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Research paper

The suitability of liposomes for the delivery of hydrophobic drugs – A case study with curcumin

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ABSTRACT

Liposomes are a popular formulation strategy for the delivery of anticancer drugs. While their benefits for formulating hydrophilic anticancer drugs have been clearly shown during the last decades, the suitability of liposomes for the delivery of hydrophobic drugs is questionable. Curcumin is a diphenolic plant compound that is extensively researched for its anticancer properties. It was chosen as a hydrophobic model drug in this study. Due to its low bioavailability, poor solubility and instability in aqueous media it is a highly problematic compound and requires particular formulation techniques. Curcumin liposomes with lipids of different rigidities were comprehensively investigated in respect to their physicochemical properties, their storage and serum stability. *In vitro* experiments were conducted with common 2D cell models and additionally with multicellular tumor spheroids (MCTS) as a more sophisticated tool to represent the physiology of avascular solid tumors. Our results indicate that liposomes containing the fluid phospholipid dioleoylphosphatidylcholine (DOPC) represent an excellent formulation to enhance the solubility and stability of curcumin. However, in presence of serum or cells, curcumin is rapidly released from the protecting and stabilizing lipid bilayer. Thus, improvement of the *in vivo* efficacy of curcumin is probably not achieved by using liposomes. Cytotoxicity and uptake experiments showed clearly a reduced effectivity of curcumin liposomes in the 3D cell model in comparison to the 2D model. This not only illustrates the limitations of monolayer cultures in predicting drug and nanocarrier interactions with solid tumors, but also further questions the use of liposomes as a formulation strategy in the treatment of solid tumors with curcumin.

1. Introduction

Since their discovery in the mid-1960s by Bangham [2], liposomes evolved into an important tool in pharmaceutical drug delivery. Their unique structure of vesicular phospholipid membranes offers the opportunity to formulate both hydrophilic drugs in their inner aqueous cavity and hydrophobic drugs in their phospholipid bilayer. Hence, liposomes can be advantageous to improve the solubility of poorly soluble drugs as well as to protect drugs from degradation or metabolic processes [1,7]. Furthermore, liposomes are often applied as circulating drug depots. This can be particularly beneficial in the delivery of cytostatics, as nanocarriers are supposed to accumulate in solid tumors due to the enhanced permeability and retention effect [28,23]. While the significance of this effect in the clinic is still under debate [22,33], several liposomal formulations of cytostatics reached drug approval and were associated with reduced side effects [5,14,40]. The most prominent example certainly is Caelix/DOXIL, where doxorubicin sulfate is actively loaded into PEGylated liposomes by a pH gradient technique [17,3]. The incorporation of hydrophobic drugs into the liposomal bilayer faces different challenges than the encapsulation of hydrophilic substances. First, the association into the bilayer can lead to perturbation of the lipid membrane and therefore alter the liposome stability and drug loading capacity [43]. Second, the residence of the drug in the bilayer is mainly based on a distribution process of the drug. Even though high incorporation efficiencies up to 100% can be reached [10], changes in the liposomal environment can lead to significant redistribution processes of the drug. Hence, after administration of such liposomes *in vivo*, the dilution process and acceptors for lipophilic molecules such as serum proteins or cellular membranes can decrease the ability of the liposomes to retain the drug until reaching the target tissue [1,36].

Evaluation of new compounds and formulations in 2D monolayer *in vitro* cell culture systems is crucial for the identification of lead candidates and for investigation of drug efficacy at the cellular level using high throughput screening (HTS) approaches. However, especially in the field of oncology, there is growing evidence that the failure of promising drug candidates and formulations in clinical studies is linked to limited physiological significance of common 2D *in vitro* cell culture systems used in

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preclinical studies [8,15,45]. 2D monolayer cell models are not able to address *in vivo* tumor features like cell-cell interactions, drug resistance due to transport restrictions associated with the tumor microenvironment and cell heterogeneity. There is a growing interest in more complex *in vitro* models that can bridge the gap between the oversimplified 2D monolayer cell models and the highly complex nature of a growing *in vivo* tumor.

Multicellular tumor spheroids (MCTS) are one of the widely used *in vitro* 3D tumor models. They are comprised of an outer rim with rapidly proliferating cells, a middle layer consisting of quiescent cells and, depending on the diameter, a core of necrotic cells. Their cell-cell interactions, metabolic characteristics such as pH, oxygen, metabolite and catabolite gradients, and their extracellular matrix development compare to avascular microtumors and micrometastases [30,42,20,11]. In addition to the metabolic similarities of the MCTS model to *in vivo* tumors, the morphological common features of MCTS and *in vivo* tumors allow the evaluation of liposome efficiency in a model that bridges traditional 2D monolayer models and xenografts [29,12].

Curcumin is a diphenolic secondary plant compound that can be isolated from the rhizome of *Curcuma longa* L. While having been used in traditional Asian medicine for a long time, it undergoes a massive growth in research as a drug for a variety of diseases today [13]. One of the most investigated indications of curcumin is cancer, where it was reported to have anti-proliferative and pro-apoptotic effects [49,32]. On a molecular level, several mechanisms of action were proposed such as its redox activity [39,35,6]or the inhbition of Nf-κB [18,35,50]. The latter plays a crucial role in the downregulation of various proteins involved in cancer progression such as Bcl-2, cyclin D1, COX-2 and matrix metalloproteinases [18,35,50], as well as of ABC transporters being responsible for multidrug resistance [48]. The good safety profile and its pleiotropic effects are often regarded as the main advantages of curcumin in cancer treatment [49,32]. However, the effectivity of curcumin in the clinics still requires proof and its application is furthermore hindered by two major drawbacks: its extremely poor aqueous solubility (< 1 µg/ml [44,21]) and its instability in aqueous environments at pH > 7 [47,9,27]. These factors lead, amongst others, also to the extremely low bioavailability of curcumin [13,26], which impedes its oral administration. Overall, these properties make curcumin a highly problematic drug and require particular formulation strategies even for parenteral administration. Besides a huge variety in proposed nanoformulations for curcumin, liposomal formulations are currently a popular strategy. However, the above-named difficulties related to hydrophobic drugs incorporated in liposomes require critical and detailed investigation. Hence, this study aimed to evaluate the suitability of liposomes for curcumin delivery regarding (a) the physicochemical properties and especially the stability of manufactured liposomes, with a systematic comparison between different phospholipids, (b) the behavior in terms of drug retention in physiological fluids and environments, and (c) the interaction of curcumin liposomes with cells. For the latter, the above mentioned multicellular tumor spheroids were applied in addition to the traditionally used 2D monolayer cell culture systems.

2. Materials

Curcumin and cholesterol (Chol) were purchased from Sigma-Aldrich (Germany). 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-mPEG2000) were kindly provided by Lipoid (Germany). 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) was obtained from Merck (Germany). Agarose, HEPES and NaCl were bought from Carl Roth (Germany). CellTiter-Glo® was from Promega (Germany), calcein-AM, propidium iodide, Rhodamine B 1,2-dihexadecanoyl-*sn*-glyerco-3-phosphoethanolamine (rhodamine-DHPE) from Molecular Probes (USA). VLE RPMI, PBS w/o Ca^{2+} and Mg^{2+}, Trypsin/EDTA, fetal calf serum (FCS) were obtained from Biochrom (Germany), Sepharose CL-4b from GE Healthcare Life Sciences (Sweden). All used solvents were of HPLC grade or higher and were from Carl Roth (Germany).

3. Methods

3.1. Curcumin quantification

The curcumin content of the liposomes was analyzed by high performance liquid chromatography (HPLC) using a Waters 2695 separations module and a Waters 2996 photodiode array detector (Waters, USA). Liposome samples were prepared by mixing either 31.6 µl curcumin liposomes (original concentration) with 600 µl MeOH, or 500 µl diluted liposomes with 500 µl MeOH. Calibrators of different curcumin concentrations were prepared in MeOH. A LiChroCART® 250-4 LiChrospher® 100 RP-18e (5 µm) or a LiChroCART® 250-4.6 Purospher® RP-18e (5 µm) column (Merck, Germany) was used. The separation was performed isocratically with a mobile phase composed of citric acid buffer 1% (m/V) pH 3 and acetonitrile in a ratio of 55:45 (V/V) and a temperature of 25 °C (LiChrospher®) or in a ratio of 50:50 (V/V) at 35 °C (Purospher®). The flow rate was set to 1 ml/min. Either 10 µl (curcumin liposomes at the original concentration) or 100 µl (diluted liposomes) were injected into the system and curcumin was detected at a wavelength of 427 nm. The curcumin peak was automatically integrated by Empower® 3.0 software (Waters, USA).

3.2. Liposome preparation

Curcumin liposomes were prepared by the film hydration method followed by hand extrusion [2,34]. Organic stock solutions of lipids and curcumin were transferred into a round bottom flask and the solvent was removed by rotary evaporation. The lipid film was hydrated with 1 ml HEPES buffered saline (HBS, 10 mM HEPES, 140 mM NaCl, pH 7.4) to a final lipid concentration of 20 mM. The formed liposomes were extruded for 41 passages through a polycarbonate membrane with a pore diameter of 80 nm (Nuclepore®, GE Healthcare Life Sciences, USA). Solvent removal, film hydration and extrusion all were performed at a temperature at least 15 °C higher than the phase transition temperature of the respective phospholipid. Eventually non-incorporated crystalline or aggregated curcumin was removed by centrifugation at 10 621*g* and 25 °C for 15 min. For cytotoxicity experiments, the liposomes were sterile filtered under aseptic conditions using Millex®-GV 0.22 µm syringe filter (Merck, Germany). Table 1 gives an overview of liposome compositions used in this study.

Table 1
Compositions of prepared liposomes with DOPC, DMPC, DPPC or DSPC as phospholipid.

	Phospholipid [mol%]	Cholesterol [mol%]	DSPE-mPEG2000 [mol%]	Curcumin [mol%]	DOTAP
CURlip-DOPC CURlip-DMPC CURlip-DPPC CURlip-DSPC	65	30	5	4.4	–
DOTAPlip-DOPC	55	30	5	–	10

3.3. Liposome characterization and stability measurements

3.3.1. Size and polydispersity index

The hydrodynamic diameter (size) and the polydispersity index (PDI) of the liposomes were analyzed by dynamic light scattering (DLS) (ZetaPals, Brookhaven Instruments Corporation, USA).

3.3.2. Curcumin/lipid ratio and incorporation efficiency

After liposome preparation, the curcumin content was measured by HPLC and the Bartlett assay was used to analyze the phospholipid content [4]. The curcumin/lipid ratio was calculated by Eq. (1), the incorporation efficiency (IE) of curcumin in liposomes was calculated by Eq. (2),

$$CUR/lipid\,[\mathrm{mol/mol}] = \frac{c_a}{L_a} \tag{1}$$

$$IE\,[\%] = \frac{c_a \times L_i}{c_i \times L_a} \times 100\% \tag{2}$$

where c_a is the actual curcumin concentration, c_i the initial curcumin concentration, L_i the initial phospholipid concentration and L_a the actual phospholipid concentration. The calculation was based on the assumption that non-incorporated curcumin can be dissolved in the aqueous phase or occurs as aggregates or crystals. Since the liposomes were centrifuged during preparation, the latter were separated from the liposomes before HPLC analysis of curcumin. The amount of free curcumin was regarded as negligible, because the aqueous solubility of curcumin is very low [44,21].

3.3.3. Liposome stability studies

For storage stability studies the liposomes were kept at 4–8 °C and characterized by DLS and their curcumin content after 4, 8 and 16 weeks. Stability of liposomal curcumin was also measured after 60-fold dilution with HBS, leading to a curcumin concentration of 5 μg/ml. The samples were stored at 20–25 °C for 0, 1, 3 and 7 d or at 37 °C for 0, 1, 3, 6 and 24 h. The stability of free curcumin was investigated in HBS with a volume fraction of 5% MeOH at a curcumin concentration of 5 μg/ml. These samples were stored at 20–25 °C for 0, 0.5, 1, 3, 6, 24 and 168 h, or at 37 °C for 0, 1, 3, 6 and 24 h. The curcumin (CUR) content of all samples was measured by HPLC and related to the initially measured content.

For stability studies of liposomal curcumin in the presence of serum, curcumin liposomes were mixed with human serum in a ratio of 1:1 (V/V). The samples were incubated at room temperature for 1 min or at 37 °C for 24 h. Size exclusion chromatography was performed with Sepharose CL-4B columns to separate liposomal and protein bound curcumin. The fractions were collected and mixed with a 5-fold volume of MeOH to precipitate the serum proteins. The samples were kept at 4–8 °C for 30 min and centrifuged at 10 000*g* at 4 °C for 15 min. The supernatant was taken, recentrifuged and its curcumin content was analyzed by HPLC. The curcumin content of the liposome and protein fraction was related to the initially measured content of the liposomes.

3.4. Cell culture

A2780 ovarian carcinoma cells (ECACC, Salisbury, UK) and LS neuroblastoma cells (a kind gift of Prof. Dr. Rupert Handgretinger, Universitätskinderklinik Tübingen, Germany) were grown at 37 °C, 5% CO_2 in a humidified incubator in VLE RPMI-1640 supplemented with 10% FCS (v/v). All cell lines were free of mycoplasma.

Multicellular tumor spheroids (MCTS) were generated by the liquid overlay method [11]. Briefly, PBS with 1.5% (w/v) agarose was autoclaved. 50 μl of the still hot agarose solution were added to the bottom of each well of 96 well plates under aseptic conditions. The plates were allowed to cool down for 30 min before seeding of 4000 cells per well in a volume of 200 μl. The plates were then centrifuged at 600*g*, 5 min and 20 °C and incubated for at least 24 h without disturbance. Spheroid formation and growth was monitored using an Axiovert 40 CFL microscope with Axiocam and Axiovision software 4.6 (Carl Zeiss, Germany). Spheroids were grown for at least three days until they reached a diameter of approximately 400 μm.

3.5. Live/dead staining of spheroids

After 4 days of culture, spheroids were washed three times with PBS and then stained with calcein-AM (1 μM) and propidium iodide (5 μM) in PBS for 2 h at 37 °C. Proliferating and necrotic cells were visualized with the Axiovert 40 CFL, 10 times objective and a corresponding filter set for calcein and propidium iodide.

3.6. Cytotoxicity

For monolayer experiments A2780 or LS cells were seeded in opaque 96 well plates in a density of 5 000 or 2 000 cells/well 24 h prior to the experiments. The cells were treated with free CUR and CURlip dilutions and after 72 h CellTiter-Glo® assay was used according to the manufacturer's protocol.

For determination of spheroid viability spheroids were cultured in opaque agarose coated 96 well plates for 3 days. Then they were treated with free CUR and CURlip dilutions and after 72 h CellTiter-Glo® 3D assay was used according to the manufacturer's protocol.

EC_{50} values were calculated using Excel solver and the principle of nonlinear least squares data fitting [19]and equation

$$y = y_0 + \frac{y_{100} - y_0}{1 + (\frac{EC_{50}}{x})^{-p}} \tag{3}$$

where y denotes the cell viability at concentration x in μM, y_{100} the viability of control cells, y_0 the lowest viability and p the hill coefficient. At least three different data sets were analyzed and the EC_{50} calculated as the mean of the different EC_{50} values.

3.7. Uptake studies

Cellular association of free CUR and CURlip-DOPC was assessed by flow cytometry after 5 min, 15 min, 1 h, 2 h and 4 h incubation. Final curcumin concentrations were 5, 20, 50 and 75 μM. CURlip-DOPC contained 0.05 mol% rhodamine-DHPE (Molecular Probes) as a fluorescent membrane label.

For monolayer experiments LS or A2780 cells were seeded in 24 well plates at a density of 60 000 cells/well and grown for 24 h. Stocks of free CUR in DMSO and CURlip-DOPC were diluted with cell culture medium immediately before use and 100 μl of the dilutions were added to the cells. At the end of the incubation period, cells were washed twice with ice cold PBS, trypsinized and detached from the wells. After washing with PBS, cell suspensions were analyzed with a LSRFortessa™ flow cytometer (Becton Dickinson, Germany) using 488 nm blue laser and 561 nm yellowgreen laser for excitation and 530/30 nm (curcumin) and 585/15 nm (rhodamine DHPE) filter sets for analysis. 10 000 events were recorded and cells were gated using forward vs. sideward scatter to determine the live cell population.

Association of free CUR and CURlip-DOPC in spheroids was investigated using 3 days old spheroids with a diameter of approximately 400 μm. After 2 h of incubation with free CUR and CURlip-DOPC, 10 spheroids as one replicate were collected into tubes to achieve the necessary cell number. The spheroids were washed twice with ice cold PBS. Subsequently, they were dispersed into single cells by incubation with Trypsin/EDTA (0.25%/0.2%) for 10 min at 37 °C with occasional pipetting. After addition of cell culture medium and washing with PBS, 5 000 events were recorded and analyzed with the same flow cytometer settings as described above.

3.8. Statistical analysis

The data were analyzed using one-way ANOVA. Statistical difference was set at p values < 0.05 (* < 0.05, ** < 0.01, *** < 0.001, **** < 0.0001). Statistical analyses were carried out with GraphPad Prism Version 6 (GraphPad Software, USA).

4. Results

4.1. Liposome characterization and stability

Curcumin liposomes were prepared by the film hydration method followed by hand extrusion. Their compositions differed in the saturation and chain length of the main phospholipid and therefore in the rigidity of the lipid bilayer.

All prepared liposomes showed appropriate physicochemical properties with hydrodynamic diameters of approximately 100 nm, PDIs below 0.1 and incorporation efficiencies between 80 and 100% (see Table 2). Nevertheless, liposomes composed of more rigid phospholipids tended to be slightly smaller and exhibited lower PDIs as well as lower incorporation efficiencies. Additionally, the formation of a sediment was observed after centrifugation of liposomes with rigid phospholipids.

After 16 weeks of storage at 4–8 °C, no significant change in size was observed in all liposome types (Fig. 1, A). Contrarily, a significant instability was observed in terms of the PDI for all liposomes except CURlip-DOPC. CURlip-DMPC and Curlip-DPPC reached a PDI of approximately 0.2 after 16 weeks (Fig. 1, B). The PDI of CURlip-DOPC remained stable below 0.1 and that of CURlip-DSPC continuously rose to a value > 0.1 over the time period. All liposomal formulations were able to protect curcumin from degradation during storage (Fig. 1, C). After 16 weeks, at least 80% of the initial curcumin content were still present in the formulations. Significant changes were only observed for CURlip-DSPC ($p < 0.05$).

The stability of curcumin was also measured after liposome dilution and compared to that of free CUR (Fig. 2). Free CUR quickly degraded to less than 50% after only 0.5 h, which is in line to the results of [47]. Even though the diluted liposomes were able to diminish this fast decay, the protective effect was clearly inferior compared to the original liposome concentration. Approximately 50% of the initial curcumin were still present after 7 d at 20–25 °C and after 24 h at 37 °C.

The liposomal formulations were incubated with human serum to investigate their ability to retain the loaded curcumin in physiological environments. The liposomal fraction of curcumin was separated from the protein bound fraction by size exclusion chromatography (SEC). As depicted in Fig. 3, already after 1 min incubation the amount of curcumin in the liposomal fraction decreased significantly for all liposomes and 20–50% of the curcumin content eluted in the protein fraction. CURlip-DOPC clearly differed from the other formulations, as 60% of the initial curcumin concentration remained in the liposomes after 1 min, whereas the more rigid formulations were only able to retain 20% (significant with $p < 0.0001$). After an incubation time of 24 h, the content in the liposomes slightly continued decreasing, whereas in the protein fraction no clear trend of increase was observed.

4.2. Multicellular tumor spheroid formation and morphology

Multicellular tumor spheroids were generated with the liquid overlay method. Spheroid morphology was assessed after [46]. A2780 ovarian carcinoma cells were not able to form the described dense spheroids with defined edges. Instead, rather loose aggregates were observed, which was also reported previously for this cell line [46,31]. In contrast, LS neuroblastoma cells could be formed into tight, dense spheroids with defined edges. In our study, the spheroid production yield was ≥ 95%. Spheroid diameters were highly reproducible with an inter plate coefficient of variation of less than 3%.

Live/dead staining of LS spheroids with calcein-AM and propidium iodide revealed the typical architecture of tight, dense spheroids with a diameter of over 400 µm. A necrotic core (red) is surrounded by a rim of rapidly proliferating cells (green) (Fig. 4). The formation of these biological zones is due to several physicochemical gradients occuring in multicellular tumor spheroids such as oxygen partial pressure, metabolites, catabolites and nutrients. These gradients are comparable to those of avascular microtumors and micrometastases *in vivo* and make the MCTS to an attractive *in vitro* tumor model.

4.3. Cellular association of CURlip-DOPC and free CUR

4.3.1. LS and A2780 2D monolayer

The cellular association of free CUR and CURlip-DOPC was evaluated time and concentration dependent in LS and A2780 monolayer cell cultures by flow cytometry.

The uptake kinetics of free CUR and CURlip-DOPC were comparable in both cell lines. Free CUR and CURlip-DOPC showed a prominent uptake after only 5 min incubation. Afterwards only a marginal increase in curcumin fluorescence was measured for CURlip-DOPC in both cell lines (Fig. 5A). Cells treated with free CUR showed a 40–60% higher curcumin fluorescence compared to cells treated with the liposomal formulation after 4 h. The fast uptake process suggests passive, diffusion-controlled uptake of curcumin into the cells, rather than an endocytic uptake of the whole liposomes.

The application of free CUR at increasing concentrations resulted in a steady increase of curcumin fluorescence in A2780 and LS cells. At a concentration of 75 µM curcumin, the fluorescence was 50–60% higher when compared to cells treated with the CURlip. A2780 and LS cells treated with CURlip-DOPC exhibited a nonlinear curcumin association profile with a clear saturation (Fig. 5B). This could be attributed to a curcumin retention in the liposomes due to formation of a distribution equilibrium.

4.3.2. LS spheroids

Spheroid cultures were used as a more sophisticated *in vitro* tumor model to assess the association of free CUR and CURlip-DOPC in comparison to the simpler 2D monolayer cell model. The association of free CUR and CURlip-DOPC was measured at various concentrations after an incubation time of 2 h by flow cytometry. DOTAPlip, a cationic formulation that should strongly associate with the cells, was used as a positive control for high liposome uptake.

Compared to the 2D monolayer cells the association of free CUR and CURlip-DOPC was clearly reduced in spheroid cultures. At low concentrations of 5 µM curcumin the association was decreased to

Table 2
Hydrodynamic diameter, polydispersity index (PDI), CUR/lipid ratio and incorporation efficiency of prepared liposomes; n = 3–4 ± SD.

	Hydrodynamic diameter [nm]	PDI	CUR/lipid ratio (mol/mol)	Incorporation efficiency [%]
CURlip-DOPC	130 ± 12	0.07 ± 0.02	0.042 ± 0.002	95 ± 4
CURlip-DMPC	104 ± 8	0.05 ± 0.02	0.041 ± 0.001	94 ± 3
CURlip-DPPC	99 ± 7	0.04 ± 0.02	0.037 ± 0.001	84 ± 3
CURlip-DSPC	91 ± 13	0.04 ± 0.01	0.035 ± 0.004	79 ± 9

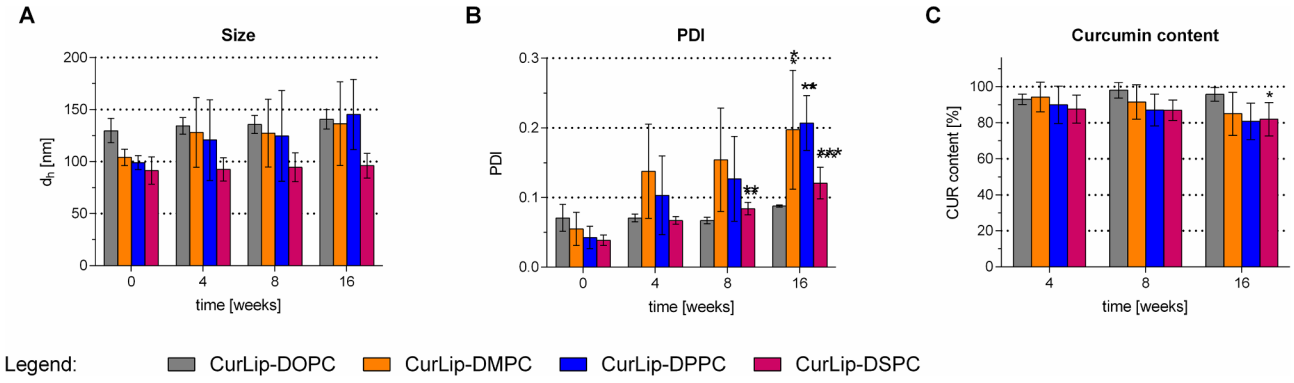

Fig. 1. Stability of size (hydrodynamic diameter, A), polydispersity index (PDI, B) and curcumin content (C) of prepared liposomes during storage at 4–8 °C for up to 16 weeks; n = 3–4, mean ± SD. Data were analyzed using one-way ANOVA and comparison of each timepoint with timepoint 0 for each liposome type.

approximately 30% of the curcumin association in monolayer cells. At higher concentrations this difference was declining for free CUR but was still pronounced in the case of CURlip-DOPC (Fig. 6A).

The uptake of liposomes was extremely low in both cell models, which was measurable by the very low fluorescence intensities of the membrane label rhodamine-DHPE. Interestingly, in spheroids even the association of the cationic control formulation DOTAPlip was decreased to 5% of the fluorescence signal in 2D monolayer cells (Fig. 6B).

4.4. In vitro effectiveness of free CUR and CURlip-DOPC

Cell viability following exposure to free CUR and CURlip-DOPC was assessed using the CellTiter-Glo® assay. The calculated half maximal effective concentration (EC_{50}) values for free CUR and CURlip-DOPC in 2D monolayer cells were almost identical (Table 3). Spheroids showed a pronounced resistance against free CUR compared to monolayer cells which resulted in a 15-fold increase of the EC_{50} value. The effectiveness of CURlip-DOPC in the spheroid culture was extremely poor. Even at the highest CURlip-DOPC concentration of 100 µM the ATP levels were hardly impacted and an EC_{50} value could not be calculated. These results were confirmed by microscopic evaluation (Fig. 7). Treatment of the spheroids with free CUR resulted in a partial reduction of spheroid growth, whereas treatment with CURlip-DOPC only slightly impacted the spheroid diameter (Fig. 7).

5. Discussion

In this study, curcumin was chosen as a model drug to investigate the suitability of liposomes for the delivery of hydrophobic drugs. Curcumin has an extremely low aqueous solubility and insufficient stability in aqueous media; therefore, it represents a highly problematic drug.

In the first part of this study, an appropriate composition of the liposomal formulation was found. The formulations were based on the lipid composition of Doxil, which contains HSPC/Chol/DSPE-PEG in a molar ratio of 57/38/5 [41]. The use of DSPE-PEG is crucial to achieve long-circulating liposomes. However, we preferred to use synthetic phospholipids instead of natural lipid blends such as HSPC, to work with defined structures. We focused on a systematic comparison between phospholipids of different transitions temperatures. Hence, the chain length as well as the saturation of the lipids was varied.

The most fluid phospholipid DOPC gave the best results in terms of physicochemical properties and stability and showed the highest incorporation efficiency. While directly after preparation PDI and size were in a good range for all tested liposomes, a pronounced instability of the rigid formulations during storage was recognizable due to an increase in their PDI. CURlip-DOPC did not show these physical changes and protected curcumin from degradation during storage very efficiently. Also in terms of serum stability this formulation showed the best results. Therefore, further *in vitro* evaluation was only carried out with this formulation.

However, even though CURlip-DOPC showed excellent physicochemical properties and storage stability, a clear drawback emerged: while being very stable at the original concentration, a strong decrease in the curcumin content after dilution gave a hint to partitioning of curcumin out of the bilayer followed by degradation. In the presence of serum proteins, 30% of the initial curcumin amount bound to serum proteins after only 1 min. A burst release *in vivo* due to diffusion processes is therefore very probable. This is important when aiming at using liposomes for passive tumor targeting. Furthermore, protection of curcumin from degradation by the liposomal formulation during blood circulation is difficult to reach, when curcumin is quickly released.

In future studies, the curcumin retention in liposomes of other lipid compositions could be investigated. Fluid natural lipid blends such as

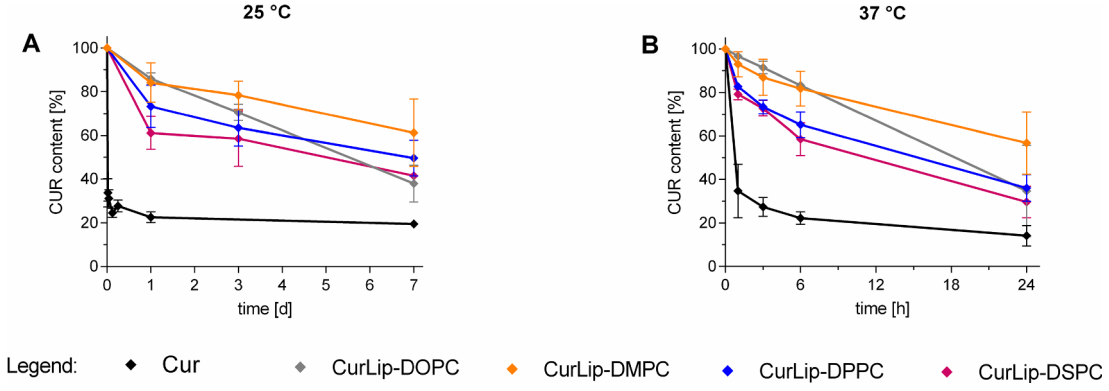

Fig. 2. Stability of free curcumin and diluted liposomal curcumin at 20–25 °C for 7 d (A) and at 37 °C (B) over 24 h; n = 3–6, mean ± SD.

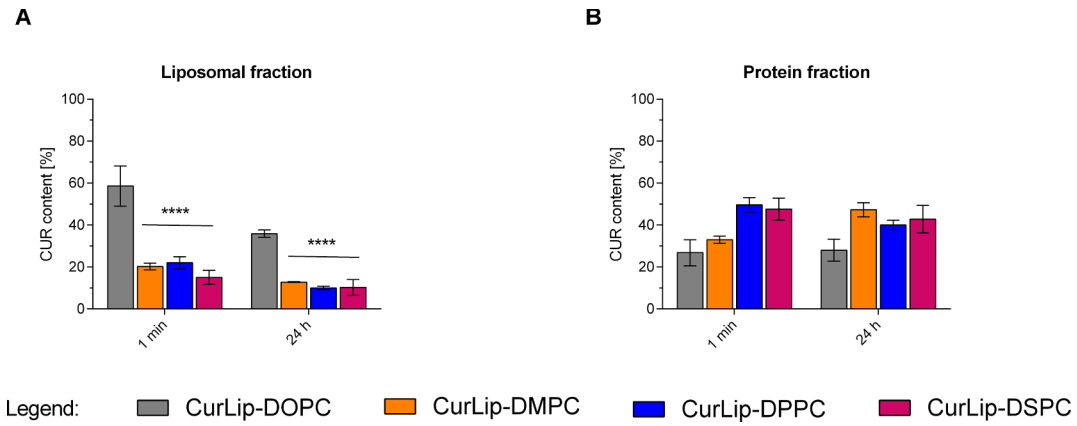

Fig. 3. Curcumin content in the liposomal fraction (A) and in the protein fraction (B) after 1:1 (v/v) incubation of curcumin liposomes with human serum and separation by size exclusion chromatography (SEC); n = 3, mean ± SD. Data (A) were analyzed using one-way ANOVA and comparison of CURlip-DOPC with each other liposome type.

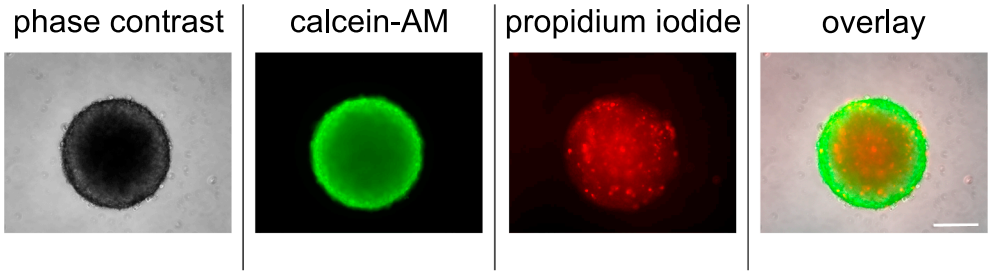

Fig. 4. Fluorescence microscopy images of 4 days old LS-spheroids after 2 h incubation with calcein-AM and propidium iodide. Calcein fluorescence (green) is mainly located at the rim of the spheroid and indicates rapidly proliferating cells. Propidium iodide (red) is located mainly in the spheroid core and reflects central necrosis. The scale bar represents 200 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

SPC or EPC could be compared with DOPC, the phospholipid of the formulation with desirable characteristics in this study. Cholesterol was included into the liposomes of the present study, since liposomes without cholesterol are prone to phospholipid removal by apolipoproteins [37,16,38]. Even though we already used a decreased cholesterol content in comparison to the above-mentioned Doxil formulation, further reduction of the cholesterol content might be favorable. Mahmud and coworkers demonstrated that a formulation composed of hydrogenated SPC/DSPE-PEG/CUR (90:5:5) retained 90% of the initial curcumin amount in the liposomes after incubation with human plasma, compared to only 60% in the corresponding cholesterol containing composition [25]. Hence, cholesterol-free curcumin liposomes could be a possibility to improve liposomal curcumin retention.

In the second part, the efficiency of CURlip-DOPC was evaluated and compared to free CUR in two cell models. Commonly used 2D monolayer cells and MCTS were used to investigate the toxicity of free CUR and CURlip-DOPC. In 2D monolayers of A2780 ovarian carcinoma and LS neuroblastoma cells the efficiency of free CUR and CURlip-DOPC was nearly the same. These findings suggest that CURlip-DOPC is not taken up by the cells as whole liposomes. It is more likely that curcumin gets released from the liposomes, distributes to the cell membranes and diffuses into the cell interior. These results were confirmed by our cellular association studies. Nearly immediately after addition of CURlip-DOPC to the cells, curcumin fluorescence could be detected and did not change significantly over 4 h. Concentration dependent association studies with CURlip-DOPC showed a saturation compared to the free drug which suggests that part of the curcumin is released whereas a portion is retained in the liposomes. Fluorescence of rhodamine-DHPE which was used as liposomal membrane marker was hardly detected at all in both cell lines.

Overall the efficiency and association studies in 2D monolayer cells are in accordance with the release experiments: CURlip-DOPC shows a

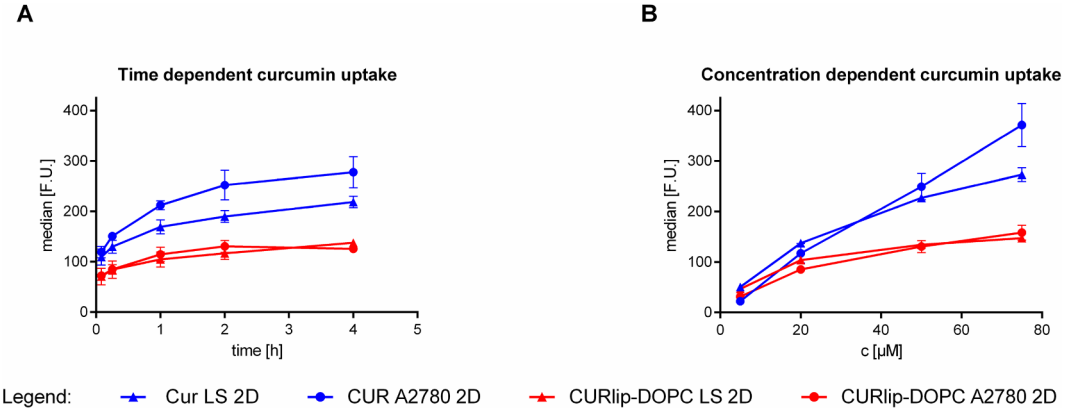

Fig. 5. Cellular association of free CUR and CURlip-DOPC in A2780 and LS cells. (A) Time dependent association at a concentration of 50 µM curcumin, (B) concentration dependent uptake after 2 h treatment. Curcumin fluorescence was measured by flow cytometry; n = 3–4, mean ± SD.

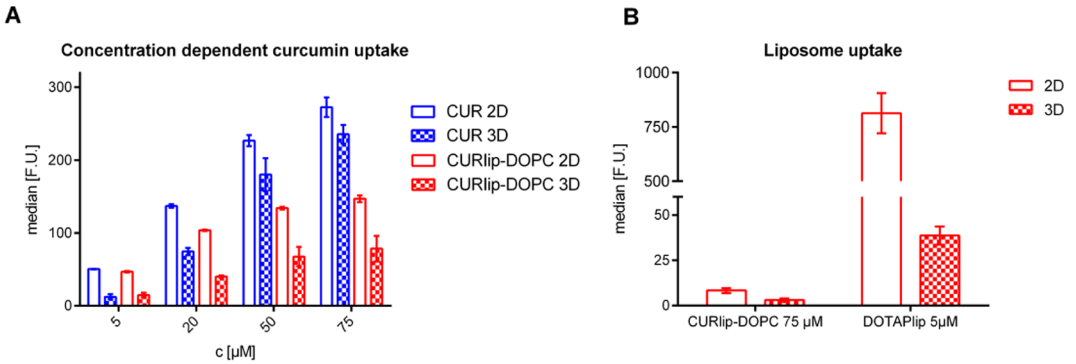

Fig. 6. Association of free CUR and CURlip-DOPC with LS 2D monolayer cells and LS 3D spheroids. (A) Concentration dependent curcumin association after 2 h incubation, (B) rhodamine DHPE intensity of CURlip-DOPC and DOTAPlip after 2 h incubation. Note that the CURlip-DOPC concentration is 15 times higher than the DOTAPlip concentration; n = 2–3, mean ± SD.

Table 3
EC_{50} values for A2780 and LS 2D monolayer cells and LS 3D spheroids treated with free CUR or CURlip-DOPC over 72 h; n = 3 mean ± SD.

Formulation	A2780 2D	LS 2D	LS 3D
Free CUR	16.9 ± 1.1 µM	2.1 ± 0.1 µM	31.4 ± 4.1 µM
CURlip-DOPC	19.0 ± 0.4 µM	1.2 ± 0.1 µM	> 100 µM

burst release and the uptake of curcumin into cells is driven by distribution of curcumin to the cell membrane and not by interaction of the liposomes with the cells.

MCTS are a more sophisticated model for avascular solid tumors. Both free CUR and CURlip-DOPC showed a decreased efficiency in the spheroid model compared to 2D monolayer cells. The increased EC_{50} value for free CUR and CURlip-DOPC respectively is very likely caused by a decreased curcumin association and penetration into the spheroids. CURlip-DOPC showed a very poor effectiveness in the MCTS model which suggests a decreased uptake and penetration into the spheroid compared to the free drug. These findings were confirmed by our association experiments with the spheroids. Association of free CUR and curcumin released from CURlip-DOPC was decreased compared with 2D monolayer cells very likely due to a decreased acceptor compartment of the spheroids compared to 2D monolayer cultures.

Overall, our results demonstrate that liposomes are an excellent tool to convert curcumin into a stable and administrable formulation. However, an improvement of the *in vivo* efficiency of curcumin by reduced degradation and enrichment in tumor tissue is certainly not achieved due to a premature release of curcumin. Even though the liposomes partly showed decreased curcumin uptake in comparison to free curcumin in 2D as well as 3D cell models, this retention is certainly based on a distribution equilibrium in the steady state conditions of the experimental setup. During circulation, always new binding sites are accessible and could continuously diminish the amount of retained curcumin in the liposomes. The accumulation of nanocarriers in solid tumors by the EPR effect requires a circulation time of approximately 6 h [24]; a long retention of curcumin is certainly not reached by the investigated liposomes. The results are likely to be similar for other hydrophobic drugs incorporated in liposomal bilayers. High affinity of

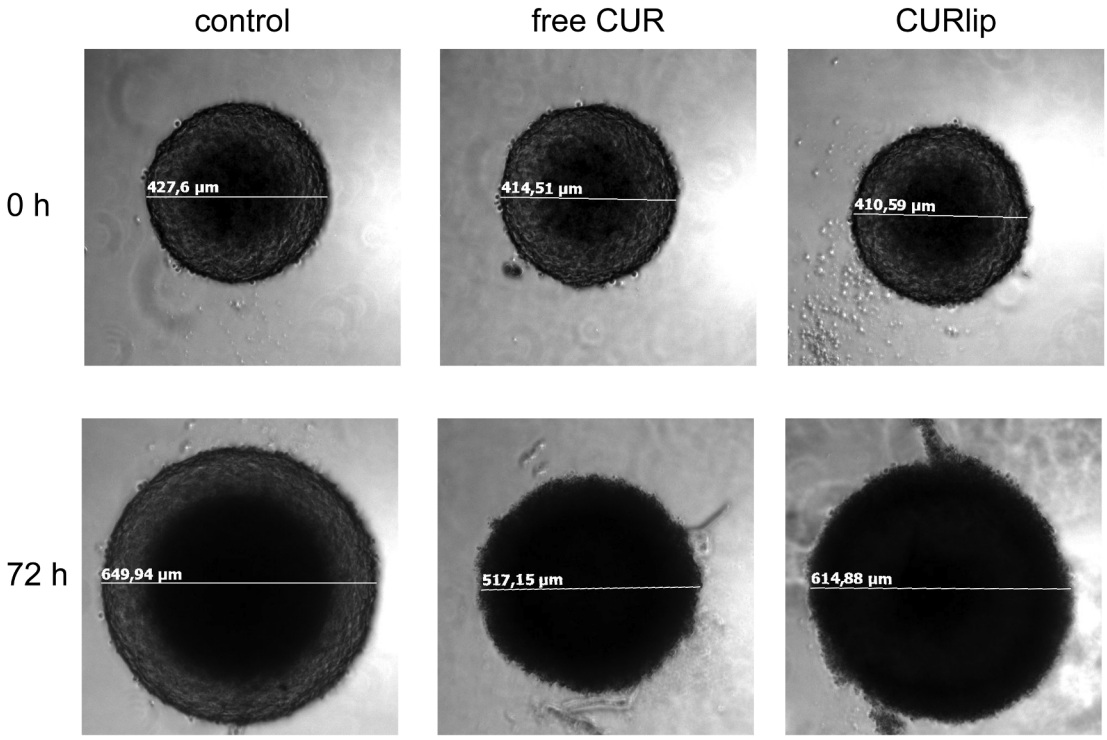

Fig. 7. Representative microscopic images of LS spheroids following treatment with free CUR or CURlip-DOPC at a curcumin concentration of 50 µM.

drugs to membranes facilitates liposome formation, but the stability *in vivo* is questionable due to the high presence of alternative biological membranes. On the other hand, total retention of the drug in a nanocarrier is not desirable neither, since finally the drug has to be released to reach its site of action. Our experiments also shed light on the problems of drug interaction in solid tumors. While EC_{50} values of curcumin were quite low in the classical 2D model, in the 3D cell culture decreased cytotoxicity and uptake were observed, even though curcumin is a lipophilic drug being able to cross biological membranes. Finally, controlling the release and uptake of hydrophobic drugs incorporated in liposomal nanocarriers for tumor therapy remains a difficult challenge. Future studies should focus on alternative nanocarriers such as nanoemulsions or nanoparticles. However, also these formulations have to be thoroughly evaluated in terms of drug retention in physiological environments.

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