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Engineering Lipid Vesicles of Enhanced Intratumoral Transport Capabilities: Correlating Liposome Characteristics with Penetration into Human Prostate Tumor Spheroids

KOSTAS KOSTARELOS,[1] DIMITRIS EMFIETZOGLOU,[2] ALEXANDROS PAPAKOSTAS,[2] WEI-HONG YANG,[3] ÅSE M. BALLANGRUD,[3] AND GEORGE SGOUROS[3,†]

[1]Imperial College Genetic Therapies Centre, Imperial College London, UK
[2]Department of Medical Physics, University of Ioannina Medical School, Ioannina, Greece
[3]Department of Medical Physics, Memorial Sloan-Kettering Cancer Center, New York, New York, USA

Liposomes have been widely used delivery systems, particularly relevant to the development of cancer therapeutics. Numerous liposome-based drugs are in the clinic or in clinical trials today against multiple tumor types; however, systematic studies of liposome interactions with solid or metastatic tumor nodules are scarce. This study is describing the in vitro interaction between liposomes and avascular human prostate (LNCaP-LN3) tumor spheroids. The ability of fluorescently labelled liposomal delivery systems of varying physicochemical characteristics to penetrate within multicellular tumor spheroids has been investigated by confocal laser scanning microscopy. A variety of liposome characteristics and experimental parameters were investigated, including lipid bilayer composition, duration of liposome-spheroid interaction, mean liposome size, steric stabilization of liposomes. Electrostatic binding between cationic liposomes and spheroids was very efficient; however, it impeded any significant penetration of the vesicles within deeper layers of the tumor spheroid. Small unilamellar liposomes of neutral surface character did not bind as efficiently but exhibited enhanced penetrative transport capabilities closer to the tumor core. Polymer-coated (sterically stabilised) liposomes exhibited almost no interaction with the spheroid, indicating that their limited diffusion within avascular tissues may be a limiting step for their use against micrometastases. Multicellular tumor spheroids were used as models of solid tumor interstitium relevant to delivery systems able to extravasate from the microcapillaries or as models of prevascularized micrometastases. This study

†Current address: Division of Nuclear Medicine, Department of Radiology, Johns Hopkins School of Medicine, Baltimore, MD, USA.

Address correspondence to Kostas Kostarelos, Centre for Drug Delivery Research, The School of Pharmacy, University of London, 29-39 Brunswick Square, London WC1N 1AX, UK; Fax: ++44-0207-753-5942; E-mail: kostas.kostarelos@ulsop.ac.uk

illustrates that interactions between liposomes and other drug delivery systems with multicellular tumor spheroids can offer critically important information with respect to optimizing solid or micrometastatic tumor delivery and targeting strategies.

Keywords liposome, tumor spheroids, intratumoral transport, diffusion, prostate, drug delivery

Introduction

Targeted cancer therapy has been clinically applicable and effective only in a limited number of malignancies. Namely, therapeutic radionuclide (β-emitting) conjugates with antibodies primarily against hematological tumors (such as non-Hodgkin's lymphoma and leukemia) and polymer-coated small unilamellar liposomes loaded with anthracyclines (doxorubicin and daunorubicin) against Kaposi's sarcomas (Allen, 2002). Delivery of targeted therapeutics to vascularized solid tumors has been impeded by the so-called 'binding-barrier' effect leading to limited intratumoral diffusion, thus elimination of cells restricted to the periphery of the tumor and an overall poor cell-kill (Saga et al., 1995; Topp et al., 1998). In the case of liposome extravasation through the leaky tumor capillaries, even though effective targeting and localization of the therapeutic agents is achieved, the end therapeutic effect is limited due to restricted intratumoral diffusion and transport.

During the last decade liposomes have been transformed from a proof-of-principle paradigm in drug delivery to an established and clinically practiced cancer therapeutic.

Still, there is plenty of room for improvement and development of new kinds of liposome systems in different therapeutic applications: chemotherapy, gene therapy, radiotherapy. Since the vast majority of applications involve the development of liposome-based treatment modalities against various types of cancer, the interaction of liposome systems with tumor cells and tissues is of seminal importance. Moreover, when taking into account the repeatedly documented poor transport of liposomes within the interstitial space of tumors leading to insufficient therapeutic indices (Ishida et al., 1999; Yuan et al., 1994), evaluation and modeling of their interaction and diffusion within avascular tumor models becomes overly valuable.

Multicellular tumor spheroids have been repeatedly used as models of micrometastatic, prevascularized tumors (Helmlinger et al., 1997). The spheroid model system offers many of the advantages in terms of experimental manipulation and analysis inherent to monolayer tissue cultures, yet exhibits many of the properties seen in prevascularized, growing, or recently disseminated tumors (Sutherland, 1988). Moreover multicellular clusters of different tumor cells are being investigated as an efficient 3D in vitro model for clinically relevant opportunistic, self-organized, malignant tumors of literally all types (ovarian, melanoma, brain, prostate) (Bjerkvig, 1992; Deisboeck et al., 2001). Three-dimensional (3-D) multicellular tumor spheroids were described in the last couple of years as the preferred in vitro model most relevant to tumor studies (Jacks and Weinberg, 2002). A lot of interest and attention has been recently placed on the advantages 3-D cellular spheroids and their importance in understanding tumor development and most importantly their utilisation in building bridges between in vitro and in vivo models (Abbot, 2003). Despite that, tumor spheroid use in the engineering and development of delivery systems for therapeutics has been lacking. Recently, tumor spheroids have been used as models toward development of clinically relevant therapeutic modalities for treatment of micrometastatic prostate tumor deposits using α-particle emitting radionuclide-monoclonal antibody conjugates (Ballangrud et al., 2001), and gene therapy vectors (Grill et al., 2001).

In the present study multicellular tumor spheroids were used as models to mimic a nonvascular tumor body, volume, and interstitial space. The present study aims to provide

Table 1
Physicochemical characteristics of liposome systems allowed to interact with tumor spheroids

Liposome systems (phospholipid compositions)	Surface characteristics	Surface charge (mV)	Liposome bilayer phase
DMPC: chol (2:1)	Slightly negative	−9.3 ± 2.2	Liquid crystalline
DMPC: DC-chol (1:1)	Positive	51.7 ± 3.9	Liquid crystalline
DPPC: chol (2:1)	Negative	−55 ± 3.2	Gel
DMPC: chol: DOPE-PEG$_{(2000)}$ (10:5:1)	Sterically stabilized	4.8 ± 0.4	Liquid crystalline

a qualitative insight of the binding and penetration profiles of liposome systems within 3-D tumor spheroid models, in relation to different important parameters in the engineering of liposomes. Evaluation of the effects of mean vesicle size, surface charge characteristics, time of interaction with the tumor mass, and lipid bilayer composition offer valuable guidelines toward engineering intratumorally diffusive liposomes relevant to effective cancer therapeutics.

Materials and Methods

A variety of different liposome types were allowed to interact with tumor spheroids consisting of the LNCap-LN3 prostate cancer cell line. The phospholipid composition of the liposome systems resulted in different surface and/or lipid bilayer properties, as shown in Table 1. Liposome systems were fluorescently labeled using the lipophilic carbocyanine dye 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) as previously described (Litzinger et al., 1994).

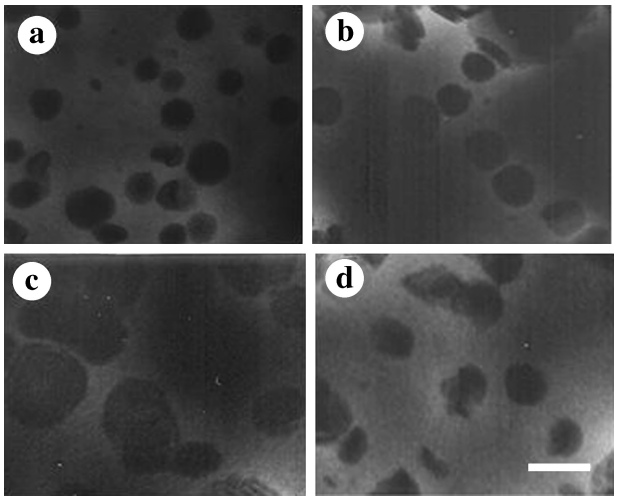

Figure 1. Negative stain transmission electron microscopy images of (a) DMPC: chol; (b) DPPC: chol; (c) DMPC: DC-chol; (d) DMPC: chol: DOPE-PEG$_{(2000)}$ SUV liposomes used in this study. The scale bar indicates 100 nm.

Tumor Spheroids

Multicellular spheroids consisting of the LNCap-LN3 prostate tumor cell line were prepared according to previously described methodologies (Ballangrud et al., 1999). Briefly, trypsinization of 10^6 LNCaP-LN3 growing in monolayer cultures were seeded into 100-mm dishes coated with a thin layer of 1% agar (Bacto Agar; Difco, Detroit, MI) with 15 mL of RPMI 1640, supplemented with 10% fetal bovine serum, 100 units/mL penicillin and 100 μg/mL streptomycin. After 3 to 5 days in the agar culture, spheroids of 200 ± 20 μm in diameter were selected under an inverted phase-contrast microscope with an ocular scale using an Eppendorf pipette. The selected spheroids were transferred to 35-mm bacteriological Petri dishes in 2 mL of medium.

Liposomes

DMPC, DC-chol, DPPC and cholesterol were purchased by Sigma-Aldrich (Poole, UK) and DOPE-PEG_{2000} was purchased by Avanti Polar Lipids (AL, USA). All liposomes were prepared following the solvent evaporation – hydration protocol in chloroform (USP). Hydration of the lipid films by addition of either PBS (in experiments not involving cells) or RPMI medium (for cellular experiments) produced multilamellar vesicles (MLVs). Extrusion cycles (Jacks and Weinberg, 2002) through polycarbonate filters (Milipore) using a LiposoFast extruder (Avestin, Canada) were used to form small unilamellar liposomes as previously described (Mui et al., 1993). The small unilamellar vesicles (SUVs) formed were studied by transmission electron microscopy (Fig. 1) using a FEI/Philips CM 120 BioTwin Transmission Electron Microscope (Eindhoven, The Netherlands). Briefly, a 300-mesh Copper Grid was had been coated with a formvar/carbon support film (Taab Labs Ltd, England). Prior to preparation the Grids were ''glow discharged'' in an Emitech K350G system for 3 min at 30 mA, negative polarity (Emitech Ltd., England). Excess sample was removed using No. 1 Watman Filter paper and consequently stained with phosphotungistic acid. Imaging was carried out using an accelerating voltage of 80 KV.

Interaction Between Liposomes and Tumor Spheroids

Liposomes were left to interact with the spheroids for 2 h and 5 h at 37°C in an orbital shaker incubator. At least five spheroids were included in each condition. The total lipid concentration interacting with the spheroids was always kept constant at 1 mg of lipid/tumor spheroid. All incubations were undertaken in an orbital shaker incubator. At the specified time points, spheroids were washed three times with PBS and placed in fresh incubation medium before fluorescence imaging was carried out; some selected spheroids were not washed prior to imaging in order to assess the relative position of liposomes that did not bind or interact with the spheroids.

Imaging Using Confocal Laser Scanning Microscopy (CLSM)

CLSM imaging was carried out by acquiring 3 μm-thick optical sections of the spheroids under study from the top toward the center of the spheroids, until approximately scanning 120 μm deep into the spheroid using a Zeiss LSM 510 microscope (Zeiss, Oberkochen, Germany). DiI fluorescence was observed red using standard rhodamine optics (excitation filter at 546 nm, dichroic mirror at 580 nm and barrier filter at 590 nm) as previously described (Claassen, 1992). Image galleries shown, depict the fluorescence signal from optical slices at the top of the spheroid toward the equatorial plane.

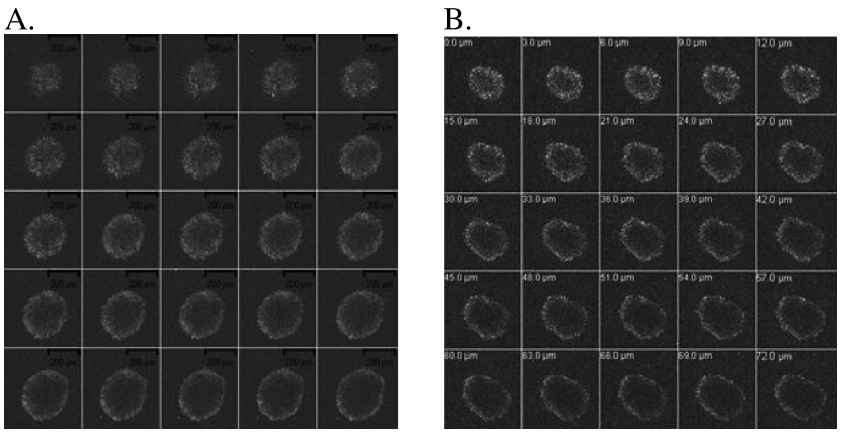

Figure 2. CLSM image gallery of DMPC:chol SUVs interacting with tumor spheroids for (A) 2 h; and (B) 5 h.

Results

All liposome systems (Table 1) were prepared as multilamellar vesicles (MLV) and small unilamellar vesicles (SUV), the two types differing in the mean particle size of their respective liposome populations. Light and electron microscopy indicated that the mean

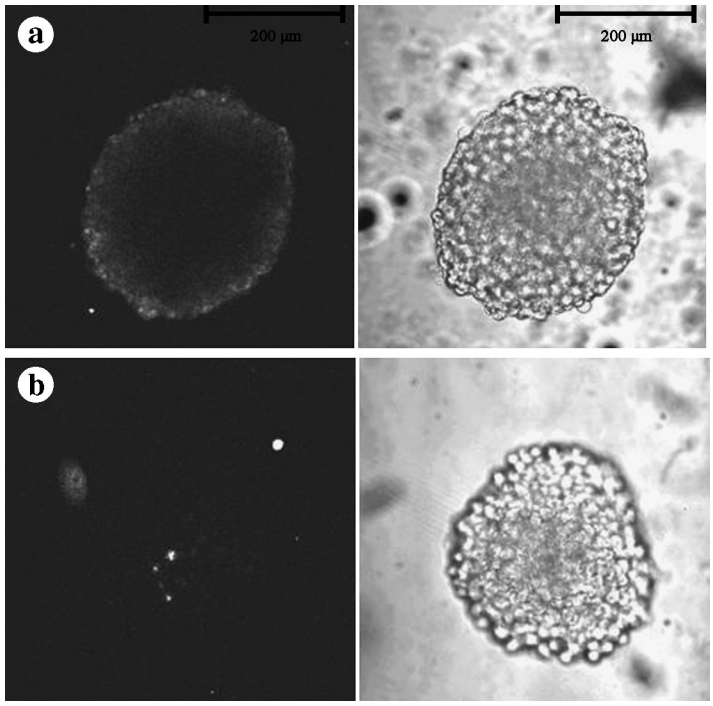

Figure 3. CLSM equatorial optical slice image of DMPC:chol (a) SUV and (b) MLV, interacting with the tumor spheroids for 2 h at 37°C. The equivalent optical microscopy image of the spheroid is shown next to the fluorescence image to colocalize the fluorescence signal in the tumor volume.

vesicle diameter for all MLV systems ranged between 800–1000 nm, and for all SUV systems between 50–150 nm (Fig. 1). Note that DMPC:DC-chol produced larger SUV liposomes (mean diameter: 120–150 nm, Fig. 1c). All other SUV systems exhibited a mean diameter below 100 nm (Fig. 1a, b, and d).

The Classic Liposome System and Its Time-Dependence of Interaction with Spheroids

Figure 2 shows the CLSM image galleries of tumor spheroids following interaction with SUVs produced using the classic liposome composition (DMPC:chol) of liquid crystalline bilayer characteristics and slightly negative charge after 2 h and 5 h. The optical slices shown reach up to approximately 80 μm deep within the spheroid volume. The images indicate that there is interaction and association with the spheroids primarily due to passive diffusion of the liposomes within the interstitial space of the spheroid and their entrapment in the extracellular space. The interaction of the SUVs with the spheroids seems to be time-independent with only a slight increase in the fluorescent signal within the spheroid after 5 h of co-incubation primarily due to accumulation of the liposomes.

The Effect of Liposome Size

The CLSM images of DMPC:chol SUV and MLV at the equatorial slice studying the effect of mean liposome size on its interaction with the spheroids is shown in Fig. 3. The respective light microscopy images are also shown to indicate the spheroid dimensions and borders. It is obvious from the images obtained that only in the case of SUVs an

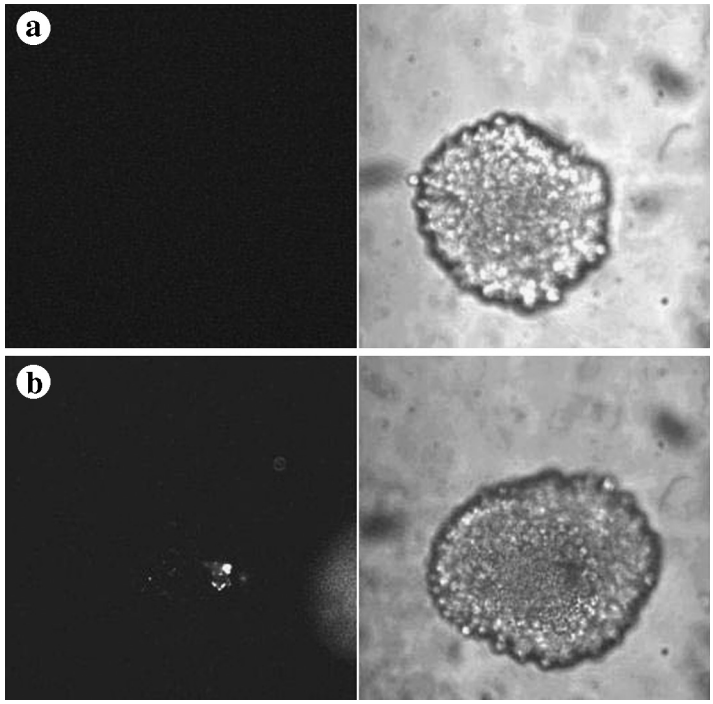

Figure 4. CLSM equatorial optical slice images of the rigid DPPC:chol liposomes interacting with tumor spheroids as (a) SUV and (b) MLV, for 2 h at 37°C.

accumulation of liposomes is obtained within the spheroid. The MLVs seem to minimally interact with the tumor cells, leading to only occasional indication of fluorescence signal on the tumor mass following washing of the spheroid prior to imaging. These results may further indicate a size-dependent mechanism of interaction between the particular liposome (DMPC:chol) system and the spheroids primarily governed by passive diffusion.

The Effect of Liposome Bilayer Characteristics and Mean Size

Figure 4 shows CLSM equatorial optical slice images of the rigid DPPC:chol liposomes interacting with tumor spheroids as MLVs and SUVs. These studies indicated that liposomes not containing a fluid bilayer (liquid crystalline phase below the phase transition temperature) were not interacting at all with the tumor cell clusters. This observation is important since rigid liposomes also exhibit an improved in vivo retention of encapsulated material and are commonly used in intravenous administration protocols. Moreover, the data provide further support that diffusion and convection are crucial mechanisms of intratumoral transport, while indicating that fluid, deformable liposomes are proven essential in optimizing intratumoral delivery of therapeutics.

The Effect of Liposome Surface Charge and Time-Dependence of Interaction with Spheroids

Interaction of positively charged liposomes with the tumor spheroids was studied next. Figure 5 shows two series of CLSM image galleries of the cationic DMPC:DC-chol MLVs allowed to interact with tumor spheroids for 2 h and 5 h. Affinity for the tumor cell surfaces is evidently enhanced for these liposomes, and a moderate enhancement in the fluorescence intensity from liposomes bound at the periphery of the spheroid was obtained when the MLVs were allowed to interact with the spheroids for longer time periods. However, the strong electrostatic binding of liposomes with the spheroids led to minimal intratumoral penetration and diffusion space independent of the duration of liposome-spheroid interaction.

Similar effects were obtained when the mean vesicle size was reduced, allowing positively charged SUVs to interact with the multicellular spheroids. CLSM equatorial

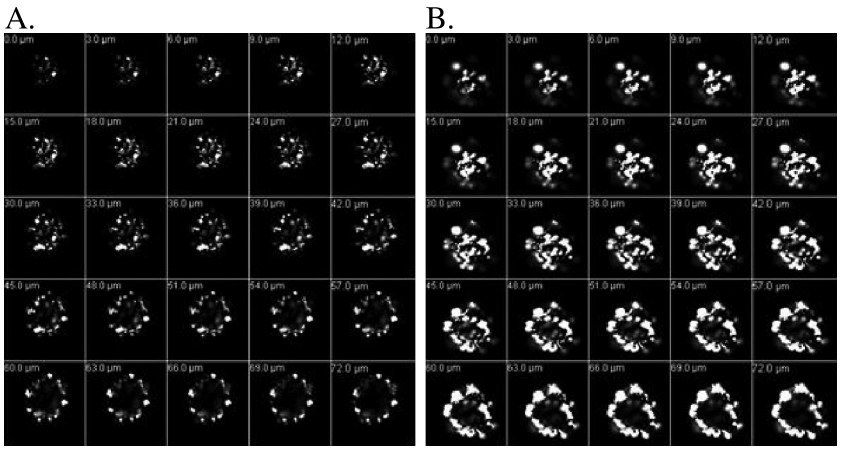

Figure 5. CLSM image galleries of the cationic DMPC:DC-chol MLVs interacting with tumor spheroids for (A) 2 h and (B) 5 h.

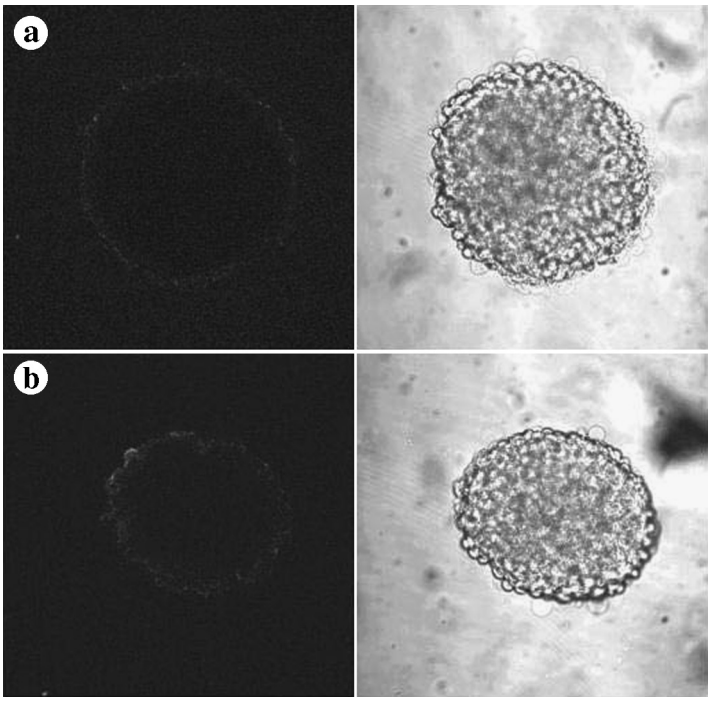

Figure 6. CLSM equatorial optical slice images of the cationic DMPC:DC-chol SUVs interacting with tumor spheroids for (a) 2 h and (b) 5 h.

optical slice images of the cationic DMPC:DC-chol SUVs interacting with tumor spheroids for 2 h and 5 h are shown in Fig. 6. An insignificant increase in fluorescence intensity was again observed at the periphery of the spheroids following interaction with the SUVs for 5 h, due to electrostatic binding of the positively charged liposomes onto the tumor cells. Both Figs. 5 and 6 using positively charged liposomes, indicate that an electrostatic binding-barrier effect may be responsible for inhibition of any notable vesicle diffusion within the tumor volume.

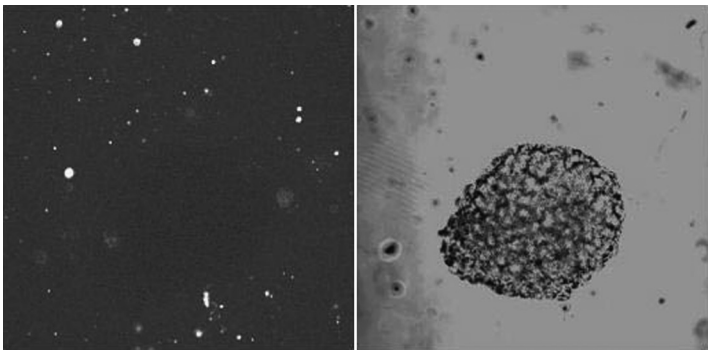

Figure 7. CLSM equatorial optical slice image of the sterically stabilized DMPC:chol:DOPE-PEG liposomes (SUVs) interacting with the tumor spheroids for 2 h. The spheroids were not washed prior to imaging in this case.

The Sterically Stabilized Liposome System Interacting with the Tumor Spheroids

Sterically stabilized liposomes are the most clinically relevant and effective systems for delivery of therapeutics to tumors. Figure 7 is a representative CLSM (and its respective optical microscopy) equatorial slice image of sterically stabilized DMPC:chol:DOPE-PEG SUVs interacting with the LNCaP tumor spheroids for 2 h. In this case washing prior to CLSM imaging was not carried out to demonstrate the presence of fluorescent signal at the spheroid periphery. Even though ample fluorescence signal from the liposomes can be observed in the optical field, there is almost complete exclusion of signal colocalization with the tumor spheroid. This data indicates that polymer-coated liposomes did not interact with the tumor cells or their clusters in any way. The polymer coat at the surface of the SUVs acts as a effective barrier against any contact or attractive force with the tumor spheroid.

Discussion

For solid tumor therapy, the distance a therapeutic moiety travels from the tumor microvasculature to the target cells is a determinant factor of its overall therapeutic effect and end-point index (Jang et al., 2003). Therefore, the fate of liposome-carried therapeutics following extravasation to a solid tumor after intravenous administration or after intratumoral injection is of seminal importance (Drummond et al., 1999; Harrington et al., 2000). Moreover, in relation to treatment development against micrometastases, successful targeting and eradication of a lung-localized micrometastatic model (approximate size of tumors 500 μm) has been reported (Ahmad et al., 1993), however no report has previously appeared studying the binding and distribution of liposomes within prevascularized tumor metastases. The present work has attempted to offer a qualitative systematic study of the surface binding and intratumoral penetration of liposomes interacting with multicellular spheroids used as in vitro models of an avascular tumor mass. The main purpose of the present study was to correlate some critical physicochemical characteristics of liposomes systems with their transport characteristics within the spheroid tumor masses.

Since their description (Moscona et al., 1957), multicellular spheroid cell cultures have gained popularity as in vitro models for the development of therapeutics against a variety of tumors, due to closer correlation with in vivo tumor models than planar monolayer cell cultures (Helmlinger et al., 1997; Sutherland, 1988). Multicellular tumor spheroids of the LNCap-LN3 cell line were first formed and reported by our group previously (Ballangrud et al., 1999) as a simple but clinically relevant model for the study of drug delivery and response of prostate carcinomas. Furthermore, we used the LNCaP spheroids as models of disseminated prostate cancer and investigated their response to treatment by ^{213}Bi radioimmumotherapeutics (Ballangrud et al., 2001). Multicellular tumor spheroids have been employed by numerous investigators toward assessment or modeling of the transport and tumoricidal characteristics of different therapeutic modalities including cisplatin (Fujiwara et al., 1994), immunotoxins (Wenning and Murphy, 1999), andiamycin (Durand, 1981), tirapazamine (Hicks et al., 1998), radiation (Stuschke et al., 1992), or other metabolically relevant molecules such as glucose (Casciari et al., 1988). More recently, the therapeutic effect and diffusion of various selectively replicative (Bauerschmitz et al., 2002; Grill et al., 2002) and nonreplicative (Enger et al., 2002; van Beusechem et al., 2002) viral gene therapy vectors have been studied. Even though systematic studies of the binding, distribution, and chemotherapeutic effect of

doxorubicin and daunorubicin (Kaaijk et al., 1996; Wartenberg et al., 1998), two therapeutic molecules clinically used in their liposome-encapsulated form for treatment of Kaposi sarcomas and being developed also for other cancer types (Tejada-Berges et al., 2002), have been published, no systematic study has described the interaction of tumor spheroids with delivery systems or liposomes in particular.

Here, the effect of various liposome properties was correlated with their penetration profiles within tumor spheroids obtained by CLSM imaging. The liposomal physicochemical characteristics varied in the present study included mean vesicle size, lipid bilayer phase, and surface charge. The classical liposome composition containing DMCP and cholesterol showed that penetration and retention within tumor spheroids were dependent on size, only occurring in the case of SUVs. This result indicates that SUVs of low anionic surface character and small size (< 100 nm) will be able to distribute a drug within a tumor volume more homogeneously, most likely by a diffusion-dependent mechanism.

Substitution of DMPC with DPPC and cholesterol with DC-cholesterol led to engineering of zwitterionic, gel liposomes and positively charged, liquid-crystalline liposomes respectively. Interestingly, completely opposite results were obtained when each of the two systems were interacted with tumor spheroids. The DPPC rigid liposomes exhibited complete lack of penetration and retention within the tumor mass irrespective of size or duration of interaction (not shown). For the DC-chol cationic liposomes very strong surface binding was observed for both the MLV and SUV vesicle systems. However, penetration in the inner tumor volume was extremely poor with the cationic liposomes, indicating lack of effective intratumoral transport capabilities. In relation to recently proposed use of cationic liposomes for targeting angiogenic tumor microvasculature (Campbell et al., 2002; Krasnici et al., 2003), our data suggest that effective binding of endothelial cell surfaces using such cationic liposomes can indeed be achieved, however, intratumoral diffusion will not be possible due to lack of any penetrative capacity exhibited in the present studies. Moreover, the present data indicate that a possible electrostatic binding site barrier effect may inhibit cationic liposomes from further binding onto target cells due to the electrostatic repulsive forces between liposomes tighly bound onto the cell surfaces and those in their vicinity.

Polymer-coated liposomes are currently used in the clinic as delivery systems for anticancer agents, thus of particular interest. In the present study, even though DMPC:chol:DOPE-PEG_{2000} sterically stabilized liposomes were of small enough mean diameter to allow diffusion within the tumor spheroids similar to the DMPC:chol classical liposomes, there was no intratumoral penetration observed. This in vitro observation correlates well with the noted immobility and restricted transport of sterically stabilized liposomes following their extravasation from the tumor vasculature into its intrestitial space in vivo (Monsky et al., 1999). It would be suggested from the current observations that improvements of sterically stabilized liposomes should incorporate a mechanism of polymer coat shedding once tumor target is reached, to allow for more efficient penetration within the avascular tumor interstitium and therefore more homogenous distribution of the delivered drug throughout the tumor mass.

Our results indicate that the physicochemical characteristics of liposome systems are critically important in determining the interaction with the tumor spheroids at the microscopic scale studied. It has been repeatedly emphasized previously that liposome physicochemical characteristics at the macroscopic level, play a determinant role in effectively targeting specific tissues once in blood circulation (Abra et al., 2002). We have further attempted to use the data offered in the present study to rationally design more

intratumorally penetrative liposomes (Kostarelos et al., 2004). The effective delivery of therapeutics to solid tumors or prevascularised metastatic nodules in circulation or residing at specific tissues, is a more complex process than initially considered, not achieved by simply enhancing the levels of drug in the tumor. Multicellular tumor spheroids offer a useful model of avascular tumor mass to optimize the engineering parameters of delivery systems for anticancer therapeutics.

Abbreviations

MLV	multilamellar vesicles
SUV	small unilamellar vesicles
DMPC	dimyristoyl-phosphatidylcholine
DC-chol	3b-[N-(N′,N′-Dimethylaminoethane)-carbamoyl]Cholesterol
DPPC	dipalmitoyl phosphatidylcholine
DOTAP	N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl-sulfate
DOPE-PEG_{2000}	1,2-Diacyl-*sn*-Glycero-3-Phosphoethanolamine-N-[Methoxy(Poly-ethylene glycol)-2000]
PBS	phosphate buffer saline
CLSM	confocal laser scanning microscope

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