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Remote loading of doxorubicin into liposomes driven by a transmembrane phosphate gradient

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Abstract

This study examines a new method for the remote loading of doxorubicin into liposomes. It was shown that doxorubicin can be loaded to a level of up to 98% into large unilamellar vesicles composed of egg phosphatidylcholine/cholesterol (7/3 mol/mol) with a transmembrane phosphate gradient. The different encapsulation efficiencies which were achieved with ammonium salts (citrate 100%, phosphate 98%, sulfate 95%, acetate 77%) were significantly higher as compared to the loading via sodium salts (citrate 54%, phosphate 52%, sulfate 44%, acetate 16%). Various factors, including pH-value, buffer capacity, solubility of doxorubicin in different salt solutions and base counter-flow, which likely has an influence on drug accumulation in the intraliposomal interior are taken into account. In contrast to other methods, the newly developed remote loading method exhibits a pH-dependant drug release property which may be effective in tumor tissues. At physiological pH-value doxorubicin is retained in the liposomes, whereas drug release is achieved by lowering the pH to 5.5 (approximately 25% release at 25 °C or 30% at 37 °C within two h). The DXR release of liposomes which were loaded via a sulfate gradient showed a maximum of 3% at pH 5.5.

Keywords: Doxorubicin; EPC/cholesterol liposome; Diammonium hydrogen phosphate; Cryo-electron microscopy; Remote loading; pH-triggered release

1. Introduction

Doxorubicin (DXR) is a widely used anticancer drug [1]. It has a broad spectrum of reactivity and shows excellent antineoplastic activity against a multitude of human cancer diseases [2–5]. However, the clinical use of DXR is hampered by acute and subacute side effects such as vomiting, bone marrow suppression, alopecia, mucositis and drug-induced dose-limiting irreversible cardiotoxicity and myelosuppression [6–8]. The mechanism of action of DXR has been extensively investigated. DXR is a DNA-intercalating agent and a topo-isomerase II inhibitor [9]. It is also known to form free radicals [10], which produce lipid peroxidation and can damage DNA with double and single strand breaks and abasic (apurin or apyrimidin) lesions, all of which may be implicated by its mechanism of action [5,7,11–14]. However it has not been shown that anthracyclines form radicals in the nucleus and therefore this mechanism is still under discussion [15]. It has been previously shown that liposomal-associated DXR delivery systems improve the therapeutic index [4,16–19]. In general, liposomal DXR exhibits efficiencies comparable to those of conventional anthracycline cytostatics, but with less general side effects especially with regard to cardiotoxicity [4,8]. It has been shown that the in vivo toxicity of DXR decreases with the increase of the drug-to-lipid ratio [20]. Therefore, achieving high encapsulation efficiencies of DXR into liposomes is desirable. Until now four different strategies of loading DXR into liposomes have been described. These different loading techniques are driven by the use of a pH-, manganese-, sulfate- or citrate-gradient. All of these concepts follow one principle, i.e., that the free DXR base diffuses inside the liposome where a modification of the drug occurs which inhibits membrane repermeation and results in accumulation inside the liposomes.

In the case of the transmembrane pH-gradient the interior of the liposome is acidic, whereas the exterior pH-value is adjusted to physiological conditions. The uncharged DXR which is incubated with these liposomes diffuses into the vesicles and

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becomes protonated intravesiculary. The positively charged DXR can no longer pass the bilayer and is trapped inside the liposomes [21–24].

The DXR loading into liposomes via a transmembrane manganese-gradient is based on the formation of intravesicular DXR–Mn^{2+}-complexes [25,26]. It has been well described that DXR forms chelate complexes with multivalent cations [27,28].

It has also been noted, that DXR can be efficiently loaded into liposomes with a transmembrane sulfate- or citrate-gradient [24,29–35]. In both concepts DXR precipitates in bundles of fibers in the liposomal interior [23,36].

Two liposomal formulations of DXR approved for human use are currently on the market, Myocet® and Caelyx® (EU) respectively Doxil® (US).

Myocet® consists of EPC and is loaded via a citric acid gradient. Doxil® is composed of MPEG-DSPE, HSPC and Cholesterol and is loaded by an ammonium sulfate gradient.

In the present study further gradients were evaluated for efficient loading of DXR and it could be shown that DXR precipitates not only in the presence of citrate and sulfate ions, but also in the presence of phosphate and acetate ions. DXR could be efficiently loaded into liposomes with a transmembrane $(NH_4)_2HPO_4$-gradient. This turns out to be a superior alternative technique of loading DXR into lipid vesicles. These liposomes show drug release rates dependent on the extra-liposomal pH. This may improve the applicability of DXR liposomes at tumor sites, which exhibit a decreased pH-value compared with the non-tumor environment. Furthermore, this study contributes to the understanding of the different loading mechanisms of DXR into liposomes.

2. Materials and methods

2.1. Materials

Egg phosphatidylcholine (EPC) was a generous gift from Lipoid (Ludwigshafen, Germany). Cholesterol (Chol) was purchased from Caelo (Caesar und Lorentz, Hilden, Germany). Doxorubicin HCl was obtained from the European Directorate for the Quality of Medicines (Strasbourg, France) and Caelyx® from essex pharma (Munich, Germany). Sepharose CL-4B was purchased from Amersham Biosciences (Uppsala, Sweden). All chemicals were of analytical grade.

2.2. Preparation of liposomes

A lipid mixture of egg phosphatidylcholine (EPC) and cholesterol (Chol) (mol/mol 7/3) was dissolved in ethanol and subsequently the solvent was removed under reduced pressure at 35 °C, followed by evaporation under high vacuum. The resulting lipid film was hydrated with the appropriate solutions by gentle mixing. The generated multilamellar vesicles (MLV) were extruded 21 times through a 80 nm pore sized polycarbonate membrane (Whatman, Maidstone, Kent, UK) using a LiposoFast-Basic ™ Extruder (Avestin, Ottawa, Canada).

2.3. Preparation of ion gradient for drug encapsulation

Liposomes were passed through a Sepharose CL-4B column equilibrated with an isotonic HEPES buffered saline (HBS), 140 mM NaCl, 10 mM HEPES ([4-(2-hydroxyethyl)-piperazino]-ethanesulfonic acid, pH 7.4) to replace the extra-liposomal solution. The eluted liposomes were diluted with isotonic HEPES buffer to yield a final lipid concentration of 5 mM. Subsequently, doxorubicin HCl was added to the liposomal dispersion to achieve a drug to lipid ratio of 1/3 (mol/mol). The loading process was carried out at 7 °C for 12 h.

2.4. Size determination

Liposomal samples were diluted with freshly filtrated isotonic HEPES buffer (0.22 μm Minisart, Sartorius AG, Göttingen, Germany) in order to yield an appropriate counting rate. All samples were placed into the specimen holder of a Zetamaster S (Malvern Instruments Ltd., Malvern, UK) 5 min prior to the start of measurement in order to allow equilibration to room temperature. Data were calculated by contin mode.

2.5. Determination of encapsulation efficiency (EE)

The separation of liposomes from free DXR was performed by size exclusion chromatography (SEC) or alternatively by ultracentrifugation.

Separation by SEC was achieved using a Sepharose CL-4B column (0.7 cm × 10 cm). Separation of 100 μl liposomes from non encapsulated DXR was carried out by elution with isotonic HEPES buffer (pH 7.4). Ultracentrifugation was performed at 130000 × *g* for 3 h at 20 °C (Optima LE-80, Beckman Instruments Inc., Palo Alto, USA), and the supernatant was removed. The liposome pellet was redispersed in HBS pH 7.4.

The DXR concentration was determined at 495 nm (Uvikon 933 A, Kontron Instruments, Italy) after lysis of the liposomes with Triton X-100 (final concentration 0.5% v/v).

2.6. Solubility of DXR

The solubility of DXR was investigated in various solutions of ammonium salts of sulfate (300 mM), hydrogen phosphate (300 mM), acetate (600 mM) and citrate (300 mM). The pH-values were adjusted with ammonia or hydrochloric acid. The salt solutions were added to lyophilized DXR. Each sample was vortexed for 1 min and incubated at 20 °C for 12 h. The precipitate was separated from the supernatant by centrifugation (10000×*g*; 10 min). DXR in the supernatants was quantified photometrically at 495 nm.

2.7. Cryo-transmission electron microscopy

Liposome formulations were diluted with the extra-vesicular buffer to reach a total lipid concentration of 4–7 mM. Copper grids (Science Service, Munich, Germany) were prepared according to a standard method [37]. After placing a drop of the sample on the grid most of the liquid was removed with filter paper, leaving a thin film stretched over the holes. The samples were shock-frozen by dipping into liquid ethane and cooled to 90 K by liquid nitrogen. The samples were transferred to the microscope (Zeiss CEM 902, Carl Zeiss, Oberkochen, Germany) as described elsewhere [38] and then examined at approximately 100 K. Zero-loss filtered images were taken by a slow scan charge-coupled device (SSCCD) camera under low-dose conditions, i.e. using the minimal dose focusing device.

2.8. Determination of the inner pH-value of liposomes

The internal pH of the liposomes was measured using the fluorescent probe HPTS (8-hydroxypyrene-1,3,6-trisulfonic acid, pyranine) [39–42]. Liposomes were prepared as described above with the exception that HPTS (0.5 mM) was added to the salt solution for hydration. Free HPTS was separated by gel filtration using a Sephadex G-50 column. The internal pH-value was determined by the excitation spectra of HPTS immediately after the separation of free HPTS from the liposomal HPTS fraction. HPTS has two excitation maxima. One occurs at 411 nm under isosbestic, pH independent conditions and the second is pH dependent and occurs at 455 nm. The calibration curve is based on the equation pH = pK + log (F455/F411), where the latter term is the logarithm of the ratio of the fluorescence intensities at 512 nm using excitation wavelengths of 455 nm or 411 nm. These measurements were performed with a Perkin-Elmer fluorescence spectrometer LS 50B (Perkin-Elmer, Rodgau Jügesheim, Germany).

2.9. DXR release experiments

The liposomal release of DXR was quantified by the fluorescence dequenching of self-associated DXR in liposomes upon dilution outside the liposomes [43,44]. Measurements were performed at λ_{exc} 480 nm and λ_{em} 590 nm [33]. Liposomal drug release was investigated in HEPES buffer (10 mM HEPES, 140 mM sodium chloride and 5 mM succinic acid) at 25 °C and 37 °C. The pH-value of the HEPES buffer was adjusted to pH-values of 5.5, 6.5 or 7.4 with sodium hydroxide. The increase of fluorescence intensity was followed in the timedrive mode. 100% dequenched DXR (total DXR) was determined after addition of Triton X-100 (0.5% v/v) [33].

3. Results

In this study solubility of doxorubicin (DXR) in different salt solutions as well as liposomal encapsulation of DXR by the use of a transmembrane gradient of these solutions was examined. In order to avoid degradation of the temperature- and light-sensitive drug, the loading of DXR into the liposomes was performed at 7 °C protected from light.

3.1. Solubility

As shown in Fig. 1, doxorubicin exhibits very low solubility at 20 °C in 300 mM phosphate, sulfate, citrate and acetate solutions over the range of pH 5–8.5. Sodium and ammonium salt solutions show approximately the same DXR solubility (data not shown). The results for DXR solubility in citrate solution are in agreement with reports on the solubility of lipophilic cationic drugs in citrate solutions [31,45].

The solubility of DXR in phosphate, citrate and acetate buffer is dependent on pH-value. The solubility of DXR decreases with increasing pH-value. The DXR solubility in phosphate solutions was not determined at pH 4 because the viscosity at higher DXR concentrations strongly increased by forming a clear, red semi-solid dispersion.

In ammonium sulfate solution the solubility of DXR was almost constant at about 0.2 mg/ml up to a pH-value of 7.5,

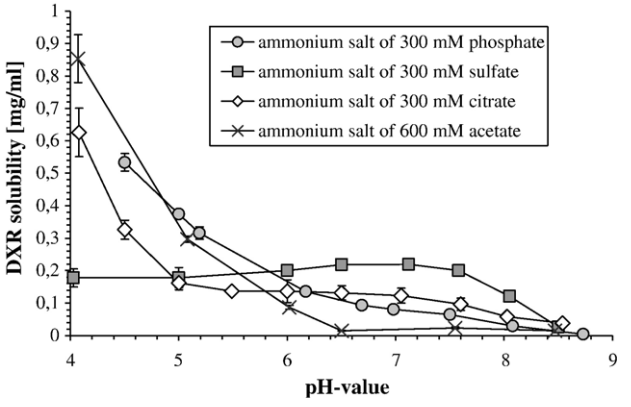

Fig. 1. Solubility of DXR in different salt solutions as a function of pH. The solutions were added to lyophilized DXR and vortexed for 1 min. After incubation of the samples at 20 °C for 12 h, the solubilized DXR in the supernatant was quantified (n=3).

which is close to the pK_a-value of 8.1 for the amino group [30]. At higher pH the solubility decreased to lower values which were comparable with those of other salt solutions.

3.2. Encapsulation of DXR by the use of several salt gradients

DXR liposomes loaded with gradients of NH_4^+-salts showed a higher EE than those loaded by sodium salts (Fig. 2). The liposomal preparations generally showed a constant particle size and polyindex during the loading process (Table 1). The EE declined in the line: citrate > phosphate > sulfate > acetate. Among the ammonium salts, only acetate showed a DXR loading below 90%. All salt gradients resulted in active DXR loading, when compared to the minimal theoretical EE, which is calculated by the ratio of inner liposomal to total aqueous volume [46].

3.3. Influence of the intravesicular phosphate concentration on the encapsulation efficiency (EE)

Dried lipid was hydrated with various buffers containing either HBS or 50, 100, 200 or 300 mM ammonium phosphate (pH was adjusted to 7.2 with 0.1 N NaOH). The size of the liposomes after extrusion was around 100 nm (±11 nm) (Table 2). External phosphate was removed by size exclusion chromatography with HBS.

After the addition of lyophilized DXR and 12 h of incubation at 7 °C, the EE increased up to 98% with a drug to lipid ratio of 1:3 (mol/mol) at a phosphate concentration of 300 mM inside the liposomes. The lowest DXR EE (2.8%) was achieved with 10 mM HEPES buffer, containing no phosphate ions.

3.4. Ammonium salt gradient

Liposomes were prepared by hydrating the lipid film with 300 mM $(NH_4)_2HPO_4$ buffer (pH 7.4) and extrusion through 80 nm membranes. The extraliposomal buffer was exchanged through different buffers (Table 3) by size exclusion chromatography. The transmembrane ammonium gradient induces a transmembrane pH-gradient, which is due to an increase of proton concentration inside the liposomes. This was measured using encapsulated HPTS as pH-sensitive probe. Table 2 shows that the intraliposomal pH-value is unmodified when the interior contains the same ammonium concentration as outside the liposome, or when no ammonium ions were present inside the liposomes. This study shows that in case of an ammonium gradient the capacity of the phosphate buffer inside the vesicles is not sufficient and therefore the intravesicular pH drops to <5.5.

3.5. Loading of DXR via sodium phosphate gradient with different interior pH-values

Liposomes were prepared with intraliposomal sodium phosphate buffer (300 mM Na_2HPO_4) of various pH-values (pH 3, 4, 5, 6 and 7.2). As shown in Fig. 3, lower pH-values resulted in a higher EE of DXR.

However, the high EE of $(NH_4)_2HPO_4$ could not be achieved with a Na_2HPO_4 gradient when combined with a pH-gradient.

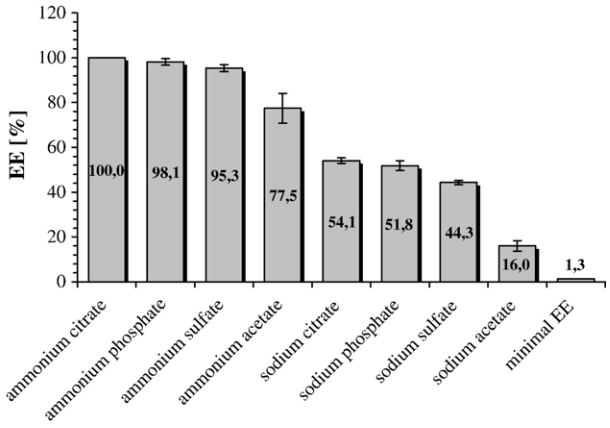

Fig. 2. Efficiency of encapsulation of DXR in EPC/Chol (7/3 mol/mol, 5 mM lipid concentration) liposomes driven by various salt gradients. The lipid formulations were prepared with a molar drug/lipid ratio of 1:3. The lipid film was hydrated with 300 mM salt solution adjusted to pH 7.4. Liposomes were incubated with DXR for 12 h at 7 °C ($n=3$).

3.6. Cryo-TEM

Cryo-TEM was used for visualization of DXR liposomes and a representative micrograph is shown in Fig. 4. The micrograph 4 reveals liposomes prepared in 300 mM $(NH_4)_2HPO_4$ solution after the extra-vesicular buffer was exchanged with HBS. Liposomes were loaded with DXR with a ratio of DXR to lipid of 1/3 (mol/mol).

As shown in Fig. 4, entrapped and precipitated DXR forms bundles that appear as linear structures and induce a change in liposomal shape, resulting in a characteristic "coffee bean"-structure. When the DXR/lipid ratio is increased to higher values than 1/3 (mol/mol), the free drug bundles can be found coexisting with empty liposomes. This was reflected by continuously decreasing EE (data not shown).

3.7. Leakage of DXR liposomes loaded via a phosphate gradient

Stability of DXR liposomes and drug release was examined under conditions approaching those which would be encountered following their application. These include contact with physiological fluids at pH 7.4, pH 6.5 of tumor tissues [47] and pH 5.5 after uptake in endosomal compartments.

The drug release properties of liposomes, which were loaded via a transmembrane phosphate gradient, differ completely from vesicles, which are loaded with a sulfate gradient.

The DXR release of liposomes which were loaded via a sulfate gradient showed a maximum of 2% at pH 5.5 and the influence of pH on the release property was negligible (Fig. 5B).

In contrast, liposomes loaded by a phosphate gradient show a DXR release dependant on the pH-value of the extraliposomal buffer. Leakage of DXR at pH 7.4 is less than 2% but leakage increases with decreasing pH (Fig. 5A). At pH 6.5 and 5.5 the drug release is influenced by the extraliposomal ratio of positively charged DXR to uncharged DXR.

Furthermore release experiments have been performed with these liposomes at 37 °C. pH dependent release from EPC/Chol liposomes was found to be slightly increased which might be due to increased DXR solubility and/or membrane fluidity (Figs. 5A and 6A).

Release experiments of DXR at 37 °C were also performed with Caelyx® and showed only maximum release of 2.5% DXR (Fig. 6B), which corresponds to our findings achieved at 25 °C with EPC/Chol liposomes which were also loaded by ammonium sulfate gradient (Fig. 5B).

Table 2
Encapsulation efficiency (EE) of DXR in EPC/Chol (7/3 mol/mol) liposomes by varying the intraliposomal ammonium phosphate concentration ($n=3$)

$(NH_4)_2HPO_4$ concentration [mM]	EE [%] (±sd)	Size [nm] (±sd)
0	2.81 (0.83)	84 (0.4)
50	23.52 (4.02)	102 (1.4)
100	61.03 (6.77)	114 (3.6)
200	83.35 (7.97)	95 (1.5)
300	97.98 (1.52)	92 (1.6)

4. Discussion

Loading liposomes with DXR via phosphate-gradient is a new, potent and alternative remote-loading method, which takes into account the thermo- and light-sensitive properties [44,48] of the drug.

Table 1
Change in particle size of the liposomes during remote loading with DXR (the intraliposomal salt concentration was 300 mM ($n=3$))

Salt-gradient	Particle size [nm] before loading (±sd)	Polyindex before loading (±sd)	Particle size [nm] after loading (±sd)	Polyindex after loading (±sd)
$(NH_4)_2HPO_4$	132.4 (±0.6)	0.094 (±0.036)	129.3 (±3.7)	0.132 (±0.044)
Na_2HPO_4	113.4 (±1.2)	0.109 (±0.044)	113.4 (±1.6)	0.034 (±0.008)
$(NH_4)_2SO_4$	128.4 (±0.9)	0.046 (±0.034)	129.2 (±2.9)	0.128 (±0.015)
Na_2SO_4	115.3 (±2.9)	0.057 (±0.039)	111.8 (±1.9)	0.101 (±0.03)
NH_4-Acetate	116.7 (±3.2)	0.105 (±0.031)	115.9 (±1.0)	0.095 (±0.03)
Na-Acetate	113.4 (±1.2)	0.109 (±0.044)	113.4 (±1.6)	0.034 (±0.008)
NH_4-Citrate	115.7 (±1.0)	0.136 (±0.133)	114.9 (±1.2)	0.061 (±0.037)
Na-Citrate	150.3 (±4.6)	0.074 (±0.05)	151.7 (±3.8)	0.351 (±0.059)

Table 3
Correlation between ammonium- and pH-gradient of EPC/Chol (7/3 mol/mol) liposomes with different intra- and extravesicular buffers ($n=3$)

Hydration buffer	Elution buffer	NH_4^+-gradient	Intraliposomal pH-value
300 mM $(NH_4)_2HPO_4$ +0.5 mM HPTS pH 7.4	HBS pH 7.4	(+)	<5.5
	300 mM $(NH_4)_2HPO_4$ pH 7.4	(−)	7.4
	300 mM $(NH_4)_2SO_4$ pH 7.4	(−)	7.4
300 mM Na_2HPO_4 +0.5 mM HPTS pH 7.4	HBS pH 7.4	(−)	7.4
	300 mM Na_2HPO_4 pH 7.4	(−)	7.4

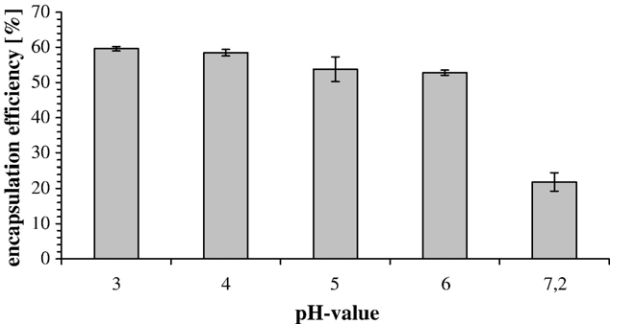

Fig. 3. Loading of DXR into EPC/Chol (7/3 mol/mol) liposomes prepared with sodium phosphate buffers of different pH-values (n=3).

Loading of DXR by means of salt gradients includes two synergistic effects, both of which result in the fact that DXR can no longer pass the lipid membrane. On the one hand loading is driven by protonation and charging of DXR within the liposome, on the other by precipitation of DXR in the hydrophilic interior of the vesicle when the concentration of DXR solubility is exceeded.

By means of a stable pH-gradient, DXR is protonated in the acidic interior of the liposome. When the extraliposomal pH is selected in that way, only a small part of the DXR is protonated. The free uncharged base is then able to pass the membrane so that there is intra- and extraliposomal adjustment in the concentration of the free base of the drug. When the intraliposomal pH-value is lower, the free DXR base is protonated intraliposomally and new extraliposomal, non-protonated DXR can diffuse into the liposome according to the concentration gradient. Therefore a "proton pool" inside the liposomes is necessary as a driving force for DXR loading.

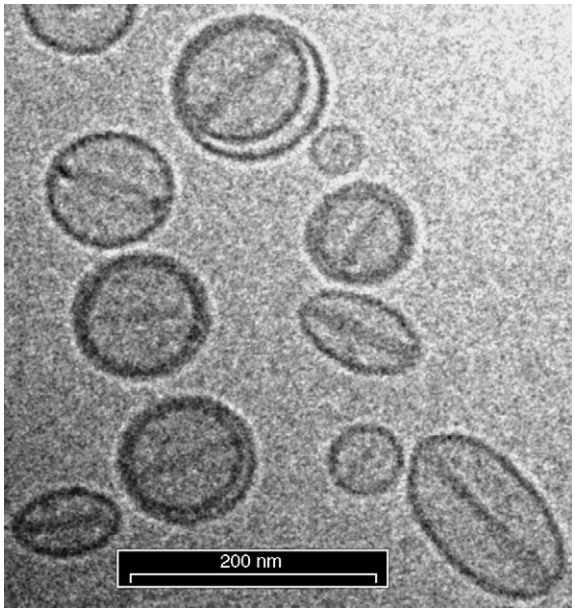

Fig. 4. Cryo-TEM micrograph of EPC/Chol (7/3 mol/mol) liposomes loaded with DXR via a $(NH_4)_2HPO_4$ gradient (intravesicular salt solution: 300 mM $(NH_4)_2HPO_4$, pH 7.4; extraliposomal buffer: 10 mM HEPES/140 mM NaCl, pH 7.4). Scale bar represents 200 nm.

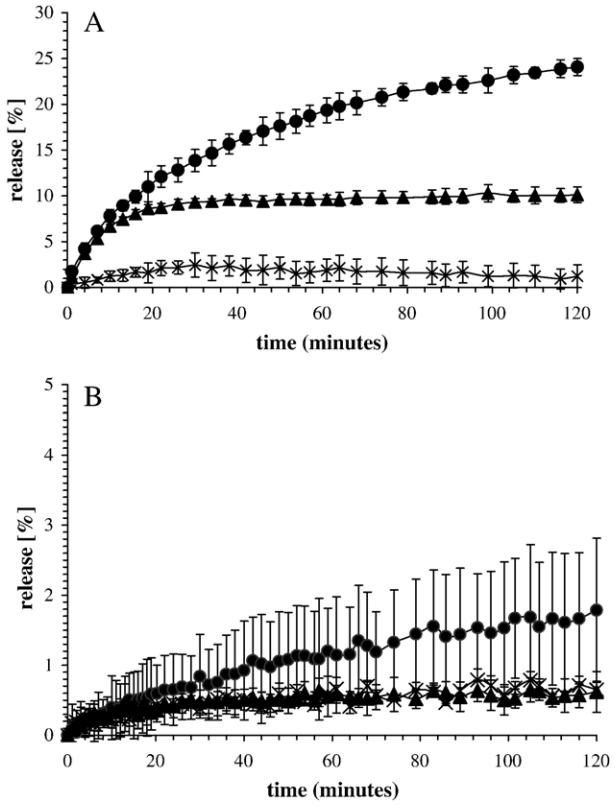

Fig. 5. Release of doxorubicin from EPC/Chol (7/3 mol/mol) liposomes at 25 °C. Doxorubicin was encapsulated by ammonium phosphate-gradient (A) or ammonium sulfate-gradient (y-axis extended) (B) and drug release was analyzed in 10 mM HEPES, 140 mM NaCl buffer pH 7.4 (x), pH 6.5 (▲) or pH 5.5 (●) at 25 °C. (n=3 in panel A, n=4 in panel B).

In the study presented a stable pH-gradient over the loading period is maintained in situ by generation of a NH_4^+-gradient, i.e. by a high intraliposomal NH_4^+ concentration and an NH_4^+ ion-free, extraliposomal buffer. Due to the NH_{4+}-gradient the pH-value decreases within the liposome from pH 7.4 to a pH lower than 5.5 (Table 2), even though the extraliposomal buffer was also set to a pH of 7.4. The in situ generation of the pH-gradient obviously results from the higher permeation coefficient of ammonia with 1.3×10^{-1} cm s^{-1} [49] as compared to the permeation coefficient of protons of 10^{-3} to 10^{-8} cm s^{-1} [50], which results in acidification of the liposome interior. In the absence of an NH_4^+-gradient there is no decrease in intraliposomal pH-value. Instead, the internal pH adjusts to that of the outer pH. Although up to 51% of DXR can be encapsulated in liposomes via a pH-gradient by a NH_4Cl-gradient (Fig. 5), higher results can be obtained by synergistic effects (Fig. 2) which include protonation and precipitation of DXR inside the liposome.

For the interaction of DXR with SO_4^{2-} anions it was shown in earlier studies that positively charged DXR precipitates with negatively charged sulfate to form a gel-like structure within the liposomes [30,32,51]. The cation–anion interaction in our solubility studies showed consistently poor solubility of DXR in a 300 mM sulfate solution in the investigated pH-range of values close to the pK_a of the amino group (pK_a=8.1 [30]) where solubility drops to almost zero.

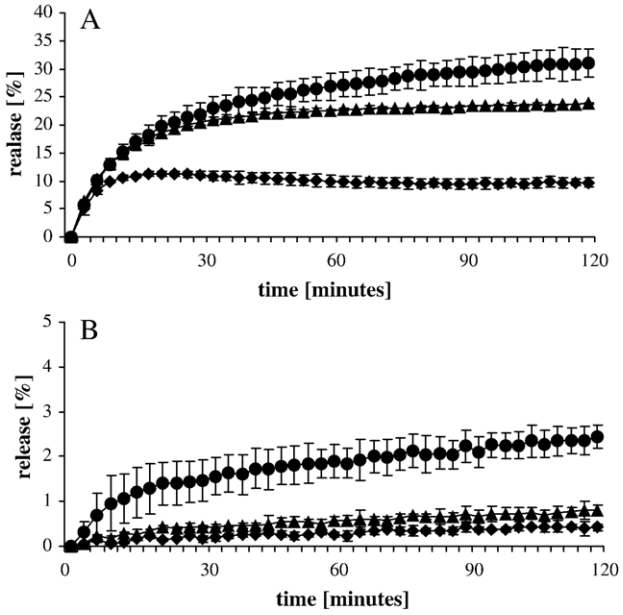

Fig. 6. Release of doxorubicin from (A) from EPC/Chol 7/3 (mol/mol) liposomes loaded via an ammonium phosphate gradient and (B) commercial product Caelyx®/Doxil® (*y*-axis extended). The drug release was analyzed in 10 mM HEPES, 140 mM NaCl buffer pH 7.4 (♦), pH 6.5 (▲) or pH 5.5 (●) at 37 °C. (n=3 in panel A, n=3 in panel B).

For the interaction of DXR with citrate intensive investigations have been carried out elsewhere [23]. However, no stable complex or salt could be found.

Our study shows that the solubility of DXR increases in 300 mM citrate, 300 mM phosphate and 600 mM acetate solution by decreasing the pH-value, although the concentration of positively charged DXR increases. At pH values >7 the solubility of DXR decreases. After deprotonation of DXR the uncharged compound is nearly insoluble in the salt solution. It seems probable that the cosmotropic (structure maker) properties of the counterion contribute to the solubility of DXR. The salts used for loading, i.e., citrate, sulfate, phosphate and acetate, are all components of the so-called Hofmeister or lyotropic series [52,53]. This series reflects the ranking of ions according to their binding strength of bulk water, and to the concomitant decrease in the hydration of other compounds. The precipitation caused by these compounds is then termed a salt-out effect.

This also explains the requirement of the high salt concentration of 300 mM for liposome loading (Table 2), since this concentration is necessary to withdraw the hydration shell of DXR. This salt-out effect plays a role not only for proteins but for amphiphilic substances as well, which has been shown in other studies [54]. The withdrawal of the hydration shell also explains the self-aggregation of DXR driven by hydrophobic interactions, which is highly dependent on the respective buffer composition [55,56].

By using Na_2HPO_4-gradients with various pH-values it could be demonstrated that with the synergism of protonation and precipitation of DXR higher encapsulation efficiencies can be achieved. The use of NH_4^+ salt-gradients is more effective than a sodium salt gradient (Fig. 2), even if the intraliposomal saline buffer solution is set to a lower pH-value (Fig. 3). In contrast to sodium, ammonium acts as a reservoir, which provides new free protons when DXR is protonated.

Aside from the hydrophilic interior of the liposome, the lipophilic compartment of the bilayer also represents a factor influencing loading. DXR consists of a hydrophobic co-planar anthraquinone structure with the amino sugar in the protonated state of a hydrophilic region. If DXR is not retained in the hydrophilic compartment by protonation or precipitation, there is the further possibility of the amphiphilic molecule being incorporated in the bilayer. The interaction of DXR with natural and model membranes could already be demonstrated by fluorescent methods [57,58].

Besides efficient loading, the release of the drug is also a critical factor for a drug delivery system. As shown in our studies, the release of DXR from liposomes which were loaded by phosphate-gradient is highly dependent on the extraliposomal pH-value. In this study pH-values 7.4, 6.5 or 5.5, similar to the situations in most tissues, tumors and endosomes, were chosen to imitate the various pH environments of the liposome from the point of application to the reaching of the target. In the case of phosphate-loaded DXR liposomes a low extraliposomal pH-value results in facilitated DXR release, whereas liposomes loaded with sulfate-gradient are not significantly influenced by the extravesicular pH-value. The fact that sulfate is a salt of a stronger acid as compared to phosphate, might explain the difference in the release behavior of both DXR salts sulfate and phosphate. The sulfate salt will not be protonated by minor pH-shifts, i.e., from 7.4 to 5.5. In contrast, the charge density of phosphate will be decreased by protonation of the salt. This results in a lower interaction between the ion and doxorubicin and a lower ability to retain DXR inside the vesicles. This interpretation is supported by the findings of Lee et al. [35].

In addition, the lyotropic series favors a higher solubility of DXR phosphate inside the liposomes and consequently a higher release rate upon acidification.

In summary, the presented remote loading process of DXR in liposomes via $(NH_4)_2HPO_4$-gradient depends on various factors: intraliposomal salt concentration, water binding potential of the salt, the pH-value in the liposome and the presence of a “proton pool”, e.g. in the form of NH_4^+ salts.

The loading of DXR in EPC/Chol liposomes via $(NH_4)_2HPO_4$-gradients represents a potent alternative procedure to the methods used to date. It allows fast and effective loading of the EPC/Chol liposomes at 7 °C as well as at RT, and results in a liposomal drug delivery system which is comparable to other liposomal DXR formulations in a physiological pH environment, whereas under the pathophysiologic pH-conditions of tumor tissue or after endosomal uptake an increased release of DXR from the vesicles is achieved. The pH-triggered release of DXR offers new perspectives for the improvement of the effectiveness of tumor treatment.

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