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Visualization of bioavailable liposomal doxorubicin using a non-perturbing confocal imaging technique

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Summary. Commonly employed tissue processing techniques can significantly alter tissue drug distribution patterns for liposomal encapsulated drugs by virtue of drug leakage via loss of membrane integrity. We report here a method that has been developed to determine the fluorescence of bioavailable doxorubicin (DOX) in tissues after administration of liposomal DOX formulations. A non-perturbing confocal fluorescence microscopy (CFM) technique with image processing analysis was used with unprocessed fresh tissues. This method takes advantage of the fact that considerable quenching occurs when DOX is within liposomes, leading to the selective visualization of the fluorescence due to DOX released from liposomes. We demonstrate that fresh tissue confocal imaging can be applied to provide detailed drug distribution information with improved accuracy and is a superior method for analyzing tissue distribution of liposome entrapped fluorescent agents.

Keywords: Tissue localization, Imaging, Liposomes

Introduction

Liposomes have been developed as drug carriers for a number of therapeutic agents such as anticancer drugs (Mayer et al., 1989, 1990, 1997; Rahman et al., 1990; Cowens et al., 1993; Adlakha-Hutcheon et al., 1999; Lim et al., 2000; Tardi et al., 2000), antimicrobial agents (Lopez-Berestein and Juliano, 1987; Vincent et al., 1992; Pinto-Alphandary et al., 2000), genes (Felgner and Ringold, 1989; Rose et al., 1991; Hyde et al., 1993; Singhal and Huang, 1994; Dass et al., 2000; Harvie et al., 2000; Kawaura et al., 2000), and antisense oligonucleotides (Bennett et al., 1992; Leserman et al., 1994; Islam et al., 2000). This is because liposomes provide the potential for 1) increased stability of the encapsulated agent, 2) a circulating microreservoir or depot of the encapsulated drug, releasing it as a function of time (sustained release), 3) increased selectivity of drug delivery to the tumor site, and 4) reduced peak free drug levels and decreased exposure to healthy tissues.

Liposomes are particularly effective in anticancer therapy because of enhanced site specific delivery to solid tumors. Vesicles of size less than 200 nm can passively extravasate through the gaps in the endothelial layer and accumulate in sites of tumor growth. This is because blood vessels associated with disease sites such as tumors are characterized by frequent interruptions along the endothelial cell lining, which is permeable to many circulating macromolecules. Macromolecules may leak through these "fenestrae" or gaps (Kohn et al., 1992) or endothelial cell facilitated transcytosis may occur (Huang et al., 1993). Liposomes with long circulation life times exhibit extended access to the openings in the endothelial lining which can often lead to increased accumulation in such extravascular sites of disease (Gabizon and Papahadjopolous, 1988; Gabizon et al., 1990; Gabizon, 1992). Liposome extravasation and accumulation in solid tumors has been well documented by numerous laboratories using a wide variety of tumor types (Gabizon and Papahadjopolous, 1988; Allen et al., 1989; Mayer et al., 1989; Gabizon, 1992; Wu et al., 1993; Yuan et al., 1994).

Tumor drug localization methods used in the past to assess intra-tissue distribution properties for liposomal drugs have generally utilized techniques wherein the excised tumor tissue is subjected to standard tissue processing methodologies for conventional immunohistochemical or fluorescence microscopy (Perez-Soler et al., 1987; Huang et al., 1992; Forssen et al., 1996). This procedure may utilize a tissue fixative, a cryofixation step and/or several washing steps (Mayer et al., 1997). Even with relatively mild processing, these conditions may disrupt the liposomal bilayer and cause drug leakage, leading to significant alterations in tissue distribution patterns for liposomal encapsulated drugs compared to distribution in the original tissue specimen. For the fluorescent anticancer compound doxorubicin

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(DOX) encapsulated in liposomes, such tissue processing could potentially lead to high nuclear DOX concentrations given it's propensity to bind to DNA. Furthermore, such studies have been unable to differentiate between released (bioavailable) and encapsulated drug (non-bioavailable).

In the current investigation, a method has been developed to determine the fluorescence of bioavailable DOX in tissues after administration of liposomal DOX formulations. A non-perturbing confocal fluorescence microscopy (CFM) technique with image processing analysis was used with unprocessed, fresh tissues. CFM analysis can be performed using thick (2-3 mm), viable tissues immersed in buffer, an approach that has been routinely utilized for other specialized applications (Masters, 1996). This method also takes advantage of the fact that considerable quenching occurs when DOX is within liposomes (Wu et al., 1997), leading to the selective visualization of the fluorescence due to DOX released from liposomes. We show that fresh tissue confocal imaging can be applied to provide detailed drug distribution information including intracellular delivery of liposome entrapped fluorescent agents.

Materials and methods

Materials

DOX hydrochloride was purchased from David Bull Laboratories (Canada) Inc., Vaudreil, Quebec, and its purity affirmed by HPLC. Polyethylene glycol 2000 coupled with distearoylphosphoethanolamine (PEG_{2000}-DSPE, >99% purity), and 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC, >99% purity) were obtained from Northern Lipids, Inc (Vancouver, BC) and cholesterol was obtained from Sigma Chemical Company (St. Louis, Missouri). Di-I (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine percholate) was purchased from Molecular Probes. Tissue-Tek OCT compound was obtained from Miles (Etobicoke, ON). Sterile supplies as well as sterile saline and water for injection, were obtained from the BC Cancer Agency stores (Vancouver, BC). Female SCID/RAG2 mice were bred in-house at the BC Cancer Agency animal facility. The MDA435LCC6 line was a generous gift from Dr. Robert Clarke, Georgetown University, Washington DC.

Liposome and drug preparation

Liposomes composed of PEG_{2000}-DSPE/DSPC/Chol (5:50:45) and DSPC/Chol (55:45; mol:mol) were prepared by initially dissolving the lipid mixtures in chloroform (100 mg lipid/ml), vacuum drying to generate the thin film, and hydrating this dried lipid film in a 300 mM citric acid pH 4.00 buffer. Briefly, the resulting multilammelar vesicles (MLVs) were subjected to five freeze-thaw cycles followed by a 10 cycle extrusion through two stacked 100 nm polycarbonate filters (Nuclepore, Pleasanton, California) using a Lipex Extruder (Lipex Biomembranes Inc., Vancouver, British Columbia, Mayer et al., 1986a). The resulting large unilammelar vesicles (LUVs) exhibited a mean diameter ranging between 100-120 nm as determined using a Nicomp 270 submicron particle sizer (Particle Sizing Systems, Inc, Santa Barbara, CA), operating at a wavelength of 632.8 nm.

DOX was encapsulated in the liposomes using the transmembrane pH gradient loading procedure (interior acidic) employing sodium carbonate as the alkalinizing agent and a drug to lipid weight ratio of 0.2:1.0 (Mayer et al., 1986b). Liposomal DOX preparations were diluted with saline as necessary prior to *in vivo* administration. Non-encapsulated DOX was dissolved in sterile saline.

Tissue cryofixation

Tissues were processed for cyrofixation as described previously (Mayer et al., 1997). Briefly, tissue fixation was performed using 3% paraformaldehyde solution in PBS at 4 °C for 30 min, washed with PBS and immersed in increasing sucrose gradients for 20 min each: 10% sucrose-PBS, 15% sucrose-PBS, and 15% sucrose-PBS containing OCT compound (1:1, v/v). The processed tissue was then embedded in OCT compound and frozen in liquid nitrogen.

Fresh tissue confocal imaging studies

Confocal images were collected on a Optiphot 2 research microscope (Nikon, Japan) attached to a confocal laser scanning microscope (MRC-600, BioRad Laboratories, Hercules, CA) using COMOS software (BioRad Laboratories). The laser line on the krypton/argon laser was 488 nm. Filterblock BHS was used to detect DOX (488 nm excitation, 515 nm emission). The numerical aperture was 0.75 on the x20 air objective and 1.2 on the x60 oil objective. The images were captured such that the xyz dimensions were 0.4 μm cubed (x20) and 0.2 μm pixel (x60). NIH Image version 1.61 was used for image analysis, and all images were based on maximum intensity projection. Projections made in the NIH Image were saved in TIFF format, then imported to Adobe Photoshop version 4.0 where the different fluorophore images were assigned to individual RGB channels and subsequently merged to provide the final image of the single or multiple sections.

For confocal imaging studies, SCID/RAG2 mice bearing MDA435/LCC6 tumors were treated with DSPC/Chol or PEG-DSPE/DSPC/Chol DOX (5 mg/kg). These studies were performed in order to assess the bioavailability of DOX administered in liposome encapsulated form as well as the intracellular uptake of DOX for both formulations. The evaluation of bioavailability relies on the fact that DOX encapsulated inside liposomes exhibiting transmembrane ion gradients is quenched with respect to its inherent fluorescence. At the indicated times following DOX administration, liver and tumor tissues were asceptically dissected, perfused

in PBS, and imaged fresh. Before imaging, thin pieces of liver lobes and tumors were placed on concave slides and observed under a 60x oil immersion lens. These were then viewed under the confocal microscope to determine DOX distribution characteristics.

In control experiments, known amounts of non-encapsulated DOX, or PEG-DSPE/DSPC/Chol-DOX liposomes were infused into freshly isolated muscle tissues and viewed for DOX fluorescence. In order to confirm quenching with liposomal DOX preparations, we compared the fluorescence properties in spiked muscle tissue of PEG-DSPE/DSPC/Chol-DOX liposomes to that obtained with empty PEG-DSPE/DSPC/Chol liposomes of identical composition but with a fluorescent lipid label. The lipid label used was Di-I and was incubated with empty PEG-DSPE/DSPC/Chol liposomes at 60 °C for 1 h with agitation.

Results

Initial experiments to evaluate the influence of cryofixation on liposomal drug distribution were performed using muscle as a relatively inert tissue with respect to liposome processing. When PEG-DSPE/DSPC/Chol liposomal DOX is spiked in freshly isolated muscle tissue, drug fluorescence is not visualized due to the fluorescence quenching of DOX that occurs inside liposomes as a result of high entrapped concentrations (Fig. 1A). However, when cryofixation is performed (Fig. 1B), a significant increase DOX fluorescence is observed, indicating that drug leakage has occurred following disruption of the liposomal bilayer caused by the sample processing steps. If concentrated liposome aliquots were treated similarly in the absence of tissue, drug release was also observed,

The results presented in Figure 2 demonstrate the fluorescence quenching observed for liposome-encapsulated DOX as well as the non-perturbing nature of evaluating fresh tissue. Figure 2A is the confocal image of PEG-DSPE/DSPC/Chol DOX liposomes injected in freshly isolated muscle tissue at a concentration of 10 μg/g. No DOX fluorescence is visualized, however, the presence of liposomes can be confirmed since PEG-DSPE/DSPC/Chol liposomes labeled with the fluorescent lipid label, Di-I (Fig. 2B) were visualized throughout the tissue. Further, when non-encapsulated DOX is injected at identical concentrations, DOX is readily visualized and appears as intense nuclear fluorescence (Fig. 2C). Therefore, the method that we have utilized here can qualitatively assess concentrations of fluorescent DOX that have been released from the liposomes and can also provide indications of the intracellular localization of DOX. Further, the method preserves cellular architecture, allows 3-D projections of multiple scans to be made and preserves liposome integrity. Despite the greater thickness of the tissue (ca. 2-3 mm), DOX visualization was possible due to the strong fluorescent signal afforded by non-encapsulated DOX and the ability of confocal microscopy to image finite sections of the tissues. It should be pointed out here that all the scans were performed on one day at identical instrument settings and imaging exposure times. Therefore, any increases in DOX signal is attributable to increased DOX levels. However, it should also be noted here that

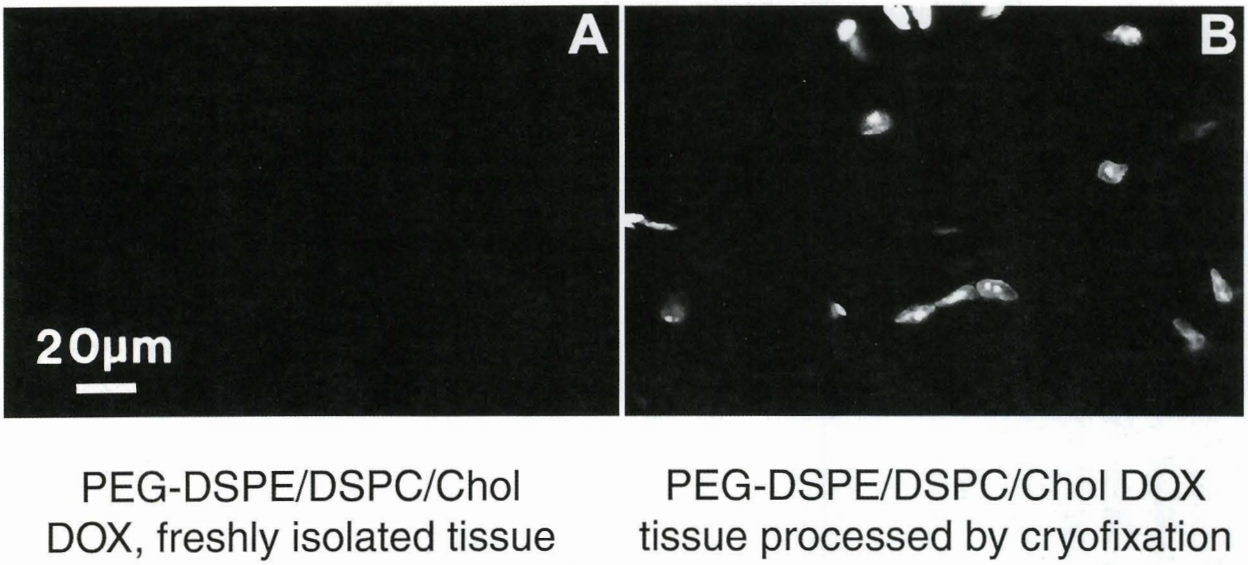
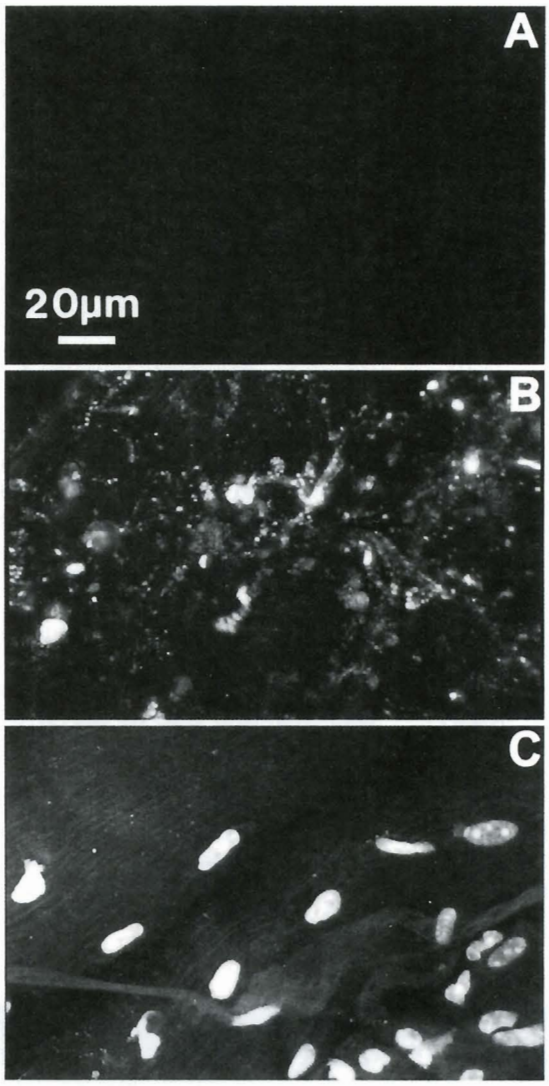


Fig. 1. A comparison of two sample treatments on DOX visualization using CFM. **A** represents the image after PEG-DSPE/DSPC/Chol -liposomal DOX is injected into a defined mass of freshly isolated muscle tissue, at a concentration of 10 μg/g, (from SCID/Rag2 mice) and visualized fresh. **B** is a representation of PEG-DSPE/DSPC/Chol-liposomal DOX at identical concentration in muscle, but subjected to a standard cyrofixation protocol before



PEG-DSPE/DSPC/Chol DOX spiked in freshly isolated muscle tissue

PEG-DSPE/DSPC/Chol (Di-I labeled) liposomes spiked in freshly isolated muscle tissue

Free DOX spiked in freshly isolated muscle tissue

Fig. 2. An illustration of DOX fluorescence quenching properties when encapsulated within liposomes. Freshly isolated muscle tissue from SCID/Rag2 mice were injected with known amounts of PEG-DSPE/DSPC/Chol - liposomal DOX (10 μg/g; **A**), empty PEG-DSPE/DSPC/Chol-liposomes labeled with DiI **(B)**, and free DOX (10 μg/g; **C**). Note the distinct strong DOX fluorescence in C, but not in B, indicating substantial quenching with PEG-DSPE/DSPC/Chol-liposomal DOX. The visualization of DiI label confirms the presence of empty PEG-DSPE/DSPC/Chol liposomes.

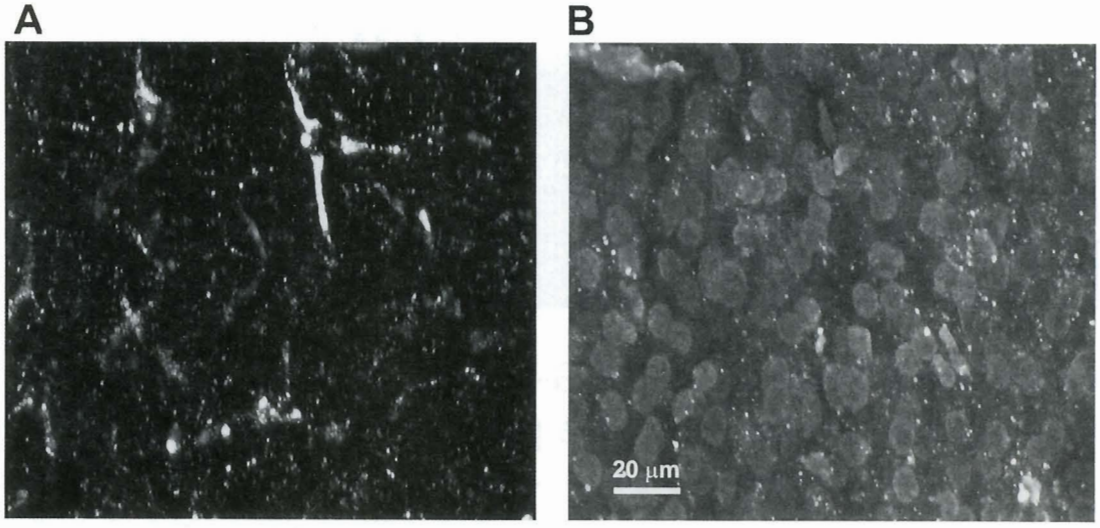


Fig. 3. Liver confocal images of DSPC/Chol-liposomal DOX (5 mg/kg). SCID/Rag2 mice were treated with liposomal DOX at a dose of 5 mg/kg, injected i.v. At 1 h post-DOX administration, liver lobes were aseptically dissected and placed in PBS containing tubes, maintained on ice. These specimens were either freshly imaged by placing un-sectioned non-fixated liver lobes on concave slides under the CFM **(A)** or subjected to a cryofixation step and un-sectioned liver lobes prior to imaging (**B**; see Materials and Methods). Identical settings on the CFM and identical processing times facilitated a comparison of DOX fluorescence intensity for the two specimen treatments.

the results presented provide qualitative information, which was not amenable to quantitative analysis, thus precluding definitive statistical comparisons to be made.

Once the initial experiments in muscle tissue provided confirmatory results on the influence of cryofixation in altering liposomal drug tissue distribution, the next set of experiments were performed in the liver (metabolically active tissue) and tumor (desired site of action) tissues in mice which received liposomal DOX injected i.v. Figure 3 presents the CFM images of liver lobes obtained from mice at 1 h post-i.v. administration of PEG-DSPE/DSPC/Chol liposomal DOX at a drug dose of 5 mg/kg. As seen in Figure 3A which shows confocal images of fresh, unsectioned, non-fixed liver lobes, localization is extracellular as well as at cell junctions. When these samples were subjected to a freeze-thaw cyrofixation procedure, DOX distribution was almost entirely intracellular and nuclear.

Figure 4 presents the CFM images of MDA435LCC6 tumors from mice administered PEG-DSPE/DSPC/Chol DOX (PEG) at a dose of 5 mg/kg. Tumors isolated from mice treated with liposomal DOX demonstrated no detectable DOX fluorescence at 1 h (Fig. 4A) even though total DOX tissue levels (determined by HPLC of extracted tissue) were 22±1.6 μg/g. Presumably the lack of any appreciable fluorescence by fesh tissue confocal microscopy in these samples was due to high concentrations of non-bioavailable DOX (DOX was still encapsulated within liposomes). Dramatic increases in DOX fluorescence were visualized at 24 h (Fig. 4 B) even though total tumor associated DOX increased moderately to 36 + 4.3 μg/g. These results were indicate the development of increased levels of bioavailable (released) DOX at the later time points. These results were consistent with observations of enhanced efficacy and reduced toxicity with Pegylated liposomal DOX (Krishna et al., 2000).

Discussion

Confocal microscopy affords a multitude of advantages for the applications of liposomal technology in anticancer therapy (Pawley, 1990). Sufficiently thin optically clear sectioning is possible with this method for relatively thick tissue specimens. In addition, high resolution and precision of microphotometry are obtained. In comparison to other microscopy techniques such as electron, light, and fluorescence microscopy which require relatively thin sections and depend on processing techniques to obtain high contrast images, confocal microscopy provides an added advantage of flexibility with thicker sections as well as reduced processing.

Liposomes are lipid bilayer vesicles composed of phospholipid and cholesterol, of sizes ranging from 30-200 nm (unilamellar vesicles) to 0.5-10 μm (multilamellar vesicles). Most efficacious liposomal drugs belong to class of large unilammellar vesicle category ranging from 100-200 nm in diameter. Whereas specimen preservation techniques are without adverse consequences for non-encapsulated drugs, adequate precaution in the processing of liposomal encapsulated drugs is necessary. As we have seen in this study; relatively mild processing such as cryofixation can significantly alter liposomal drug distribution via increased drug leakage due to disruption of the liposomal membrane. Such disruption of liposomal integrity could yield misleading interpretation of data such as enhanced intracellular accumulation of a

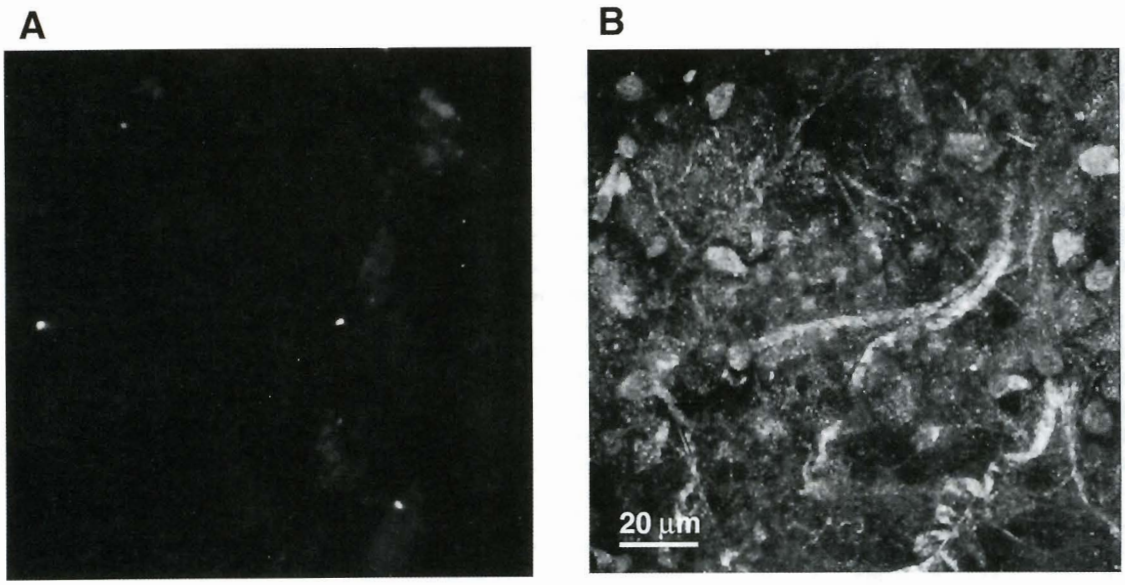


Fig. 4. Tumor confocal images of PEG-DSPE/DSPC/Chol-liposomal DOX (5 mg/kg). SCID/Rag2 mice were treated with liposomal DOX at a dose of 5 mg/kg, injected i.v. Following 1 and 24 h post-DOX administration, tumor samples were aseptically dissected and placed in PBS containing tubes, maintained on ice. These samples were directly imaged fresh by lacing them on concave slides under the CFM (see Materials and Methods). Identical settings on the CFM and identical processing times facilitated a comparison of DOX fluorescence intensity at the two different time points.

liposomal drugs/oligonucleotides that have been reported previously for various liposomal formulations (Perez-Soler et al., 1987; Huang et al., 1992; Thierry and Dritscilo, 1992; Forssen et al., 1996; Hatta et al., 1997).

The important purpose of a tissue fixation method is to preserve structural specimen integrity. A common problem with tissue fixatives is that of shrinkage for immunofluorescence protocols (Bacallao et al., 1990). Specimen skrinkage was shown to be dependent upon the total osmolarity of the fixative and type of buffer used (Lee, 1984). In addition to these inherent disadvantages of tissue fixatives, the implications for liposomal drugs are in cases where the fixative itself can cause an effect on membrane integrity. For example, the use of common fixatives such as paraformaldehyde has been shown to cause morphological distortion and disruption of the plasma membrane (Sato et al., 1976). In 1978, Poste et al. reported a potential artifact caused by specimen preparation techniques in the application of electron microscope autoradiography in studying the uptake and intracellular localization of liposomes containing radiolabeled saturated phospholipids. Significant translocation and intracellular redistribution of radiolabeled lipids was observed when using glutaraldehyde as a specimen fixative. Recently, Pichon and co-workers (1999) have highlighted the importance of fixative protocols in the study of intracellular localization of fluorescently labeled oligonucleotides and their derivatives. Interestingly, whereas oligonucleotides were localized in vesicular compartments in non-fixated cells, enhanced nuclear localization was observed when the specimens were fixated with commonly employed tissue fixatives, such as methanol and acetone.

In summary, we have shown that commonly employed processing techniques can significantly alter tissue drug distribution patterns for liposomal encapsulated drugs by virtue of drug leakage during processing, compared to fresh tissue confocal imaging. Determination of bioavailable drug levels was possible given the quenching of DOX fluorescence within liposomes. It should be noted that given the potential quenching of DOX fluorescence which occurs upon binding to DNA (Gigli et al., 1988), the fluorescence observed in fresh tissue sections may reflect a minimum level of drug that has been released from the liposomes. Nonetheless, this procedure alleviates the overestimation of DOX delivery to tumor cells that clearly occurs when using standard tissue fixation procedures. Therefore, fresh tissue confocal imaging may provide detailed drug distribution information with improved accuracy for liposome entrapped fluorescent agents whose distribution and imaging properties are altered by processing steps employed in conventional tissue fixation procedures.

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