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Novel prodrug of doxorubicin modified by stearoylspermine encapsulated into PEG-chitosan stabilized liposomes.

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KEYWORDS liposomes, FTIR spectroscopy, doxorubicin.

ABSTRACT: Here, we report a new modification of doxorubicin based on an amphiphilic stearoylspermine anchor, enabling loading into liposomal membranes. Doxorubicin is coupled to stearoylspermine via an acid-labile hydrazone linker in order to ensure the release of the drug in the acidic interstitium of tumors. By ATR-FTIR spectroscopy (attenuated total reflectance Fourier transformed infrared spectroscopy), the mechanism of interaction of doxorubicin with the anionic liposomal membrane was studied: incorporation of stearoyl chains leads to an increase in local microfluidity and the amino groups of spermine interact with the phosphate groups of lipids. To stabilize liposomes against aggregation, we have applied the copolymer PEG-chitosan as a coating: complex formation leads to charge neutralization and the liposomes grow in size. According to MTT tests and confocal microscopy for cell lines A459 and Caco-2, PEG-chitosan coated liposomes are as effective as neutral liposomes but much more stable.

INTRODUCTION

Doxorubicin remains one of the most important drugs in therapy of a wide range of tumors: the list includes breast, liver and lung cancers and some types of leukemia. Due to high cardiotoxicity *(1)*, doxorubicin is usually loaded into PEGylated liposomes, so-

called stealth liposomes *(2)*; liposomes are relevant drug delivery systems for a wide range of drugs due to their biocompatibility and low immunogenicity. However, the tendency to aggregate still limits their application in medicine. PEGylation is a common method to stabilize liposomes and prolong their blood circulation, but application of PEGylated lipids can exert influence on cellular uptake and was reported to cause hand-foot syndrome *(3,4)*. Doxil, a drug based on this technology, is successfully applied in ovarian cancer and multiple myeloma treatment. Still, effective loading of doxorubicin into liposomes is a challenge. A number of methods that require additional loading steps subsequent to liposome formation have been developed such as loading by pH gradient *(5)*, $(NH_4)_2SO_4$ gradient *(6)*, phosphate gradient *(7)* and $MnSO_4$ gradient *(8)*. The majority of these methods usually offers less than 80% of efficacy and involves purification steps usually by chromatography or by dialysis. In this study, we demonstrate that chemical modification of doxorubicin with a stearoylspermine anchor (CPPa = cell penetrating polyamine) *(9)* will improve its lipophilic properties and facilitate the loading. Moreover, the amphiphilic moiety will firmly anchor the chemotherapeutic within the liposomal membrane minimizing any uncontrolled, diffusion mediated release. In addition, we have previously demonstrated that the branched copolymer PEG-chitosan is a promising noncovalent stabilizing agent for anionic liposomes *(10)* and can be used instead of PEG. In this study, we further stabilized the DOXCPPa liposomes by a PEG-chitosan modification, and characterize their structure, physicochemical properties and cytotoxic activity in A549 (lung carcinoma) and Caco-2 (colon carcinoma) lines.

MATERIALS AND METHODS

ATR-FTIR spectroscopy was performed on a Bruker Tensor 27 equipped with a BioATR Cell II, sampling accessory and a liquid-nitrogen cooled photovoltaic MCT-detector. FTIR spectra were acquired from 900 to 4000 cm^{-1} with 2 cm^{-1} spectral resolution. For each spectrum, 100 scans were accumulated at 20 kHz scanning speed and averaged. All spectra were registered in PBS Gibco buffer solution at pH 7.4 and 22 °C. Spectral data were analyzed with the Opus 7.0 (Bruker Optics, Ettlingen, Germany) software. Spectra were evaluated using linear blank subtraction, straight-line baseline correction and atmosphere compensation. If necessary, seven- or nine-point Savitsky - Golay smoothing was used to remove white noise. Exact band positions in the spectra were identified using the peak picking algorithm of the OPUS software *(11)*. Curve-fitting was performed in Bruker OPUS 7.0 software. The center positions of band components were found by the second derivative production. Bands were fitted by components of Lorenz shape with correlation at least 0.995.

Synthesis of DOXCPPa (9). The synthetic procedure was recently published by Hahn *et al*. (Scheme 1) and described in details in *(12)*.

***Synthesis of 6*.** 485 mg (1.00 mmol, 1.00 eq.) of the 2-chlorotrityl chloride resin were swollen in 12 mL dry CH_2Cl_2 and treated with 0.57 mL (425 mg, 3.30 mmol, 3.30 eq.) DIPEA and 1.83 g (3.30 mmol, 3.30 eq.) allyl (4-(((allyloxy)carbonyl)-(3-(2-nitrophenylsulfonamido)propyl)amino)butyl)(3-aminopropyl)carbamate (**1**). The reaction vessel was closed and shaken for 15 h at room temperature. Afterwards, 3 mL of methanol were added and the mixture was shaken for another 15 minutes. The solvents were removed and the resin was washed with CH_2Cl_2/MeOH/CH_2Cl_2/MeOH/CH_2Cl_2/MeOH/CH_2Cl_2 and again CH_2Cl_2 (3×). The reaction yielded the yellow resin **2**.

Reaction control was performed via a test cleavage of a few resin beads with 20 µL TFA and subsequent mass analysis: MS(ESI-TOF): 556.2 $[(M+H)^+]$.

1.00 mmol (1.00 eq.) of the resin **2** were swollen in 12 mL dimethylformamide (DMF) and treated with 0.75 mL (5.00 mmol, 5.00 eq.) DBU and 0.70 mL (10.00 mmol, 10.0 eq.) 2-mercaptoethanol. The reaction vessel was closed and shaken for 1 h at room temperature. Afterwards, the solvents were removed and the resin was washed with DMF until the filtrate remained colorless. The whole procedure was repeated with only 30 minutes of reaction time until the reaction mixture remained colorless subsequent to the reaction. Afterwards, the resin was washed with DMF, CH_2Cl_2/MeOH/CH_2Cl_2/MeOH/CH_2Cl_2 and again CH_2Cl_2 (3×). The reaction yielded the yellow resin **3**.

Test cleavage: MS(ESI-TOF): 371.3 $[(M+H)^+]$.

1.00 mmol (1.00 eq.) of the resin **3** were swollen in 80 mL DMF/CH_2Cl_2 (1:1) and treated with 766 mg (5.00 mmol, 5.00 eq.) HOBt and 1.42 g (5.00 mmol, 5.00 eq.) stearic acid. The reaction vessel was shaken for 5 minutes. Then, 770 µL (5.00 mmol, 5.00 eq.) DIC was added. The reaction vessel was closed and shaken for 15 h at room temperature. Afterwards, the solvents were removed and the resin was washed with DMF, CH_2Cl_2/MeOH/CH_2Cl_2/MeOH/CH_2Cl_2 and again CH_2Cl_2 (3×). The reaction yielded the yellow resin **4**.

Test cleavage: MS(MALDI-TOF, Matrix: DHB): 637.1 $[(M+H)^+]$; 659.0 $[(M+Na)^+]$; 674.9 $[(M+K)^+]$.

1.00 mmol (1.00 eq.) of the resin **4** were swollen in 12 mL abs. tetrahydrofuran (THF) and treated with 1.56 g (10.0 mmol, 10.0 eq.) dimethylbarbituric acid and 116 mg (0.100 mmol, 0.100 eq.) tetrakis(triphenylphosphine)palladium(0). The reaction vessel was closed and shaken for 15 h at 35 °C. Afterwards, the solvents were removed and the resin was washed with THF, CH_2Cl_2/MeOH/CH_2Cl_2/MeOH/CH_2Cl_2 and again CH_2Cl_2 (3×). The reaction yielded the yellow resin **5**.

Test cleavage: MS(ESI-TOF): 469.5 $[(M+H)^+]$.

In a vial, 1.00 mmol (1.00 eq.) of the resin **5** were treated with 14 mL 5% TFA solution in CH_2Cl_2. The reaction vessel was closed and shaken for 15 h at room temperature. Afterwards, the solvents were removed and the resin was washed with CH_2Cl_2, MeOH (2×), CH_2Cl_2/MeOH/CH_2Cl_2/MeOH/CH_2Cl_2/MeOH/CH_2Cl_2 and again CH_2Cl_2 (3×). The organic phases were combined and the solvent was removed under reduced pressure to yield the crude product **6**. Purification was performed using HPLC. MS(ESI-TOF): 469.5 $[(M+H)^+]$, 235.3 $[(M+2H)^{2+}]$.

***Synthesis of 8*.** 10 mg (17.0 µmol, 1.00 eq.) doxorubicin hydrochloride were dissolved in 200 µL abs. methanol. A solution of 5 mg (17.0 µmol, 1.00 eq.) BMPH×TFA (*N*-[ß-maleimidopropionic acid] hydrazide) in 200 µL abs. methanol was added under stirring. The mixture was stirred for 90 min in the dark at room temperature. Afterwards, the reaction mixture was poured into 10 mL abs. ether/cyclohexane (1:2). The precipitate (mixture of mainly **8** and **7**) was filtrated and used without further purification. MS (MALDI-TOF, Matrix: THAP): 708.5 $[(M+H)^+]$; 730.5 $[(M+Na)^+]$; 746.5 $[(M+K)^+]$.

***Synthesis of 9*.** The activated doxorubicin **8** and 16 mg (34.0 µmol, 2.00 eq.) polyamine **6** were dissolved in 1 mL ethanol. The solution was cooled to 0 °C. 2 mg (17.0 µmol, 1.00 eq.) 2-iminothiolane dissolved in 17 µL deionized water were added under stirring in the dark. After 16 h, the solvent was removed under reduced pressure and the obtained crude product (Scheme S2) was purified using HPLC and characterized by ATR-FTIR spectroscopy (Fig. S6).

Synthesis of PEG-chitosan branched copolymer. 50 mg chitosan (MW 60-120 kDa, Sigma-Aldrich Chemie GmbH) were dissolved in 7 mL 0.2 M sodium acetate buffer pH 4.5 during 1 hour of stirring. 25 mg mPEG-NHS (N-hydroxylsuccinimide (NHS) functionalized polyethylene glycol) MW 5000 (Nanocs, USA) were dissolved in 2 mL DMF. The solutions were mixed, 6 mL NaOH 0.2 M was added till pH 8.0. The mixture was stirred for 15 h at room temperature. Dialysis in Serva tubing (cut-off mass 13 kDa)

against PBS pH 7.4 (Gibco® Life TechnologiesTM) was conducted for 3 days. The final concentration of polymer was 3 mg/mL.

Liposome preparation. Liposomes were prepared with the thin-film/hydration method and extrusion. PC Egg (phosphatidylcholine) (Sigma-Aldrich Chemie GmbH) and CL (cardiolipin) were dissolved in chloroform (Sigma-Aldrich Chemie GmbH) in the desired concentrations. DOXCPPa was added in the desired concentrations. The solvent was removed under low pressure. Then the lipid film was hydrated in 1 mL of 60 °C preheated PBS (Gibco® Life TechnologiesTM) over night at 60 °C. The solution was sonicated twice for 5 min (Branson sonifier cell disruptor B15, G. Heinemann Ultraschall- und Labortechnik) and afterwards firstly extruded 30 times through a 1 μm pore sized membrane and secondly 40 times through a 0.1 μm pore sized membrane (Avanti® Mini-Extruder, Avanti Polar Lipids, Inc.). The extrusion steps were also carried out at 60 °C. The solutions were stored at 4 °C.

DOXCPPa loading efficiency has been evaluated by liposome lysis followed by UV-VIS measurements. Firstly, the initial liposome suspension was transferred into a dialysis membrane (MWCO 3500, Serva) and dialyzed versus PBS under 24 hours of stirring to remove free DOXCPPa. Liposomes were lysed by addition of 5% Triton X-100; DOXCPPa concentration in the lysate was evaluated by measurement of absorbance at 480 nm (Molecular Devices Spectramax M5, USA).

Complex preparation. Liposomes PC:CL 80:20 were mixed with solution of polymer in PBS (Gibco® Life TechnologiesTM). The mixture was heated to 60 °C by water bath for 10 minutes. The final concentration of liposomes was 1 mg/mL, the final concentration of polymer was $1x10^{-6}$M.

Dynamic light scattering and ζ-potential measurement. To measure the size and ζ-potential of the liposomes and their complexes 0.5 mL of the solution was transferred into a cuvette (DTS1060, Malvern Instruments) and the scattered light was detected in an angle of 90° (ZetasizerTM nanoseries, Malvern Instruments). With each measurement a number of 10 sub runs were detected and the average size was calculated. With each measurement a number of 100 sub runs were detected and the average ζ-potential was calculated.

Cell culture. Caco-2 cells were grown in DMEM (Dulbecco's modified Eagle medium, Gibco® Life TechnologiesTM) containing 25 mM HEPES, 10% Fetal Bovine Serum, 1% Myco- 3 (AppliChem), 1% Penicillin – Streptomycin (liquid (10,000 units of penicillin/mL, 10,000 μg of streptomycin/mL): Gibco® Life TechnologiesTM). A549

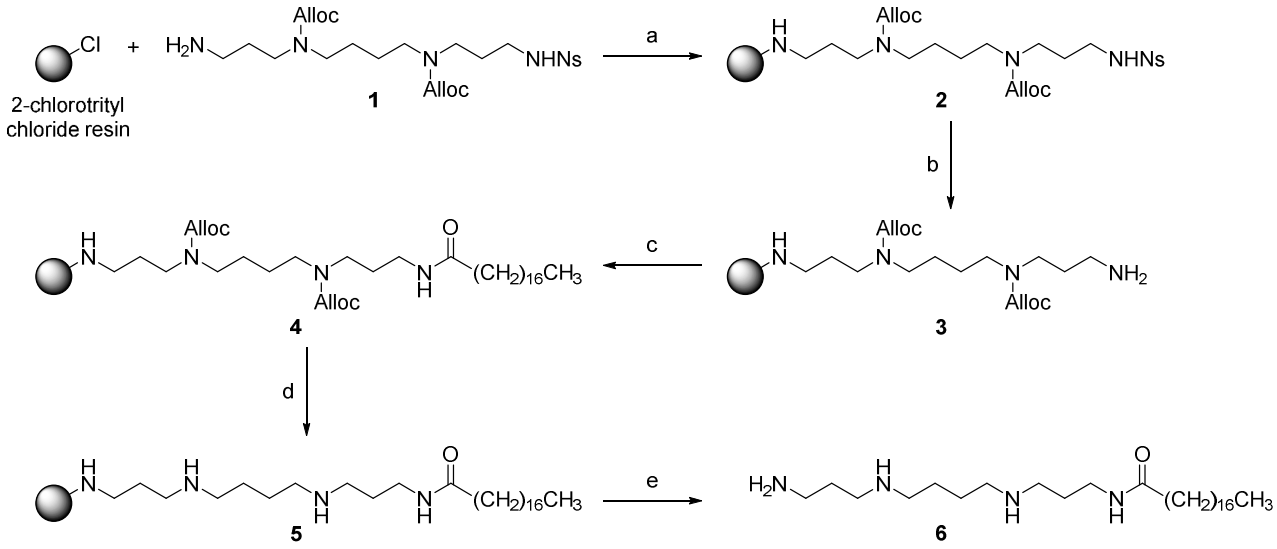
cells were grown in RPMI-1640 (Roswell Park Memorial Institute medium, Gibco® Life TechnologiesTM) containing 10% Fetal Bovine Serum, 1% Myco-3 (AppliChem), 1% Penicillin – Streptomycin (liquid (10,000 units of penicillin/mL, 10,000 µg of streptomycin/mlL): Gibco® Life TechnologiesTM). Cells were cultivated at 37 °C in a humidified incubator with 95% air/ 5% CO_2.

Confocal microscopy. Two hours after seeding, $1x10^5$ Caco-2 or A549 cells/well were transferred into 8 well ibiTreat chamber slides (ibidi, Martinsried, Germany) in 0.2 mL medium. After 24 hours, cells were treated with the liposomes in desired concentrations. After 4 and 24 hours of incubation, the cells were investigated by confocal microscopy. Details: Leica TCS SP5 Tandem DM6000 Microscope, pinhole 111.5 µm, resolution 8 bit, line average 16, format 1024x1024 pixels, 200 Hz, objective HCX PL APO CS 63.0x1.20 water UV, laser 488 argon 11.4%, emission bandwidth PMT 555 – 721 nm.

MTT assay. Short term toxicity of the complexes was determined using the MTT assay. Caco-2 or A549 cells were seeded in 96 well plates, $1x10^4$ cells per well. After incubation for 72 h with the liposomal complexes, cells were treated with MTT reagent solution for 2 – 3 h at 37 °C in a humidified incubator with 95% air/ 5% CO_2. After addition of 100 µL solubilization solution, the absorbance was detected at 595 nm (ELX808IUUltra Microplate Reader, BIO-TEX Instruments). Results are shown as percentile of control cells in 100 µL.

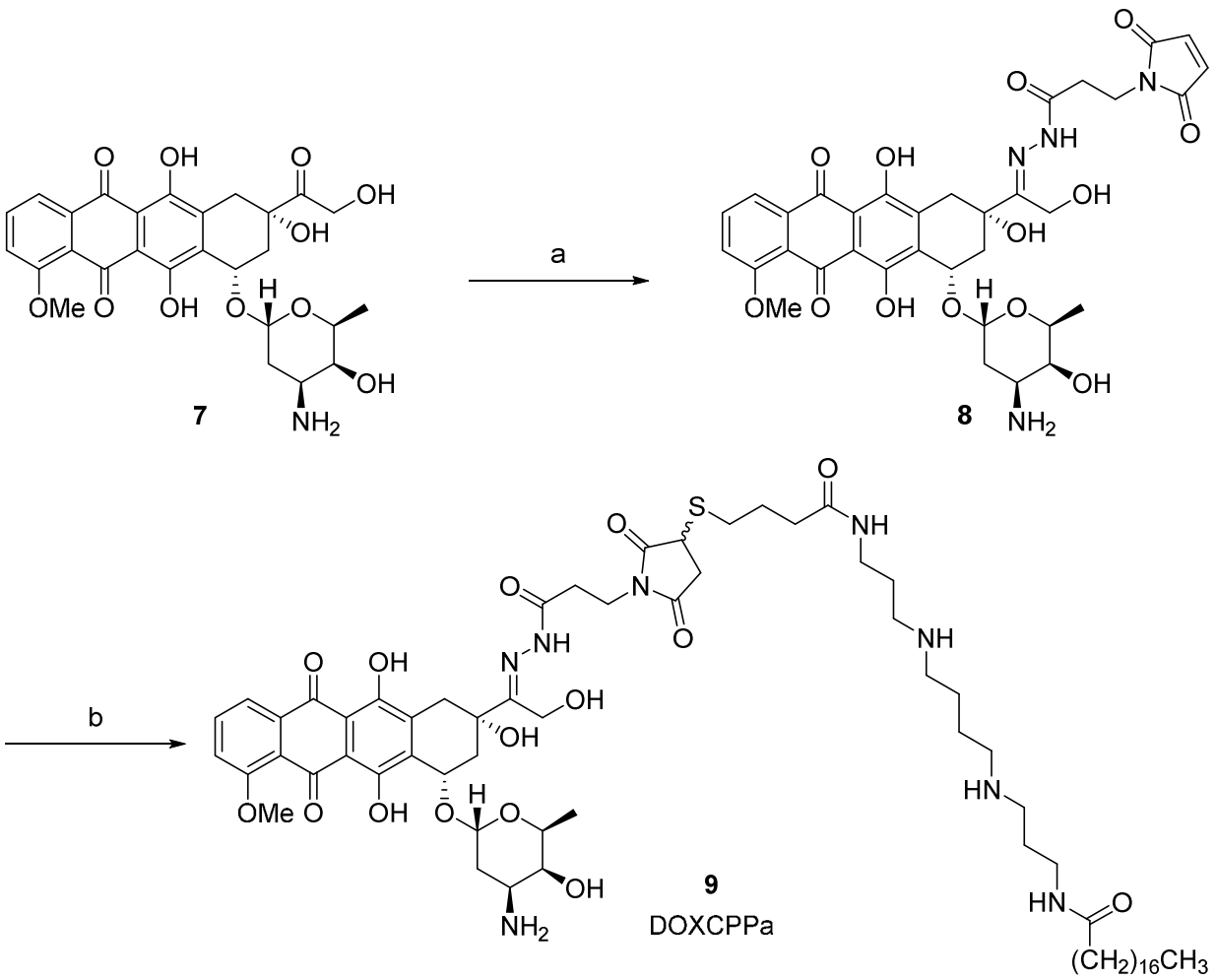
RESULTS AND DISCUSSIONS

For a more efficacious integration of doxorubicin into the liposomal formulation we covalently linked the doxorubicin **7** to an amphiphilic stearoyl polyamine (CPPa), which was also shown to act as a cell penetrating moiety. In Scheme 1, the synthesis of stearoylspermine anchor **6** is presented. The preparation of asymmetric polyamines requires extensive protection group manipulation of orthogonally protected building blocks, and therefore the respective synthesis is preferentially performed on solid supports to facilitate the removal of excess reagents and byproducts. The Alloc-protected spermine **1** was prepared as previously described *(9)*. The spermine building block is able to participate in electrostatic interactions with anionic groups because of the presence of amino groups and increases the solubility of stearoyl chains in aqueous media.



Scheme 1. Synthesis of the amphiphilic spermine-derivative **6** on 2-chlorotrityl chloride resin *(9)*. Reaction conditions: **a** DIPEA, abs. CH_2Cl_2, RT, 15 h; **b** β-mercaptoethanol, DBU, DMF, RT, 2 h; **c** stearic acid, DIC, HOBt, DMF, RT, 15 h; **d** $Pd(PPh_3)_4$, dimethylbarbituric acid, abs. THF, 35 °C, 15 h; **e** 5% TFA in CH_2Cl_2, RT, 15 h.

Eventually, the conjugation of stearoylspermine **6** with doxorubicin (**7**, Scheme 2) was performed employing an acid-sensitive linker containing hydrazone and thioether groups. Usually, cancer cells suffer from acidosis *(13)* and linker hydrolysis within the acidic interstitium of the tumor is considered to facilitate drug release and transfer to cell nucleus.



Scheme 2. Synthesis of the doxorubicin-polyamine-conjugate **9** (DOXCPPa). Reaction conditions: **a** *N*-β-maleimidopropionic acid hydrazide, abs. MeOH, dark, RT, 2 h; **b** spermine **6**, 2-iminothiolane, EtOH/H_2O, dark, 0 °C - RT, 16 h.

Subsequently, the DOXCPPa should be integrated into the liposomal formulation, e.g. neutral liposomes consisting of phosphatidylcholine or anionic liposomes consisting of phosphatidylcholine and cardiolipin. The presence of stearoyl chains obviously increases the affinity between the liposomal membrane and DOXCPPa. However, the outer membrane of liposomes usually suffers from oxidation and aggregation during storage. To prevent the oxidation and therefore this aggregation, stabilizing additives, e.g. PEG-chitosan, are frequently applied. Here, PEG-chitosan was synthesized and characterized as described before *(10)*. Chitosan is conjugated with monomethoxypolyethyleneglycolyl-*N*-hydroxysuccinimidyl-succinate via deacylated amino groups. The degree of modification was estimated as 25%; in each copolymer approximately 300 amino groups are unmodified and considered as potential sites of binding.

DOXCPPa easily incorporates into liposomes: we have estimated the loading efficiency as 98.5±0.3% for anionic liposomes PC/CL according to UV-VIS measurements (absorbance at 480 nm, data not shown), which is much higher than for doxorubicin (ca. 60 – 70%) simple loading. Loading capacity was determined as at least 25 mol %.

Here, we have studied neutral and anionic liposomes and as the main method to characterize the structure of liposomes we have chosen ATR-FTIR spectroscopy. It is a powerful and highly informative method to study the physicochemical properties of lipids and liposomes upon drug incorporation *(14-16).* As we have demonstrated before *(17),* ATR-FTIR spectroscopy allows determining the fine structure and physico-chemical parameters of biosystems in aqueous solution, which is in contrast to common IR methods (e.g. absorption or transmitting IR-spectroscopy), which lack the necessary sensitivity (*18*).

ATR-FTIR revealed a number of intensive bands of a typical liposomal spectrum (Fig. 1): they comprise bands of asymmetric and symmetric valence oscillations of methylene groups CH_2 as and CH_2 s (2915 – 2930 cm^{-1} and 2850 – 2855 cm^{-1}, respectively), which are sensitive to changes of lipid package in the bilayer *(16).* Here, we emphasize that these bands are very sharp and conserved with no significant shifts: e.g. the phase transition of PC (egg phosphatidylcholine) /CL (cardiolipin) (80/20 w/w) liposomal gel-like bilayer – fluid-like bilayer is accompanied by a shift of CH_2 as band from 2919 cm^{-1} to 2924 cm^{-1} and CH_2 s band from 2851 cm^{-1} to 2853 cm^{-1}. Bands of carbonyl groups (1720 – 1750 cm^{-1}) and phosphate groups (1220 – 1270 cm^{-1}) are multicomponent bands. High-frequency components correspond to low-hydrated groups, low frequency bands correspond to highly-hydrated groups *(10).* A decrease of hydration degree is usually caused by formation of electrostatic interactions with charged ligands.

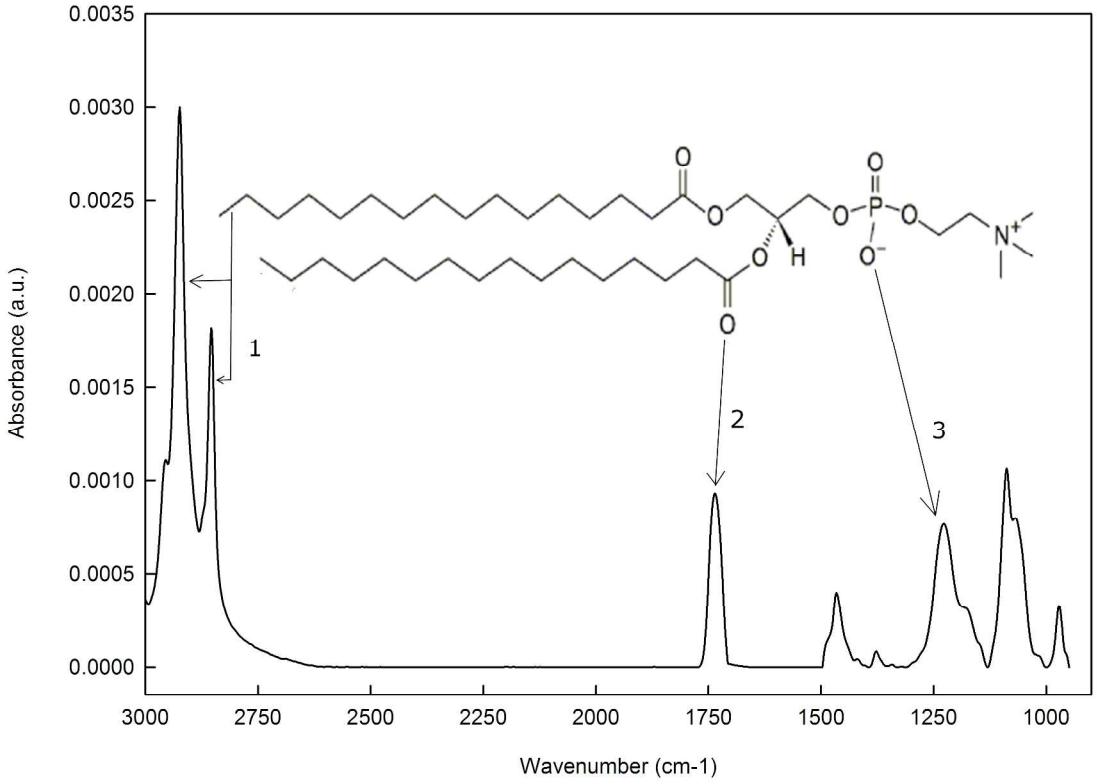


Figure 1. FTIR-spectra of liposomes (3 mg/mL), consisting of 80/20 w/w PC/CL in borate buffer system 0.05 M, pH 8.5, 22 °C. The most important bands are numbered: (1) CH_2 asymmetric and symmetric oscillations, (2) carbonyl group oscillations, (3) phosphate group asymmetric oscillations.

Eventually, we have investigated how DOXCPPa embedded into the liposomal membrane depending on the lipid composition: PC 100 % or PC/CL 80/20 % w/w. Table 1 displays the main band positions. PC 100 % liposomes are in a fluid-like state, which is characterized by high-mobile acyl chains (2923 – 2925 cm^{-1} for CH_2 as and 2851 – 2852 cm^{-1} for CH_2 s bands) as well as by highly hydrated carbonyl (1720 – 1725 cm^{-1}) and phosphate groups (1219 – 1222 cm^{-1}). Presence of 20% of cardiolipin stimulates a gel-like state of bilayer characterized mainly by low-mobile acyl chains (2917 – 2920 cm^{-1} for CH_2 as and 2849 – 2850 cm^{-1} for CH_2 s).

Loading of DOXCPPa leads to a high-frequency shift of the CH_2 as band in both cases. In case of PC/CL liposomes, this effect is more pronounced. We have observed that an embedding of DOXCPPa leads to a phenomenon close to phase transition: bands of CH_2 as and CH_2 s underwent high-frequency shifts (Table 1), which indicates an increase of local membrane fluidity.

We assume that this high-frequency shift observed in both cases is caused by disturbance of the membrane due to stearoyl chain embedding, proving the incorporation of DOXCPPa. The embedding is also accompanied by significant changes in the structure

of the phosphate and carbonyl group bands, namely redistribution of groups on hydration degrees.

Table 1. The main band positions on ATR-FTIR spectra of PC 100%, PC/CL 80/20 liposomes and the same loaded by DOXCPPa (1 μM).

Composition /Band	PC 100% / cm^{-1}	PC 100% + DOXCPPa / cm^{-1}	PC/CL 80/20 / cm^{-1}	PC/CL 80/20 + DOXCPPa / cm^{-1}
ν_{as} CH_2	2923	2925	2919	2923
ν_s CH_2	2853	2853	2851	2853
νC=O	1737, 1727	1735	1740, 1723	1745, 1723
$\nu_{as}PO_2^-$	1232	1226	1225	1251, 1219

In order to disclose these fine changes and to investigate their molecular details we have applied curve-fitting analysis as it is described elsewhere *(19)*. The distribution of phosphate groups on the degree of hydration in PC 100% is presented in Figures 2A, B. Embedding of DOXCPPa led to interrelated changes in the phosphate groups' state. We have observed a decrease of the contribution from highly-hydrated PO_2. The integrated portion of component 1225 cm^{-1} diminished from 65% to 41%. On the other hand, contribution from low-hydrated groups (1236 cm^{-1} and 1247 cm^{-1}) increased equally from 11% to 24%. For super low-hydrated phosphate groups (component 1257 cm^{-1}), we have observed only a marginal effect of DOXCPPa. In sum, these changes allow us to propose that the spermine moiety of DOXCPPa interacted with the phosphate groups because of electrostatic forces. However, we emphasize that changes are not as pronounced as in multipoint interactions of anionic liposomes and cationic polymers (*10*).

It is well-known that carbonyl groups in liposomes are slightly immerged in the bilayer and, in contrast to phosphate groups, possess only partial negative charge. Therefore, carbonyl groups are considered to be less active in electrostatic interaction with cationic ligands. On the other hand, carbonyl groups are sensitive to changes in lipid package in the bilayer. Increased membrane fluidity is accompanied by higher accessibility of carbonyl groups for water molecules because of a looser structure. Here, we have observed that embedding of DOXCPPa led to an increased acyl chain mobility, i.e. a looser bilayer structure. Curve-fitting analysis demonstrated that contribution of low-hydrated carbonyl groups (1744 cm^{-1}) decreased from 52% to 21%; while contribution of

high-hydrated (1733 cm^{-1}), on the contrary, increased from 30% to 62% (Fig. 2 C, D). These changes demonstrate that carbonyl groups did not participate in electrostatic interactions with the spermine moiety but greatly depend on bilayer fluidity.

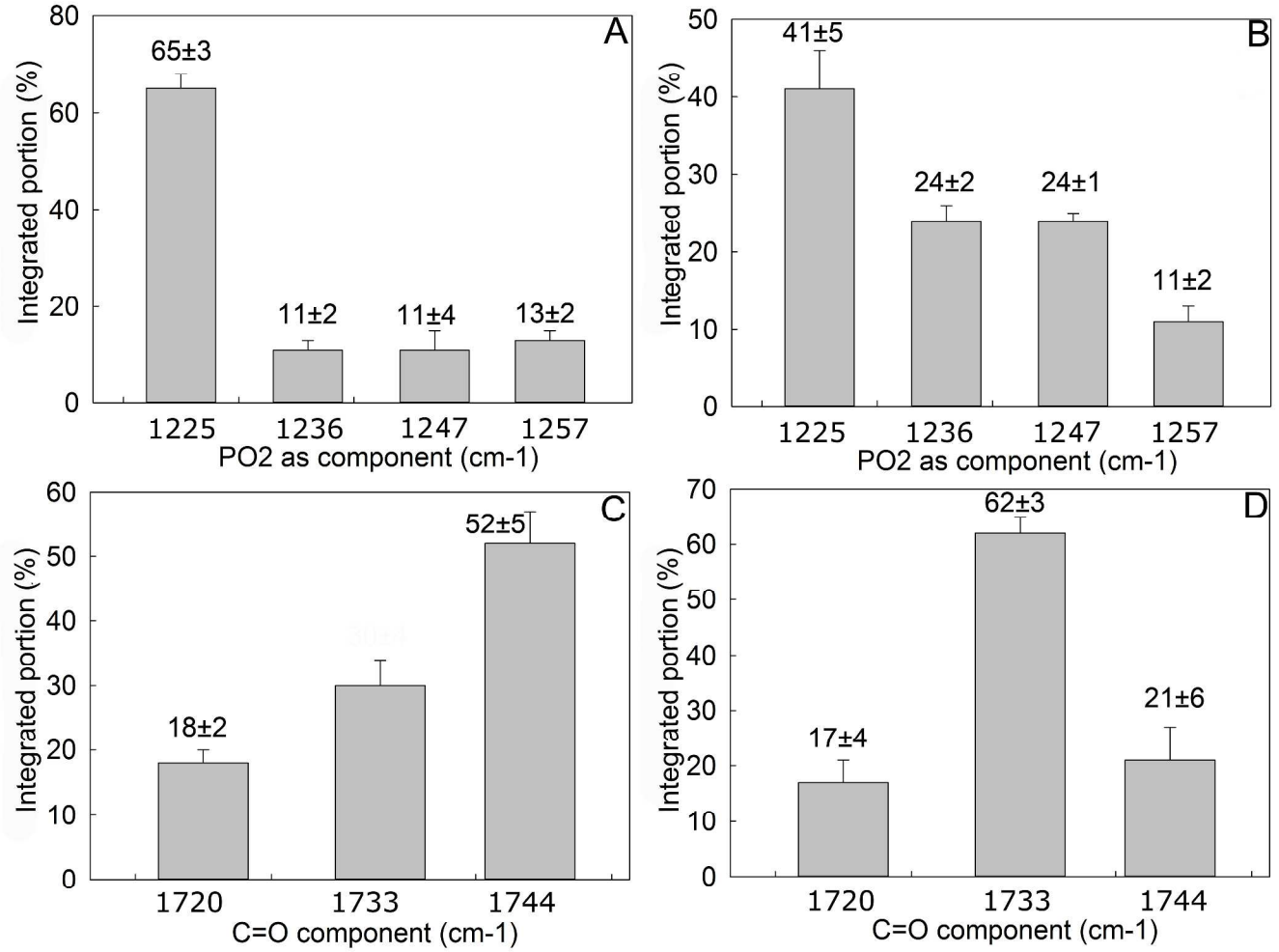


Figure 2. Integrated portion of components of phosphate group band. A. Liposomes 100% PC 1 mg/mL. B. Liposomes PC 100% 1 mg/mL loaded with DOXCPPa. Integrated portion of components of carbonyl group band. C. liposomes 100% PC 1 mg/mL. D. Liposomes PC 100% 1 mg/mL loaded with DOXCPPa. All spectra were recorded in phosphate buffered saline pH 7.4, 22 °C .

A dramatically different pattern is observed for PC/CL liposomes. Embedding of DOXCPPa led to changes in the phosphate groups' hydration degree, which is typical for interaction with polycations (*10*). For non-loaded PC/CL liposomes, the major part of phosphate groups (76%) was highly-hydrated (Fig. 3A), while loading of DOXCPPa led to a decrease of this value to 35% (Fig. 3B). Contribution of 1229 cm^{-1} component stayed practically unchanged, whereas the integrated portion of low-hydrated groups (1242 cm^{-1}, 1251 cm^{-1}, 1256 cm^{-1}) increased significantly from 3% to 40%. These changes

strikingly pointed to an interaction of cardiolipin phosphate groups with amino groups in the spermine moiety.

Another potential site of liposomes' interaction with cationic ligands are carbonyl groups. In this case, we observed an increase of low-hydrated carbonyl groups contribution (component 1745 cm^{-1}) from 17% to 32% upon interaction with DOXCPPa (Fig. 3 D, E).

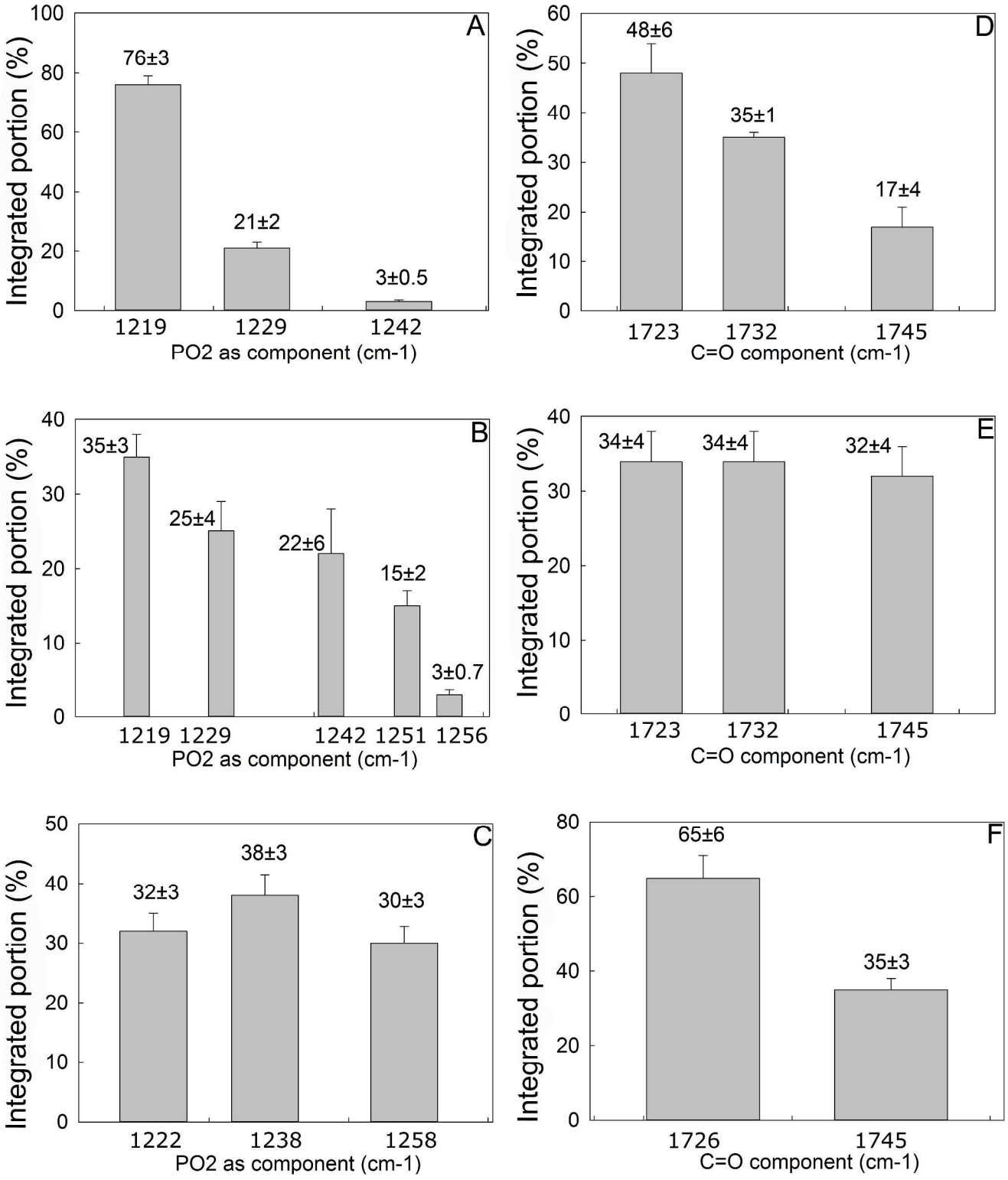


Figure 3. Integrated portion of components of phospate group band. A. Liposomes PC/CL 80/20 1 mg/mL. B. Liposomes PC/CL 80/20 1 mg/mL loaded with DOXCPPa. C. Liposomes PC/CL 80/20 1 mg/mL loaded with DOXCPPa and covered by PEG-chitosan. Integrated portion of components of carbonyl group band. D. Liposomes PC/CL 80/20 1 mg/mL. E. Liposomes PC/CL 80/20 1 mg/mL loaded with DOXCPPa. F.

Liposomes PC/CL 80/20 1 mg/mL loaded with DOXCPPa and covered by PEG-chitosan. All spectra were recorded in phosphate buffered saline pH 7.4, 22 °C.

Thus, we suggest the following structure of the DOXCPPa loaded liposome. The doxorubicin part is located in the water media of liposomes, the spermine moiety interacts with the phosphate and carbonyl groups of the membrane and the stearoyl chain is embedded into the hydrophobic area.

We propose that, due to more effective binding, PC/CL liposomes are more suitable as a DOXCPPa carrier. However, anionic liposomes should be coated in order to prevent oxidation and membrane fusion *(20)*. In order to stabilize this system, we have used PEG-chitosan as a coating. As it was previously shown *(10)*, PEG-chitosan interacts with the outer leaflet of the membrane of PC/CL 80/20 liposomes by forming electrostatic interactions with the phosphate and carbonyl groups. In case of drug-loaded liposomes, the complex is formed by the same mechanism. The main band positions are presented in Table 2. The PEG-chitosan coating led to a considerable increase in low-hydrated phosphate groups (1258 cm^{-1}) (Fig. 3 C). While, in drug-loaded liposomes, the integrated portion of 1258 cm^{-1} component was 3%, in coated liposomes this value was about 30%. Perhaps, PEG-chitosan was able to cause lateral segregation or flip-flop effects in fluid-like state as this was shown for some synthetic polymers *(21)*. By this mechanism, some cardiolipin molecules that have not interacted with spermine can move from the inner to the outer leaflet to bind the polymer. Interaction of these molecules with the PEG-chitosan amino groups could explain the significant growth of the 1258 cm^{-1} component contribution. As for carbonyl groups, the portion of the low-hydrated component 1745 cm^{-1} stays almost unchanged, while two components 1723 cm^{-1} and 1732 cm^{-1} combine in one band at 1726 cm^{-1} (Fig. 3 E, F). Perhaps this confluence was caused by a more uniform distribution of water molecules on the outer surface of the bilayer because of the water coat of PEG-chitosan.

In summary, the obtained data display the strong interaction between the outer leaflet of liposomes and PEG-chitosan. The absence of changes in CH_2 as and CH_2 s band position after coating of loaded liposomes with PEG-chitosan indicates a surface interaction with the polymer (Table 2).

Table 2. The main band positions on ATR-FTIR spectra of PC/CL 80/20 liposomes, PC/CL 80/20 liposomes loaded with DOXCPPa (1 μM) and PC/CL 80/20 liposomes loaded with DOXCPPa (1 μM) and coated with PEG-chitosan.

Composition /Band	PC/CL 80/20 / cm^{-1}	PC/CL 80/20 + DOXCPPa / cm^{-1}	PC/CL 80/20 + DOXCPPa + PEG-chitosan / cm^{-1}
ν_{as} CH_2	2919	2923	2924
ν_s CH_2	2851	2853	2852
νC=O	1740, 1723	1745, 1723	1743, 1729
$\nu_{as}PO_2^-$	1225	1251, 1219	1259, 1238

Data obtained by FTIR were confirmed by DLS and ζ-potential measurements (Table 3). Complex formation increases the diameter from 108 to 151 nm, which is preferable in terms of EPR (enhanced permeability and retention) and tumor targeting *(22)*. Charge neutralization of PC/CL 80/20 from -23 mV to -12 mV indicates saturation in terms of cardiolipin phosphate groups. The results obtained are in a good agreement with those obtained previously *(17)*.

Table 3. Hydrodynamic diameters and ζ-potentials of drug-loaded liposomes (DOXCPPa concentration 1 μM) and drug-loaded liposomes with PEG-chitosan coating.

Composition	Z Average diameter / nm	ζ-potential / mV
PC 100 % + DOXCPPa	107±1	-12.0±0.5
PC/CL 80/20 + DOXCPPa	108±1	-23±2
PC/CL 80/20 + DOXCPPa + PEG-chitosan	151±1	-12±2

In order to test the efficacy of the developed system as a suitable doxorubicin carrier, the DOXCPPa encapsulated into PC/CL 80/20 liposomes coated by PEG-chitosan was tested in cells. Cytotoxicity studies were performed in lung carcinoma (A549) and colon

carcinoma (Caco-2) cell lines. We have investigated the influence of DOXCPPa concentration from 0.25 μM to 1.5 μM and the effect of the different liposomal containers as well as the coating itself. Figure 4 shows the MTT tests for PEG-chitosan and for the liposomal formulation of DOXCPPa 1 μM.

According to MTT tests (Figure 4), the non-loaded liposomal containers from PC and PC/CL (data not shown) as well as the PEG-chitosan coating were not toxic at concentrations up to 1 mg/mL. After loading with the DOXCCPa, the highest cytotoxic effect was observed for non-coated PC/CL liposomes. In terms of LD_{50} values, PC 100% DOXCPPa liposomes were characterized with an LD_{50} of 1.3 μM. For PC/CL DOXCPPa liposomes the LD_{50} is 0.7 μM, and for PC/CL DOXCPPa liposomes with PEG-chitosan coating the LD_{50} is 1.1 μM. On the other hand, stability of PEG-chitosan coated liposomes is much higher than of free liposomes *(10)* and it can be expected to have prolonged blood circulation time. As it was previously demonstrated, non-coated liposomes possess shorter blood circulation time than PEG-coated liposomes because of the entrapment into the mononuclear phagocyte system. Accordingly, application of PEG-chitosan coating seems to be the preferable approach. The same results were obtained in studies of other liposomal DOXCPPa concentrations (0.25 – 1.5 μM), and the corresponding data is presented in SI.

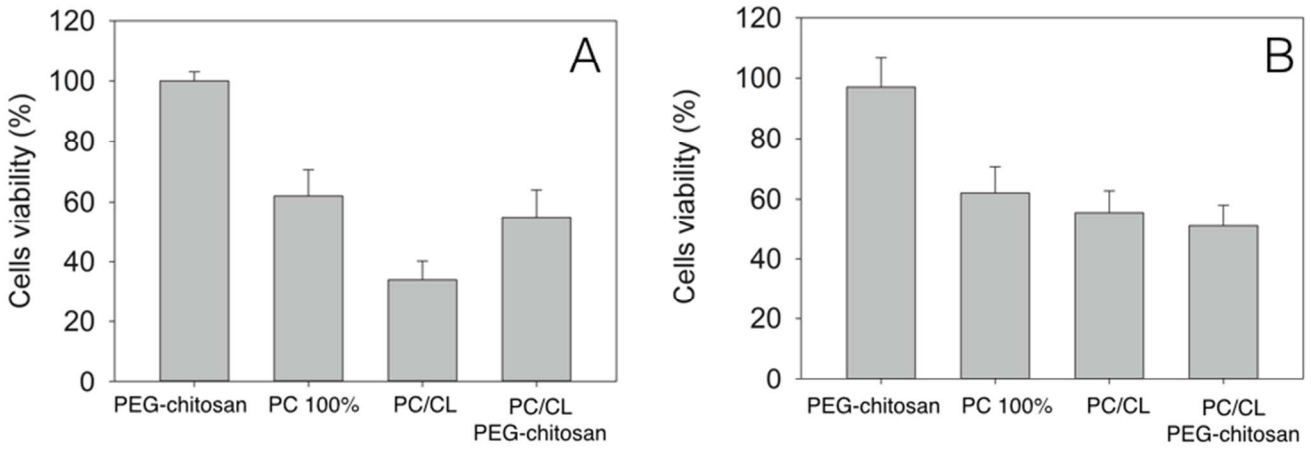


Figure 4. A. Results of MTT assay for A549 cells after 48 hours of incubation with PEG-chitosan or liposomal formulation of DOXCPPa 1 μM. B. Results of MTT assay for Caco-2 cells after 48 hours of incubation with PEG-chitosan or liposomal formulation of DOXCPPa 1 μM. Statistical errors bars were calculated from triplicates of n = 3 experiments; significance was determined according to Student's test with $p < 0.05$).

For a detailed study of cellular uptake and nuclear drug release we have applied confocal microscopy of the cells after 4 and 24 hours of incubation with the respective liposomes. For A549 cell line, after 24 hours of incubation, doxorubicin effectively accumulates in

the cells (Fig. 5). Further data on 4 hours of incubation (A549) and on Caco-2 (4 and 24 hours of incubation) is presented in SI.

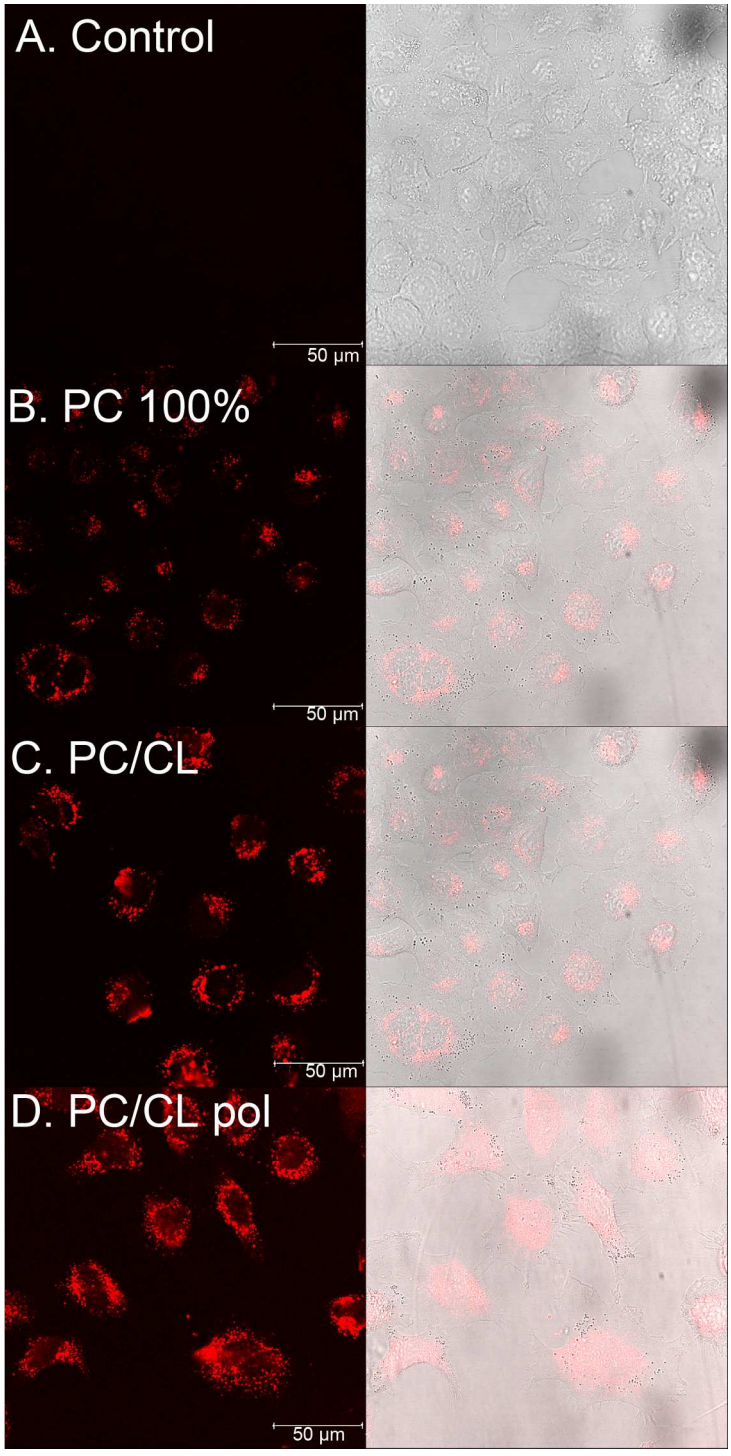


Figure 5. Confocal images of A549 cells after 24h of incubation with liposomal DOXCPPa 1 µM. A. Control. B. PC + DOXCPPa. C. PC/CL + DOXCPPa. D. PC/CL + DOXCPPa + PEG-chitosan. Scale bar - 50 µm. Leica TCS SP5 Tandem DM6000 Microscope, pinhole 111.5 µm, resolution 8 bit, line average 16, 200 Hz, objective HCX PL APO CS 63.0x1.20 water UV, laser 488 argon 11.4%, emission bandwidth PMT 555

– 721 nm.

CONCLUSIONS

In conclusion, we have synthesized DOXCPPa (doxorubicin, coupled with a stearoylspermine anchor via an acid-sensitive linker) and successfully loaded it into liposomes of different composition. The structure of the obtained liposomal formulations was determined by ATR-FTIR spectroscopy. The doxorubicin moiety is located in the water area of the liposomes, the spermine moiety is interacting with the phosphate groups of lipids, and the stearoyl chain is integrated into the hydrophobic area of the bilayer. In order to increase the stability of the liposomal system, PEG-chitosan was used. The study of cytotoxicity on two cell lines, A549 and Caco-2, demonstrates that DOXCPPa-loaded, anionic liposomes coated with PEG-chitosan have almost equal cytotoxic activity to neutral, DOXCPPa-loaded liposomes. Anionic liposomes coated by PEG-chitosan, however, demonstrate higher storage stability.

ABBREVIATIONS

PC, egg phosphatidylcholine; CL, cardiolipin; FTIR, Fourier transformed infrared spectroscopy.

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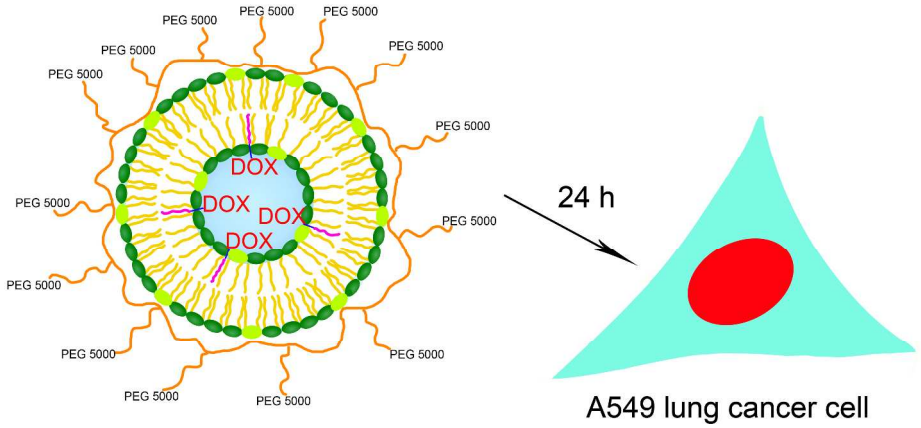

Graphical abstract

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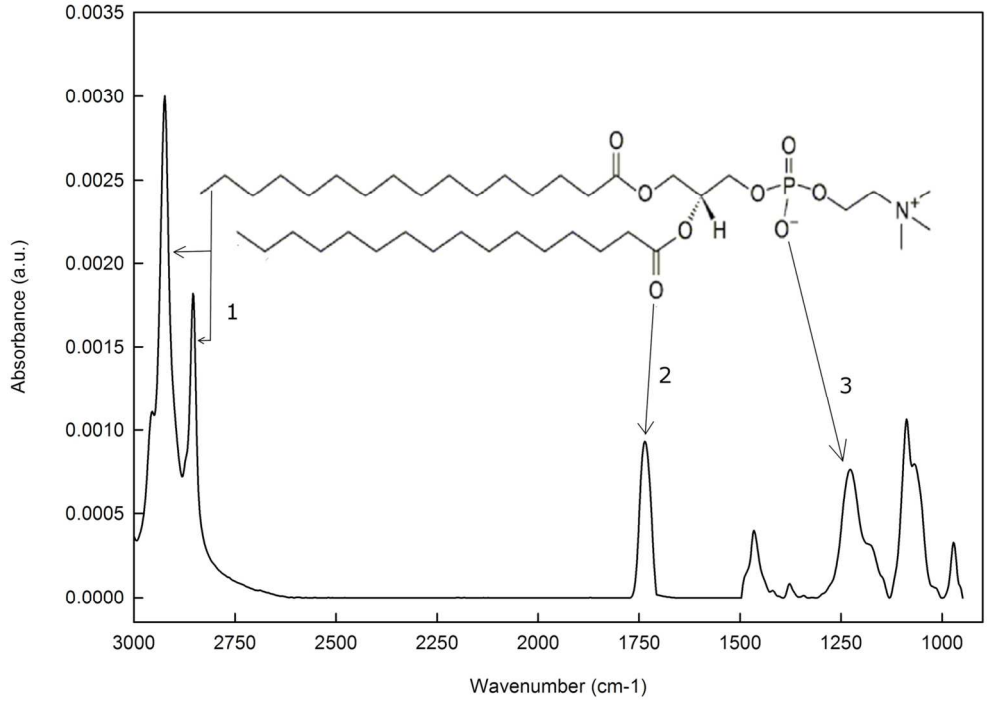

Figure 1 FTIR-spectra of liposomes (3 mg/mL), consisting of 80/20 w/w PC/CL in borate buffer system 0.05 M, pH 8.5, 22 °C. The most important bands are numbered: (1) CH2 asymmetric and symmetric oscillations, (2) carbonyl group oscillations, (3) phosphate group asymmetric oscillations.
ATR-FTIR revealed a number of
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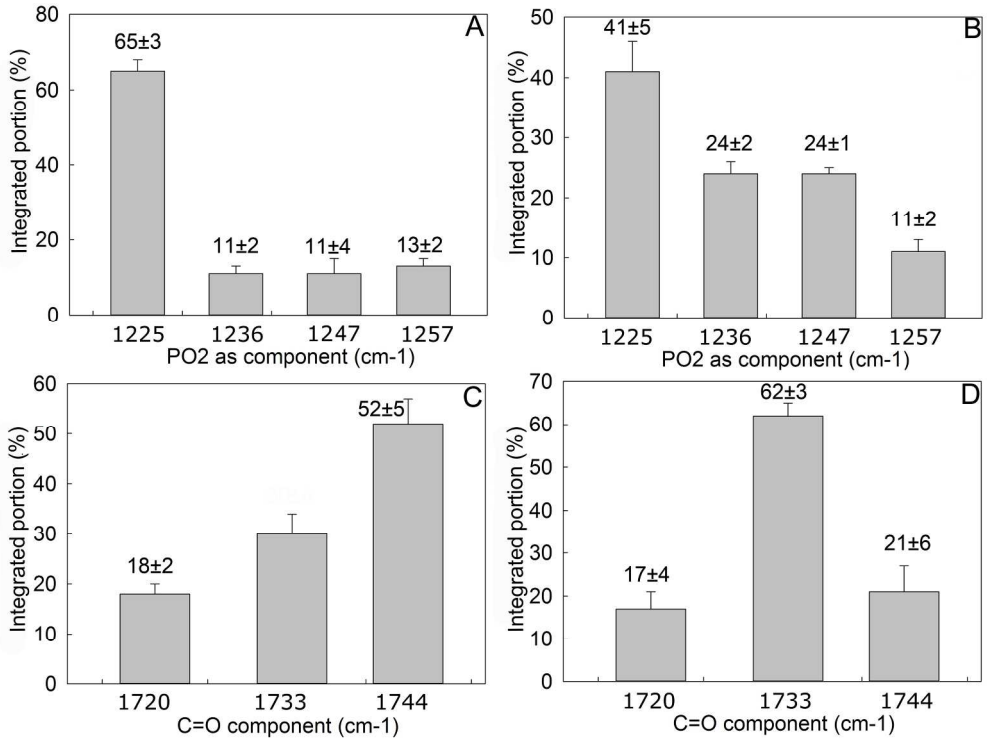

Figure 2. Integrated portion of components of phosphate group band. A. Liposomes 100% PC 1 mg/mL. B. Liposomes PC 100% 1 mg/mL loaded by DOXCPPa. Integrated portion of components of carbonyl group band. C. liposomes 100% PC 1 mg/mL. D. Liposomes PC 100% 1 mg/mL loaded by DOXCPPa. All spectra were recorded in phosphate buffered saline pH 7.4, 22 °C .
The distribution of phosphate
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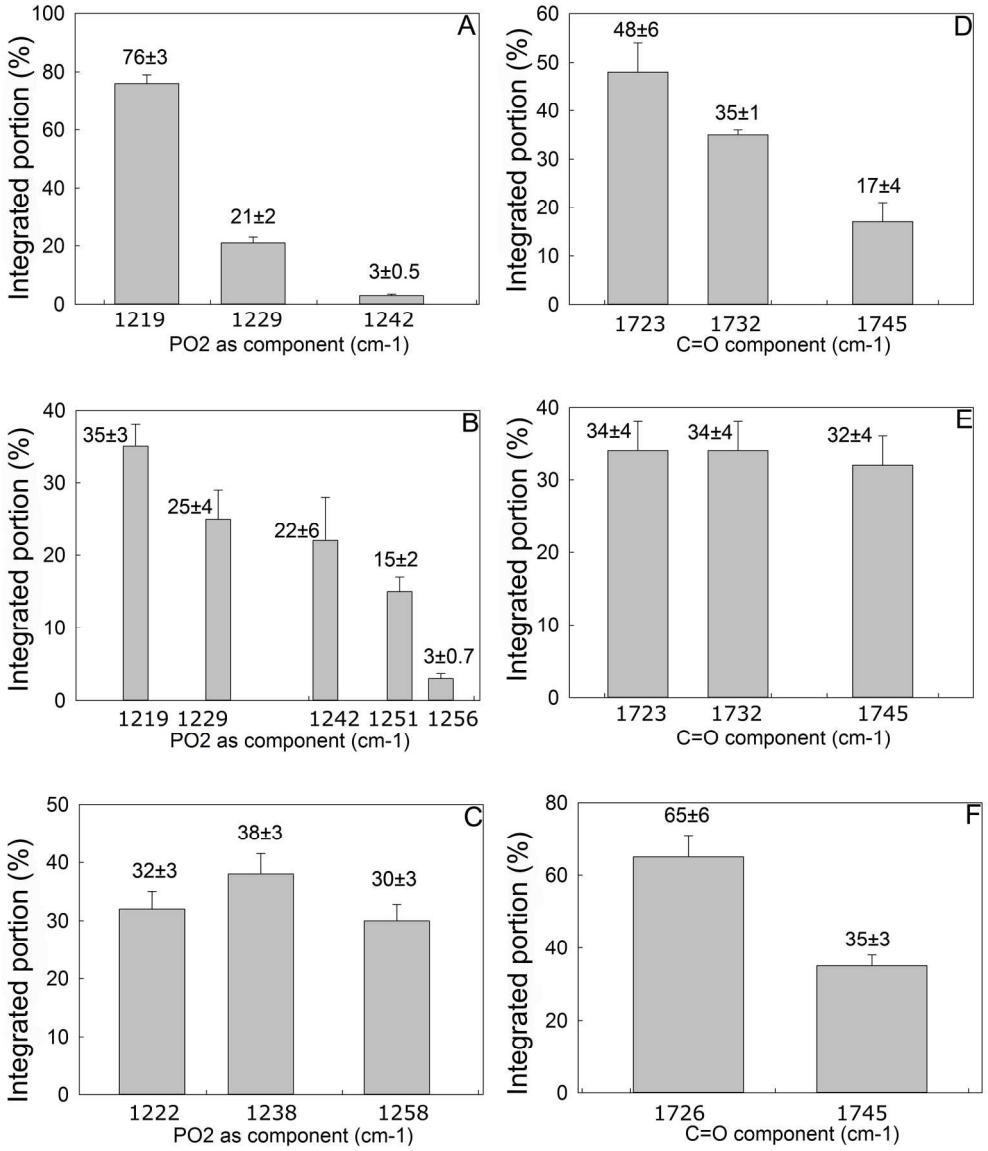

Figure 3. Integrated portion of components of phospate group band. A. Liposomes PC/CL 80/20 1 mg/mL. B. Liposomes PC/CL 80/20 1 mg/mL loaded by DOXCPPa. C. Liposomes PC/CL 80/20 1 mg/mL loaded with DOXCPPa and covered by PEG-chitosan. Integrated portion of components of carbonyl group band. D. Liposomes PC/CL 80/20 1 mg/mL. E. Liposomes PC/CL 80/20 1 mg/mL loaded by DOXCPPa. F. Liposomes PC/CL 80/20 1 mg/mL loaded by DOXCPPa and covered by PEG-chitosan. All spectra were recorded in phosphate buffered saline pH 7.4, 22 °C.
For non-loaded PC/CL liposomes
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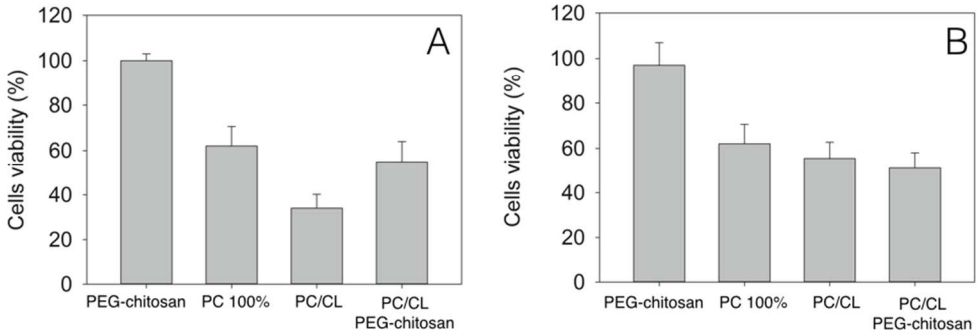

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Figure 4 shows the MTT tests f
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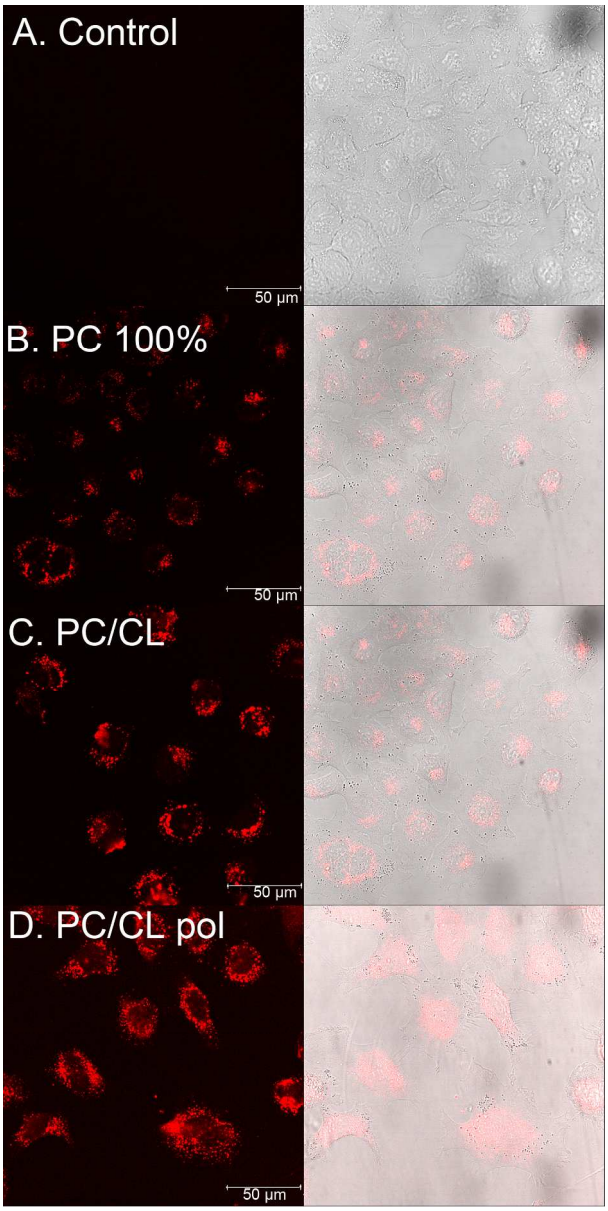

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409x819mm (96 x 96 DPI)