBS (pH7.4), fixed in formaldehyde 4% (in PBS) with Hoechst33342 stain (1 μg/mL) for 3 h. Fluorescent images of cells were acquired using a LSM 510 Zeiss confocal microscope (Carl Zeiss, Jena, Germany) at excitation and emission wavelengths of 543 nm and 560 nm respectively.

3. Results

3.1. Preparation and characterization of liposomes

In this work, we used the extrusion method to generate monodisperse populations of unilamellar vesicles (Fenske and Cullis, 2007). Whatever the preparation, the mean particle diameter was 110 nm with a polydispersity index was 0.046.

DOX loading efficiency obtained by the transmembrane pH gradients procedure was found to be greater than 90% at the drug-to-lipid ratio of 1:20 (w/w) used in this study. Final drug-to-lipid ratio was in excess of 1/2 (mol/mol). This high level of EE can be explained by the formation of an insoluble drug precipitate inside liposomes (Li et al., 2000).

3.2. Chemical stability of the entrapped DOX

The chemical stability of the drug in different solvents was investigated by recording the UV–visible absorbance spectra (Fig. 1a). There were no noticeable differences between the absorbance spectra obtained in the different buffers (PBS pH7.4, DMEM pH7.4, Phosphate Buffer pH5.3), suggesting no significant changes in the physical properties of the drug. The UV–visible spectra of the free and encapsulated DOX and empty liposomes are shown in Fig. 1b. The UV–visible spectrum of empty liposomes clearly indicates the absence of absorbance of lipid at 483 nm. The resemblance of the spectra corresponding to free DOX and entrapped DOX confirms the presence of drug in the loaded liposomes (Chouhan and Bajpai, 2009).

3.3. In-vitro release of DOX from liposomes

The profiles of DOX release from liposomes in vitro in PBS pH7.4, DMEM and phosphate buffer pH5.3 are presented in Fig. 2. The release of the entrapped DOX was very weak and similar in culture medium (Fig. 2a) and in PBS (Fig. 2b) very little encapsulated DOX was released and more than 98% and 90% respectively of the encapsulated DOX was retained in liposomes after 24 h of incubation at 37 °C. In contrast, in phosphate buffer pH5.3 (Fig. 2c), DOX release from liposomes reached 37% after 24 h of incubation. Thus, only a small amount of drug was be released at physiological pH, while more efficient release was observed in acidic conditions.

It is possible that the acidic pH destabilizes the lipid carrier (Minotti et al., 2004) but it has also been suggested that the physical state of DOX plays a role in drug release (Li et al., 2000). The results suggest that liposomes would be able to retain their cargo of DOX in the plasma but release it in the endosomes after capture by cells (Barenholz, 2001).

3.4. Cellular uptake of DOX by flow cytometry

The fluorescent properties of DOX were used to investigate its uptake by the cardiac cells when it was presented as free drug or as liposomes, by flow cytometry experiments. Empty liposomes labeled with a fluorescent lipid were used to study the interaction of the carrier with the cells. The results are shown in Fig. 3. Fig. 3A1 and B1 shows the background fluorescence of untreated cells at 3 h and 20 h, respectively. Treatment with free DOX (Fig. 3A4 and B4) led to a high level of cell-associated fluorescence, particularly after 20 h (fluorescence intensity of 175 arbitrary units after 3 h and 273 after 20 h, as compared with about 13 units in the control cells at either time, Table 1). When the same concentration of DOX was incubated with the cells in the form of liposomes (Fig. 3A2 and B2) the mean fluorescence intensity was lower than that of free DOX (133 arbitrary units after 3 h and 129 after 20 h). The interaction of the liposomes themselves with the cells was determined using rhodamine labeled empty liposomes (Fig. 3A3 and B3). The fluorescence intensity was only slightly higher than the untreated cells at 3 h, but increased after 20 h, suggesting a gradual accumulation of the PEG-liposomes within the cells. However, it is not possible to make direct comparisons between the empty liposomes and the DOX-loaded ones because the properties of the fluorophores are different. The uptake of DOX in H9c2 cells treated with encapsulated DOX was not a result of release of DOX from liposomes, because the DOX release in DMEM was very weak, as shown in Fig. 2. However, DOX-loaded liposomes may interact more with cells than empty liposomes because of their increased density.

The uptake of free DOX by the cells was accompanied by an increase in cell debris, which increased from 2% of the total fluorescence intensity at 3 h to 23% at 20 h.

3.5. Intracellular uptake and distribution of DOX by confocal microscopy analysis

Confocal microscopy was used as a complement to flow cytometry, to examine liposome-cell interactions in more detail. Phase contrast images of H9c2 cells adhering to glass

| Table 1 | Uptake of liposomal DOX in H9c2 cells derived from flow cytometry measurements (arbitrary units). |
|---------|--------------------------------------------------|
|          | Fluorescence intensity after 3 h | Fluorescence intensity after 20 h |
| Free DOX | 175 ± 61 | 273 ± 99 |
| Entrapped DOX | 133 ± 39 | 129 ± 38 |
| Empty liposomes | 19 ± 34 | 170 ± 89 |
| Untreated cells | 13 ± 12 | 13 ± 5 |
coverslips and incubated for 3 h and 20 h are shown in Fig. 4A and B respectively. Fig. 4C and D shows the results of incubation with free DOX for 3 h and 20 h respectively. Intense fluorescence can be observed in the nucleus, with only diffuse staining in the cytoplasm. When encapsulated DOX was used at the same concentration (Fig. 4E and F) the nuclear fluorescence was much less intense.

Fig. 5 shows the results of incubation with rhodamine-labeled liposomes. Phase contrast images of untreated cells are shown in Fig. 5A and D (3 h and 20 h incubation respectively). Treatment with unloaded liposomes did not affect cell morphology (results not shown). The accumulation of fluorescence associated with the cells was slow (compare images B and C, recorded after 3 h, and images E and F, recorded after 20 h). Furthermore, the fluorescence was limited to the cytoplasm and the nuclei remained unstained. Thus, intact liposomes are unable to cross the nuclear membrane and the DOX observed in the nuclei in Fig. 4E and F was the result

Figure 4  Confocal microscopy images of H9c2 cells treated with free and entrapped DOX. Images A and B show the phase-contrast appearance of untreated cells after 3 h and 20 h of incubation respectively. Images C and D show cells incubated with free DOX for 3 h and 20 h respectively. Images E and F depict cells incubated with entrapped DOX for 3 h and 20 h respectively.
of release from the liposomes, probably in the endosomes. The observation that DOX release was increased at pH 5.3 (Fig. 2c) was consistent with this interpretation.

3.6. Cytotoxicity of free and liposome-encapsulated DOX

The effects of free and liposome-encapsulated DOX on the viability of H9c2 cells were compared. Two well established tests were used. The MTT test measures the activity of mitochondrial dehydrogenases and therefore reflects both cytostatic and cytotoxic effects. On the other hand, the release of the cytosolic LDH enzyme evaluates membrane integrity and thereby indicates cell death by necrosis (Bernuzzi et al., 2009; Sardão et al., 2009). The tests were performed after 48 h of exposure to free or encapsulated DOX, which represents twice the cell doubling time. The results are shown in Fig. 6.

In the MTT test, DOX showed a dose-dependent inhibition of dehydrogenase activity, with an IC50 value of about 30 μM (Fig. 6A). At concentrations of 30 μM and higher, the liposomal form induced significantly less inhibition than the free

![Figure 5](image-url)
drug ($P > 5\%$, Student’s two-tailed $t$ test). The effect of empty liposomes at an equivalent concentration was always less than that of the loaded liposomes; however some inhibition of mitochondrial dehydrogenase activity was observed.

LDH release under the same conditions is shown in Fig. 6B. Even at low concentrations, free DOX causes considerable LDH leakage (around 80% for concentrations between 10 and 30 $\mu$M, rising to 100% for 50 and 80 $\mu$M). The LDH release after treatment with encapsulated DOX was always significantly lower. LDH release provoked by empty liposomes was quite similar to that provoked by loaded liposomes.

4. Discussion

For many years, drug delivery strategies have been developed to improve the effectiveness and reduce the cardiotoxicity of anthracyclines. In a first approach, association with colloidal nanosystems such as liposomes and nanoparticles allows the distribution to be modified avoiding the heart. In a second step, carriers specifically directed toward cancer cells have been developed (Barenholz, 2001).

For the first strategy, liposomes have been the most frequently studied carrier system and were therefore chosen for the present work. Our results for DOX encapsulation are in agreement with previous studies, showing that the use of a transmembrane pH gradient yields high trapping efficiency (Woodle, 1995; Fenske and Cullis, 2007; Ogawara et al., 2009) and encapsulation close to 100% (Fenske and Cullis, 2007; Ogawara et al., 2009). In our case more than 90% of added DOX was incorporated. This slightly lower value compared with the literature can be explained by the difference in composition of lipids entering in the structure of the liposomal membrane as well as by the difference salts used for the generation of the gradient of pH (Abraham et al., 2002; Fritze et al., 2006).

Figure 6  Cytotoxicity of entrapped and free DOX was measured in H9c2 cells after 48 h of incubation, measured by two classical tests: (A) MTT conversion; (B) LDH release. The results are expressed as a percentage of the value in untreated cells and are the mean ± SD of four wells.
Several studies have shown that the organ distribution of liposome-encapsulated DOX is different from that of the free drug, and this contributes to a reduction in toxicity to normal tissues (Berry et al., 1998; Gabizon et al., 2003; Batist, 2007). Our work focused on the interactions between free and encapsulated drug and cardiac cells. Observations made by confocal microscopy and flow cytometry suggest that DOX-liposomes can be taken up by these cells by endocytosis, despite the presence of PEG on their surface. H9c2 cells treated with empty fluorescent liposomes displayed rhodamine fluorescence in a punctuate pattern in the cytoplasm (Fig. 5) and flow cytometry showed that the peak of fluorescence shifted toward higher values with increasing time of incubation (Fig. 3A and B3). We were also able to use the intrinsic fluorescence of DOX to follow its uptake by cells. In confocal microscopy the fluorescence of the DOX, localized mainly in the nucleus, was more intense in cells treated with the free DOX compared with cells exposed for the same time to the same concentration in liposomal form. This result was corroborated by the flow cytometry results and is in accordance with earlier observations (Morisco et al., 2011). The high affinity of DOX for DNA appears to be the major driving force for the drug to enter cells by diffusion (Frezard and Garnier-Siullerot, 1991). Confocal microscopy revealed that the fluorescence of DOX is mainly in the nuclear compartment while the cytoplasm shows a weak intensity of fluorescence (Xiong et al., 2005). Administration of DOX to a sensitive cell line results in rapid nuclear uptake, with >99.8% of nuclear drug becoming directly associated with DNA (Cutts and Phillips, 1995). It appears that endocytosis of the liposomes is slower and therefore limits accumulation within the cells. However, DOX fluorescence inside these liposomes may be greatly quenched (Lee and Low, 1995), and the cytoplasm may contain significantly more Lipo-DOX than indicated by their relative fluorescence intensity.

The measurement of in vitro drug release from the liposomes enabled us to understand the mechanism of DOX uptake from liposomes. Very little release was observed when the liposomes were incubated in pH 7.4 buffer or in complete cell culture medium for 24 h. Therefore, the DOX uptake seen when loaded liposomes were incubated with the cells was probably due to the uptake of intact liposomes rather than release into the medium. This weak release of DOX from liposomes is the result of their lipid composition (saturated phospholipid, cholesterol). Both leakage and diffusion of drug across the cell membrane after contact are limited (Hu et al., 1995). On the other hand, more drug release was seen at pH 5.3. Thus DOX could have been released at the acid pH of the endosomes and then enter the nucleus by diffusion. The pH of tumor stroma has been reported to be lower than that of normal tissue (Ganta et al., 2008), and therefore this pH-dependent release could allow help to deliver the drug to tumor cells.

Cytotoxicity studies carried out after 48 h (two cell doubling times) of exposure to free and liposome-encapsulated DOX. The results (Fig. 6) showed that the toxicity of the anthracyclines was reduced by encapsulation. This could probably be explained by the reduced uptake by the cells. Therefore, the reduced cardiac toxicity of the encapsulated drug, which leads to a better therapeutic index for anticancer treatment, can be attributed to lower uptake by cardiac cells as well as an altered organ distribution of the drug.

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