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Study of Functional Qualities of Different Types of Tailored Liposomes with Encapsulated Doxorubicin using Electrochemical and Optical Methods

Marketa Kominkova[1], *Roman Guran*[1], *Miguel Angel Merlos Rodrigo*[1], *Pavel Kopel*[1,2], *Iva Blazkova*[1], *Dagmar Chudobova*[1], *Lukas Nejdl*[1], *Zbynek Heger*[1], *Branislav Ruttkay-Nedecky*[2], *Ondrej Zitka*[1,2], *Vojtech Adam*[1,2], *Rene Kizek*[1,2*]

[1] Department of Chemistry and Biochemistry, Faculty of Agronomy, Mendel University in Brno, Zemedelska 1, CZ-613 00 Brno, Czech Republic, European Union
[2] Central European Institute of Technology, Brno University of Technology, Technicka 3058/10, CZ-616 00 Brno, Czech Republic, European Union
[*]E-mail: kizek@sci.muni.cz

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This study points to changes in the properties of liposomes and its functional qualities in task of release of encapsulated drug doxorubicin according to the content of cholesterol in the phospholipid bilayer. The influence of sodium dodecyl sulphate (SDS) addition to liposomal variants was also evaluated too. Three variants of liposomes differing in various concentrations of cholesterol were assessed. Firstly, we focused on the toxicity of all liposomal variants to *Staphyloccocus aureus* and it was found that the content of cholesterol increases the IC_{50} values of encapsulated doxorubicin in liposome with higher concentration of cholesterol than in liposome without cholesterol even four times. Further, the new approach to comparing the influence of various liposomes on detection of encapsulated doxorubicin using the electrochemical detection with construction of differential hydrodynamic voltammograms was used. Finally, the fluorescence detection was used to confirm the release of encapsulated doxorubicin from liposomes after the addition of detergent SDS. In this work we demonstrated the suitability of number of methods including electrochemistry for studying of effect of substances such substance as SDS and cholesterol on the lipid functional qualities.

Keywords: Liposome; Cholesterol; Doxorubicin; Sodium Dodecyl Sulphate; Drug Delivery; Drug Encapsulation;

1. INTRODUCTION

Since liposomes were discovered in the 1960s [1], these phospholipidic structures have been studied intensively for their potential to serve as the vehicles for drug transportation. Liposomes are

particles with lipid bilayer enclosing a vesicular space wearing a range of attractive properties including the ability to encapsulate aqueous solutions within the liposome core, sequester lipophilic compounds within the bilayer, and support tailored surface chemistries of the liposomes for targeted delivery [2], however, the most common drug carriers are currently pegylated liposomes which have an immobilised polyethylene glycol on their surface [3,4]. When using liposomes, particularly the pegylated ones, it is possible to achieve prolonged persistence of the drug in the body and to reduce the degradation of drug in the liver, which leads to reduction of the negative effects on the organism while the antitumor efficacy is maintained [5]. For using of liposomes as transporters in anticancer therapy, their ability to aggregate, open and release the drug at the place of interest is necessary. However, this has not been completely resolved yet [3,6].

The release of drug from the liposomes is based on the fusion with its membrane, but the newly appearing option, e.g. sonication, through which the drug is released, can be also used [4,7,8]. Pharmacokinetic properties of the liposomes are given by their physicochemical properties [9,10]. These attributes can be significantly affected by the change of the composition of the lipid bilayer and by the addition of other substances to this structure. The predominant ingredients used in their preparation are glycerophospholipids, sphingolipids and cholesterol [11]. These components give the liposomes a similarity to natural cell membranes [11]. The cholesterol present in the structure of liposomes supports their stability and enables the control of permeability and solubility of the liposome membrane [12,13]. Amphiphilic ends of cholesterol in the lipid bilayer are oriented as parallel with the carbon chains of lipids and carboxyl groups of the aqueous phase [14]. Besides medical applications liposomes can also be used for analytical purposes. Recently, a number of methods based on liposomes have been developed including immobilised liposome chromatography (ILC) [15,16], liposome capillary electrophoresis (LCE) [17,18], biosensors [19] and liposome immunosorbent assay (LISA) [20-22].

In this article we focused our attention on studying of properties of synthesized liposomes, which differed in the content of cholesterol in the lipid bilayer, and on the possibilities of opening these liposomes after addition of sodium dodecyl sulphate (SDS).

2. EXPERIMENTAL PART

2.1. Chemicals and pH measurement

Cholesterol, 1,2-dioleoyl-sn-glycero-3-phospho-rac-(1-glycerol) sodium salt, chloroform, doxorubicin·HCl, sodium dodecyl sulphate (SDS) and water were purchased from Sigma-Aldrich (St. Louis, MO, USA) in ACS purity. Hydrogenated phosphatidylcholine from soybean was obtained from Lipoid GMBH (Ludwigshafen, Germany). The deionised water was prepared using reverse osmosis equipment Aqual 25 (Aqual s.r.o., Brno, Czech Republic). The deionised water was further purified by using MiliQ Direct QUV apparatus equipped with the UV lamp from Millipore (Billerica, MA, USA). The output resistance was 18 MΩ. The pH was measured using pH meter WTW inoLab (Weilheim, Germany).

2.2. Liposomes

2.2.1. Preparation of liposomes

Liposome 8: cholesterol (100 mg), 1,2-dioleoyl-sn-glycero-3-phospho-rac-(1-glycerol) sodium salt (100 mg) and phosphatidylcholine (100 mg) were dissolved in chloroform (4.5 ml). A lipid film was obtained by rotary evaporation of solvent and residual chloroform was blown out by nitrogen.

Liposome 9: cholesterol (50 mg), 1,2-dioleoyl-sn-glycero-3-phospho-rac-(1-glycerol) sodium salt (100 mg) and phosphatidylcholine (100 mg) were dissolved in chloroform (3.75 ml). A lipid film was obtained by rotary evaporation of solvent and residual chloroform was blown out by nitrogen.

Liposome 10: 1,2-dioleoyl-sn-glycero-3-phospho-rac-(1-glycerol) sodium salt (100 mg) and phosphatidylcholine (100 mg) were dissolved in chloroform (3.75 ml). A lipid film was obtained by rotary evaporation of solvent and residual chloroform was blown out by nitrogen.

2.2.2. Preparation of encapsulated doxorubicin

Solutions containing 0, 6.25, 12.5 and 25 µl of doxorubicin·HCl (2 $mg.ml^{-1}$) in 0.5 ml of water were added to liposomes (10 mg). Samples were homogenized in ultrasonic bath Sonorex Digital 10P (Bandelin, Berlin, Germany) for 15 min. The homogenized mixtures were then heated and shaken for 15 min at 60 ºC at Thermomixer Comfort (Eppendorf). The samples were then washed several times with Britton-Robinson buffer (pH = 10) on Amicon 3k (Millipore). Final volume of samples was 0.5 ml.

2.2.3. The analysis of cholesterol amount in liposomes

200 µl of reagent R1 (Greiner, Germany, 0.3 mM 4-aminoantipyrine, 5 mM phenol, peroxidase 3.0 $kU.l^{-1}$, cholesterol esterase 200 $U.l^{-1}$, cholesterol oxidase 100 $U.l^{-1}$ in 50 mM Good's buffer, pH 6.7) were pipetted into the cuvette, then 2 µl of measured sample were added. The three samples of liposomes (8, 9 and 10) with concentration of encapsulated doxorubicin 0, 25, 50 and 100 $\mu g.ml^{-1}$ were used for measurement. Absorbance was measured for 6 minutes at 505 nm. To calculate the absorbance of sample the absorbance values of reagent R1 and absorbance values after 6 minutes of incubation with the sample were used.

2.3. Fluorescence photography

Fluorescence monitoring was performed using an In vivo Xtreme system by Carestream Health Inc. (Rochester, NY, USA). This instrument was equipped with a 400 W xenon light source. Emitted light was captured by 4 MP CCD detector. The excitation wavelength was set at 480 nm and the emission was measured at 600 nm. The exposure time was 2 s, binning – 2 x 2 pixels, fStop - 1.1, field of view – 17.5 × 17.5 cm. Samples (100 µl) were placed in a Nunc MaxiSorp® flat-bottom 96 well plate (Thermo Fisher Scientific, Roskilde Denmark).

2.4. Fluorescence microscopy

Bacterial culture of *Staphylococcus aureus* was observed, after the incubation with doxorubicin in liposome, by fluorescence microscope. The bacterial culture (250 µl) was incubated with 50 µl of doxorubicin (100 $\mu g.ml^{-1}$) in liposome (liposome 8, 9 and 10). After the incubation (24 hours, 37 °C), the culture was washed with PBS buffer (0.24 $g.l^{-1}$ KH_2PO_4, 1.44 $g.l^{-1}$ $Na_2HPO_4.2H_2O$, 8 $g.l^{-1}$ NaCl, 0.2 $g.l^{-1}$ KCl) two times. The culture was stirred with 1 ml of PBS buffer and centrifuged (20 °C, 6000 g, 10 min). Supernatant was removed and procedure was repeated. Finally the cells were stirred in 100 µl of PBS.

The cells obtained were pipetted (5 µl) on the microscope slide and covered by cover slip. The sample was placed by coverslip down and the immersion oil was used. The objective (PlanFLN; Mag. 100x; NA 1,3; F.N. 26.5) and the magnification lens 1.6x was used, and the total magnification was 1600x. The inverted research fluorescence microscope Olympus IX71S8F-3 (Olympus Corporation, Tokyo, Japan) was used. The images were captured by Olympus Camera DP73 and processed by Olympus Stream Basic 1.7 Software. The images resolution was 4800 x 3600 pixels. The parameters for the ambient light images were following: exposure time – 2.2 ms and ISO 200. Fluorescence of the doxorubicin was detected using excitation filter – 520 - 550 nm and emission filter – 580 nm; exposure time: 530.8 ms, ISO 100.

2.5. Matrix-assisted laser desorption/ionization time of flight mass spectrometry

Matrix-assisted laser desorption/ionization time of flight mass spectrometric (MALDI-TOF MS) experiments were performed on a MALDI-TOF/TOF mass spectrometer Bruker ultrafleXtreme (Bruker Daltonik GmbH, Germany) equipped with a laser operating at wavelength of 355 nm with an accelerating voltage of 25 kV, cooled with nitrogen and a maximum energy of 43.2 µJ with repetition rate 2000 Hz in linear and positive mode, and with software for data acquisition and processing of mass spectra flexControl version 3.4 and flexAnalysis version 2.2. The matrix used in the MALDI method was 2,5-dihydroxybenzoic acid (DHB) (Sigma-Aldrich, USA). The saturated matrix solution was prepared in 50% methanol and 0.1% trifluoroacetic acid (TFA). Mixture was thoroughly vortexed and ultrasonicated using Bandelin 152 Sonorex Digital 10P ultrasonic bath (Bandelin electronic GmbH, Germany) for two minutes at 50% of intensity at room temperature. Sample preparation crystallization method for MALDI-TOF was dried-droplet method (DD) as the sample solutions for analysis were mixed with matrix solution in volume ratio of 1:1. After obtaining a homogeneous solution, 2 µl was applied on the MTP 384 polished steel target plate (Bruker) and dried under atmospheric pressure at room temperature. A mixture of peptide calibration standards (Bruker) was used to externally calibrate the instrument. The preparation yielded relatively large crystals on the target surface as well as regions without matrix or analyte. All measurements were performed in the reflector positive mode in the m/z range 0-1200 Da. The MS spectra were typically acquired by averaging 500 sub spectra from a total of 500 shots of the laser (Smartbeam 2. Version: 1_0_38.5) with laser power of 60-75 %.

2.6. Growth curves of Staphylococcus aureus treated with doxorubicin in liposomes

To determine the antimicrobial activity of doxorubicin encapsulated in liposomes and doxorubicin released from liposomes after the addition of SDS, the evaluation of antimicrobial effect of tested compounds on bacterial culture of *Staphylococcus aureus* was performed on Multiskan EX (Thermo Fisher Scientific, Germany). 24-hour grown bacterial culture was diluted with Luria Bertani medium (10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl; Sigma-Aldrich, St. Louis, MO, USA) in spectrophotometer Specord 210 (Analytik Jena, Germany) and the absorbance was measured at a wavelength of 600 nm to absorbance 0.1. This diluted culture was pipetted into the microplate in various combinations with tested samples or separately as a control measurement. The ratio of bacterial culture to tested sample was 5:1 (250 µl of bacterial culture and 50 µl of sample). The total volume in the microplate wells in microplate was therefore always 300 µl. Measurements were carried out at starting time 0, then at each half-hour intervals for 24 hours, at 37 °C and at wavelength of 600 nm. The achieved values were evaluated in a graphic form of growth curves for each variant individually.

Software STATISTICA (data analysis software system), version 10.0 (Tulsa, Oklahoma, USA) was used for data processing. Half-maximal concentrations (IC_{50}) were calculated from logarithmic regression of sigmoidal dose-response curve. General regression model was used to analyze differences between the combinations of compounds.

2.7. High performance liquid chromatography with electrochemical detection for determination of doxorubicin

The samples were analyzed using high performance liquid chromatography with electrochemical detection (HPLC-ED). HPLC system consisted of two solvent delivery pumps operating in the range of 0.001-9.999 $ml.min^{-1}$ (Model 582 ESA and Model 584 ESA; ESA Inc., Chelmsford, MA), with reversed-phase chromatographic colony Zorbax eclipse AAA C18 (150 × 4.6; 3.5 nm particles, Agilent Technologies, USA) and a Coulochem electrochemical detector. The electrochemical detector includes one low volume flow-through analytical cell (Model 5040, ESA, USA), which is consisted of glassy carbon working electrode, hydrogen-palladium electrode as reference electrode and auxiliary electrode, and Coulochem III as a control module. Both the detector and the reaction coil/column were thermostated.

The sample (20 µl) was injected using autosampler (Model 542 HPLC, ESA, USA). Samples were kept in the carousel at 8 °C during the analysis. The column was thermostated at 30 °C. The flow rate was 1 $ml.min^{-1}$. Mobile phase consisted of: (A) aqueous solution of 0.05 M Na_2HPO_4 with 0.05 % triethylamine (pH 4.6 was adjusted by citric acid) and (B) acetonitrile. Analysis time was 20 minutes. Samples were diluted 10 times prior to the analysis.

2.8. Releasing of doxorubicin from liposomes

All three variants of liposomes prepared according to the procedure above were divided into aliquots and used for preparing variants of opened liposomes. These were prepared by adding 30 mM SDS to the liposomes in volume ratio 1:1 and vortexed for several seconds.

2.8.1. Absorption and fluorescence spectra of doxorubicin in liposomes

Absorption and fluorescence spectra were measured by multifunctional microplate reader Tecan Infinite 200 PRO (TECAN, Switzerland). The absorption scan was measured within the range from 230 to 800 nm per 5 nm steps. The detector gain was set to 100. For fluorescence spectra measurement, 480 nm was used as an excitation wavelength and the fluorescence scan was measured within the range from 510 to 850 nm. The detector gain was set to 100, too.

Two sets of liposomes 8, 9 and 10 with concentration of doxorubicin 0, 25, 50 and 100 $\mu g.ml^{-1}$ were used. The first set contained liposomes with addition of ACS water in volume ratio 1:1, and the second set contained liposomes with addition of 30 mM SDS in volume ratio 1:1. Both tested sets were placed in UV-transparent 96 well microplate with flat bottom by CoStar (Corning, USA). To each well 50 µl of sample was pipetted. All measurements were performed at 30 °C controlled by Tecan Infinite 200 PRO (TECAN, Switzerland).

2.8.2. Flow injection analysis with electrochemical detection

Flow injection analysis system consisted of a chromatographic pump Model 584 ESA (ESA Inc., Chelmsford, MA) (working range 0.001-9.999 $ml.min^{-1}$) and of an electrochemical detector Coulochem (ESA, USA), to which the amperometric cell (model 5040, ESA, USA) was connected. The cell contained a working electrode made from glassy carbon. The 20 µl of sample was injected automatically by an autosampler (Model 542, ESA, USA). During the analysis the samples were stored in the carousel. Flow rate of a mobile phase was 1 $ml.min^{-1}$. Other conditions were optimized.

2.9. Descriptive statistics

Data were processed using MICROSOFT EXCEL® (USA) and STATISTICA.CZ Version 8.0 (Czech Republic). Results are expressed as mean ± standard deviation (S.D.) unless noted otherwise (EXCEL®). Statistical significances of the differences were determined using STATISTICA.CZ. Differences with $p < 0.05$ were considered significant and were determined by using of one way ANOVA test (particularly Scheffe test), which was applied for means comparison.

3. RESULTS AND DISCUSSION

We focused this study on comparing the properties of differently prepared liposomes with encapsulated doxorubicin. These liposomes differ in the amount of cholesterol contained in phospholipid bilayer (0, 50 and 100 mg of cholesterol used during the preparation) and this fact has a significant influence on physicochemical properties of liposomes [11]. Schemes of prepared liposomes are shown in Fig. 1A-C. Verification of different cholesterol concentrations in liposomes were determined spectrophotometrically with utilization of R1 reagent. In this way the applied amount of cholesterol was confirmed for all liposomes with each concentration of encapsulated doxorubicin (Fig. 1D).

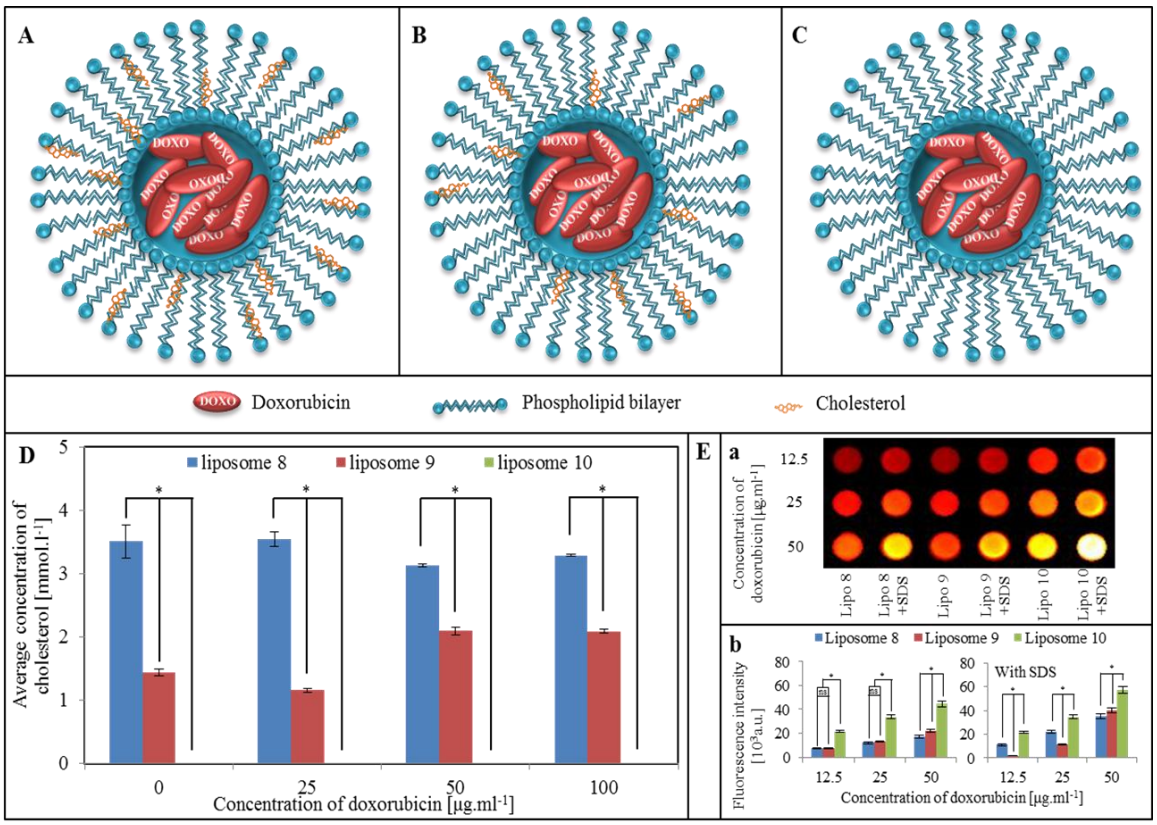

Figure 1. Schemes of studied liposomes, determination of cholesterol in liposomes on BS-400 device and measurements of doxorubicin's fluorescence in liposomes using In-vivo Xtreme device. Absorbance of cholesterol was measured for 6 minutes at 505 nm. For calculation of cholesterol concentration the absorbance values of R1 reagent and of the solution after 6 minutes incubation with sample were used. (**A**) Liposome 8; it contains 100 mg of cholesterol. (**B**) Liposome 9; it contains 50 mg of cholesterol. (**C**) Liposome 10; it doesn't contain cholesterol. (**D**) Concentrations of cholesterol determined in liposomes 8, 9 and 10 with different concentrations of encapsulated doxorubicin (0, 25, 50 and 100 µg.ml-1). (**E**) Fluorescence of liposomes with concentrations of doxorubicin diluted twice. 200 microliters of sample and water (or SDS 30 mM) at volume ratio 1:1 was pipetted on NuncTM 96-well microplate so final concentrations of doxorubicin were 12.5, 25 and 50 µg.ml^{-1}. Wavelength of excitation radiation was 600 nm. The fluorescence of liposomes without doxorubicin was within the range from 38 to 57 a.u. and so was insignificant. (*) Differences between measured values are statistically significant (at the significance level $\alpha = 0.05$). (ns) Not significant differences between measured values are statistically insignificant (at the significance level $\alpha = 0.05$).

The fluorescence intensity of doxorubicin encapsulated in liposomes was evaluated in terms of characterization of the liposome influence on the detection of doxorubicin. Fluorescence photographs (Fig. 1Ea) and dependence plotted in the graph (Fig. 1Eb) indicate an increased fluorescence of doxorubicin when using the liposome that doesn't contain a cholesterol. This suggests that cholesterol contained in phospholipid bilayer of liposomes reduces the fluorescent properties of doxorubicin. Same effect was also observed for doxorubicin released from liposome by addition of sodium dodecyl sulphate (SDS), when the doxorubicin from liposome 10 (doesn't contain cholesterol) still provided the highest fluorescence. This applies to all used concentrations of doxorubicin.

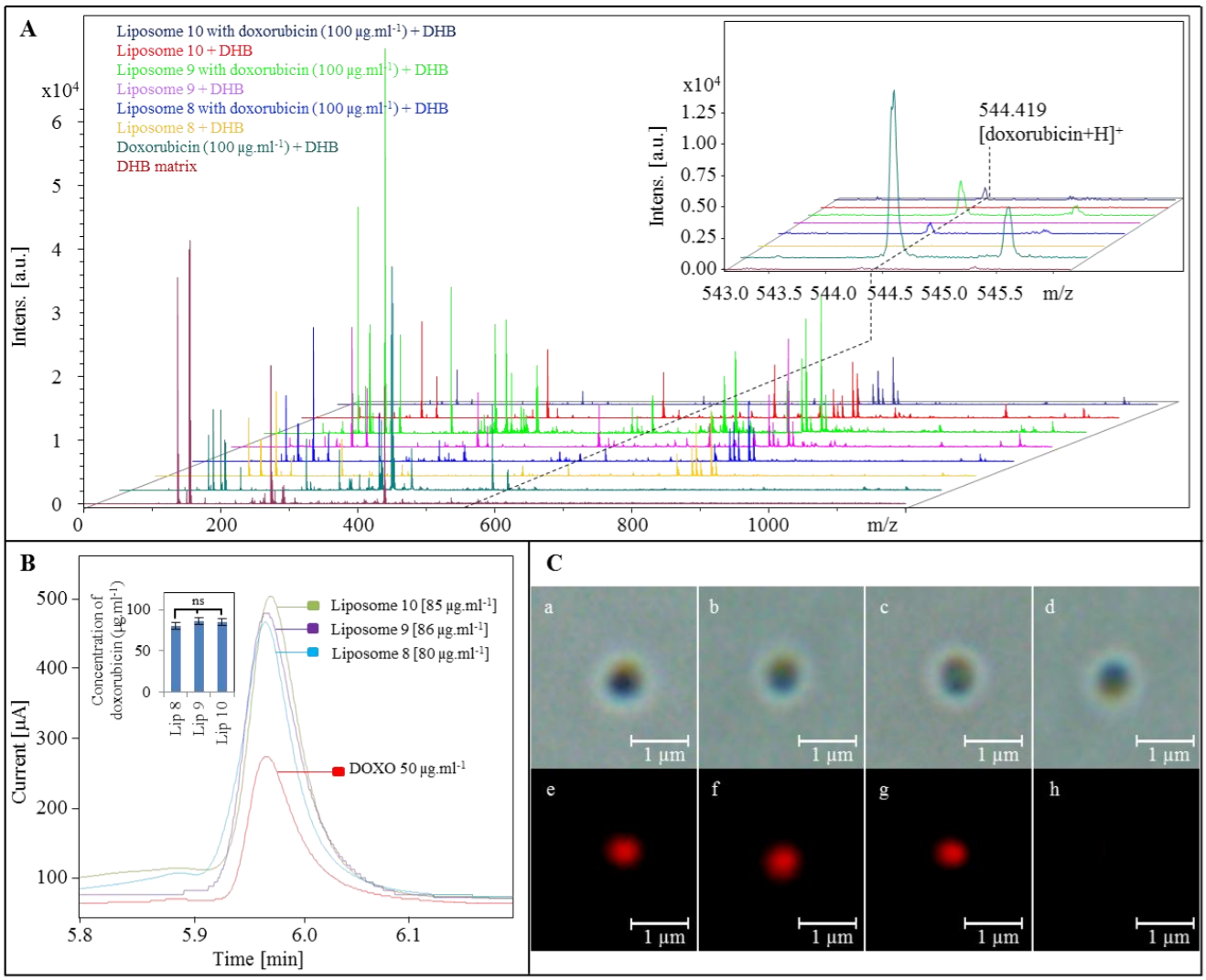

Figure 2. Liposomes with the highest loaded concentration of doxorubicin (100 µg.ml^{-1}) were analysed by mass spectrometry. (**A**) MALDI-TOF mass spectra of liposomes with and without encapsulated doxorubicin, and control spectra of free doxorubicin and DHB matrix. The detail of $[doxorubicin+H]^+$ peak of doxorubicin is presented in inset. (**B**) The total content of encapsulated doxorubicin was determined by HPLC-ED. The comparison of determined concentration of doxorubicin is shown in inset. (C) Microscopic photos of *Staphylococcus aureus* after the incubation of doxorubicin encapsulated in liposomes (Olympus IX71, Tokyo, Japan): **a, b, c, d**: ambient light; **e, f, g, h**: fluorescence photos (excitation: 520 -550 nm; emission: 580 nm); **a**, **e**: Liposome 8; **b**, **f**: Liposome 9; **c**, **g**: Liposome 10; **d**, **h**: *Staphylococcus aureus* without liposome (control sample).

For ensuring that doxorubicin is really encapsulated in liposomes we used several methods as matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF), high performance liquid chromatography with electrochemical detection (HPLC-ED) and fluorescence microscopy. It is obvious from the obtained mass spectra (Fig. 2A) that the presence of doxorubicin was confirmed. Furthermore, there are obvious changes between mass spectra of liposomes with or without cholesterol (386.6 Da). Spectra of liposomes 8 and 9 most likely contain fragments and adducts of cholesterol – peaks with m/z 352.9, 398.9 and 420.9 – which weren't observed in spectra of

liposome 10. After the confirmation of doxorubicin presence, the concentration of doxorubicin was determined by HPLC-ED (Fig. 2B), which was done for all variants of encapsulated doxorubicin with maximal loaded concentration 100 $\mu g.ml^{-1}$. Due to lower recovery, which is probably due to destruction of liposome capsules when it comes to the chromatography system using reverse phase column and high pressure, it provides the determined concentrations within the range of 80 - 86 $\mu g.ml^{-1}$ without significant differences between applied concentrations of cholesterol. Besides the quantitative ability of liposomes to encapsulate applied substance was shown, the visual confirmation of the ability of all three different liposomes to carry the target substance into cells was verified by fluorescence microscopy (Fig. 2C). The results show that all applied liposomes were able to carry encapsulated doxorubicin into cells.

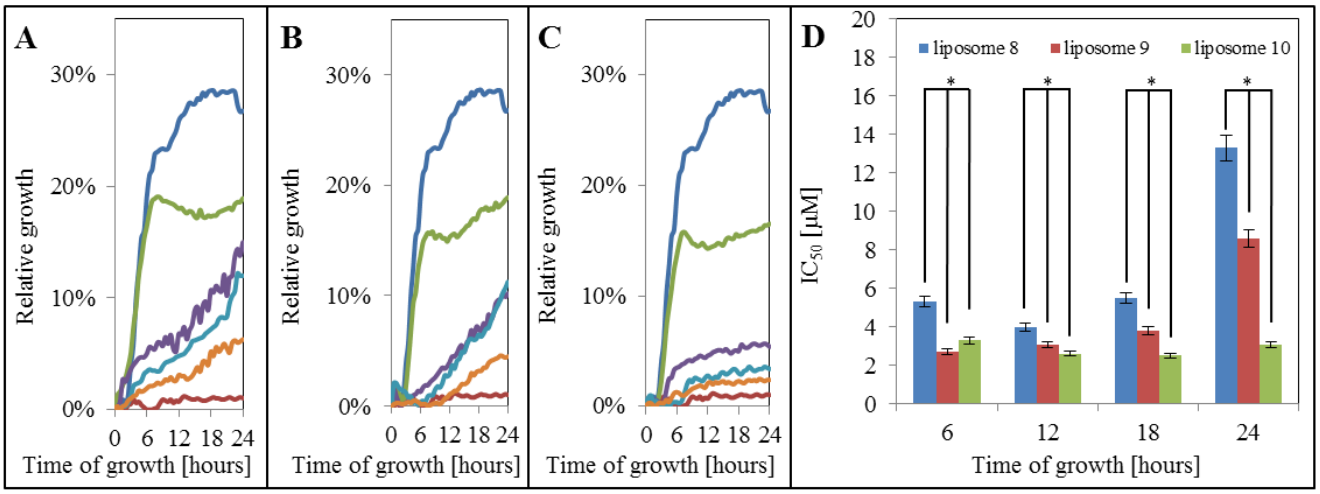

Figure 3. Growth curves, evaluation of antimicrobial effect of doxorubicin encapsulated in liposomes. The effect of substance was tested on bacterial culture of *Staphylococcus aureus* (S.a.) using Multiskan EX device. Measurements were carried out at 30 minutes intervals at 37 °C and at 620 nm. All graphs contain growth curves for doxorubicin (100 $\mu g.ml^{-1}$, red curve) and *S.a.* (dark blue curve). (**A**) Growth curves of bacterial culture treated with doxorubicin encapsulated in liposome 8. (**B**) Growth curves of bacterial culture treated with doxorubicin encapsulated in liposome 9. (**C**) Growth curves of bacterial culture treated with doxorubicin in liposome 10. (**D**) IC_{50} values (μM) for doxorubicin encapsulated in liposomes. Concentrations of doxorubicin in samples were 0 (olive green curve), 12.5 (purple curve), 25 (azure curve) and 50 (orange curve) $\mu g.ml^{-1}$ – it's 0, 23, 46 and 92 μM after conversion. (*) Differences between measured values are statistically significant (at the significance level $\alpha = 0.05$).

Characterization of doxorubicin encapsulated in liposome also included determination of growth curves with utilization of *Staphylococcus aureus* culture and determination of half maximal inhibitory concentration IC_{50} (represents the concentration of a drug that is required for 50% inhibition of *Staphylococcus aureus in vitro*) (Fig. 3). The influence of doxorubicin on the growth of *Staphylococcus aureus* was assessed only for liposomal doxorubicin and for doxorubicin itself because 15 mM concentration of SDS after addition to liposomes was inhibitory for the used bacterial culture. The highest toxicity for bacterial cells showed liposome 10 in the case of all evaluated times (6, 12, 18

and 24 hours) and the IC_{50} was within the range from 2.5 to 3.3 µM. It means that the concentration of encapsulated doxorubicin didn't have a big impact on IC_{50}. In contrast, both variants of liposomes with cholesterol showed dependence of toxicity on the concentration of cholesterol. For liposome 8 the IC_{50} was 13.3 µM at 24 hours and for liposome 9 it was 8.6 µM. This is in correlation with behaviour of cholesterol in phospholipid bilayer, in which it was described that cholesterol strengthens the bilayer and decreases bilayer's permeability [11]. The increased IC_{50} can also be explained by the positive influence of cholesterol on the growth of *Staphylococcus aureus*. Stimulation effect of lower cholesterol concentrations on microorganisms was also previously published [23,24].

3.1. Optimization of FIA-ED conditions for detection of doxorubicin releasing

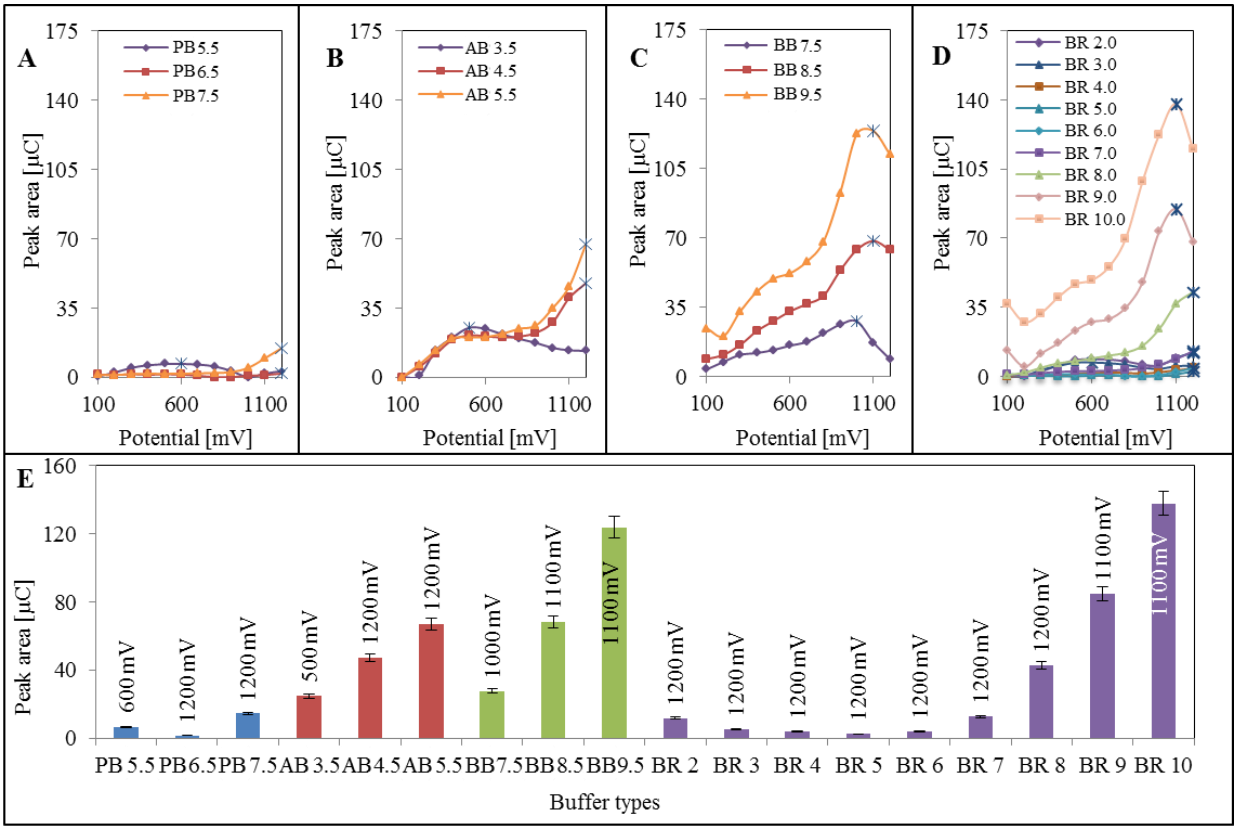

Figure 4. Analysis of doxorubicin (50 $mg.ml^{-1}$) in different buffers by FIA-ED. The buffer, which was used for dilution of doxorubicin aliquot, was used also as the mobile phase. The potential range was from 100 to 1200 mV with 100 mV step. Blue marks in graphs represent maximal measured values. (**A**) Phosphate buffer (PB) with pH 5.5, 6.5 and 7.5. (**B**) Acetate buffer (AB) with pH 3.5, 4.5 and 5.5. (**C**) Borate buffer (BB) with pH 7.5, 8.5 and 9.5. (**D**) Britton-Robinson buffer (BR) with pH 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0. (**E**) The highest detector responses considering different buffers and applied potential.

After that we found the negative effect of all three prepared liposomes on the target bacterial culture, we decided to study way how to open doxorubicin externally. For doxorubicin determination, flow injection analysis (FIA-ED) with electrochemical detection was optimized. Before that we made necessary optimization of different buffers used for doxorubicin detection because it is crucial for this

type of detection [25]. Standard solution of doxorubicin was always diluted to 50 $\mu g.ml^{-1}$ with a buffer, which was also used as mobile phase in FIA-ED. Each buffer was used in its natural buffering range: Britton-Robinson buffer (pH 2, 3, 4, 5, 6, 7, 8, 9, 10), acetate buffer (pH 3.5, 4.5, 5.5), phosphate buffer (pH 5.5, 6.5, 7.5) and borate buffer (pH 7.5, 8.5, 9.5). In each buffer with each pH the hydrodynamic voltammograms (HDVs) were measured with a 100 mV step within the potential range from 100 to 1200 mV (Figs. 4A, B, C, and D).

The largest peak area was achieved using Britton-Robinson buffer at pH 10. With decreasing pH the peak area of doxorubicin measured by FIA-ED was also decreasing. This effect of pH was surprising because with other types of electrochemical detection the low pH is preferably used [26-29]. For similar types of detection in HPLC the lower pH is used as well. Often a phosphate buffer with addition of triethylamine is used at pH lower than 5 [29-31]. These conditions are mainly used for separation but the signal of detector is distinctly lower than with use of our optimized conditions of Britton-Robinson buffer at pH 10 (Fig. 4E).

3.2. Electrochemical monitoring of doxorubicin releasing

According to the results obtained above the electrochemical characterization of all liposomes was performed on glassy carbon electrode under the optimized conditions. Differential HDVs of all samples, including samples with added 30 mM SDS, are shown in Fig. 5. Measurements were performed within the potential range from 100 to 1000 mV with a 100 mV step. Differential HDV's curves were obtained by subtracting the peak area of blank samples (liposomes without doxorubicin) from the peak area of doxorubicin encapsulated in liposomes and are shown in Figs. 5A, B and C for tested liposomes with doxorubicin only and in Figs. 5E, F and G for liposomes with doxorubicin affected by SDS.

In Figs. 5D and H there are shown the maximal differences of peak area at 900 mV potential, which provided the highest response of detector. The statistically significant differences were acquired between individual liposomes with the same concentration of doxorubicin, including those liposomes with addition of SDS. The biggest difference showed liposome 9 with all concentrations of doxorubicin. It is interesting that liposome 8 with higher concentration of cholesterol showed smaller difference of peak areas than liposome 9. Cholesterol probably has a role in the improvement of electrochemical detection of encapsulated doxorubicin, but this improvement has a limitation factor in concentration of cholesterol (critical concentration). It is possible that cholesterol enhances the electron transfer at the applied conditions, but further experiments are necessary to prove it. In Figs. 5E, F, G and H the electrochemical detection was influenced by the addition of SDS in the way that increased differences were obtained in the case of the highest applied concentration of doxorubicin. A significant increase from 13.2 to 29.4 μCin maximal difference of peak areas was determined at liposome 10. In contrast, liposomes with cholesterol provided decreased differences of peak areas and thus the detection was deteriorated. Decreasing trend is in correlation with a concentration of cholesterol in phospholipid bilayer of liposomes.

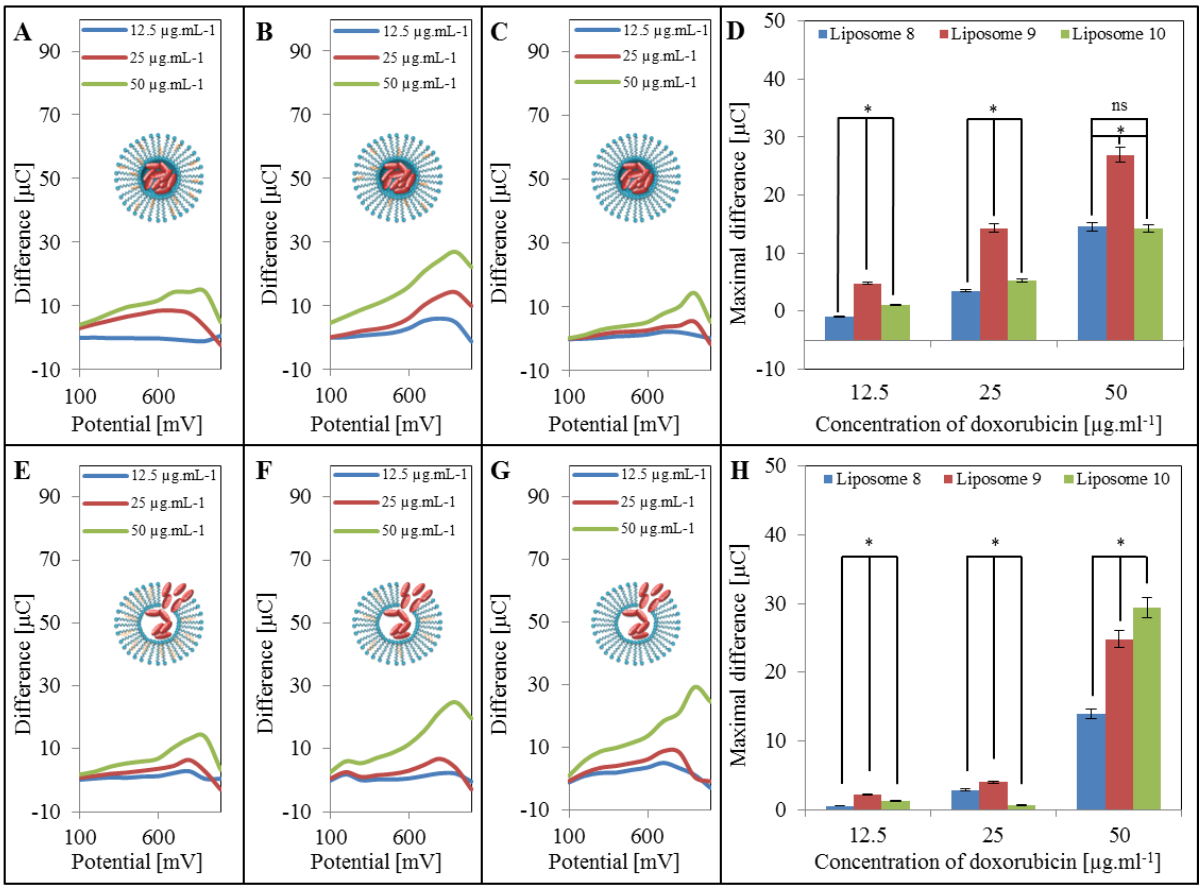

Figure 5. Differential hydrodynamic voltammograms (HDV) of doxorubicin encapsulated in liposomes and of doxorubicin encapsulated in liposomes after the addition of SDS. Differential curves were obtained by subtracting the values of blank samples from the values of samples with doxorubicin. The analysis was performed with FIA-ED. All measurements were done within the range from 100 to 1000 mV with 100 mV step. Samples were prepared in Britton-Robinson buffer with pH 10.0. (**A**)-(**D**) Concentrations of doxorubicin in liposomes were 12.5, 25 and 50 µg.ml^{-1}. (**E**)-(**H**) 30 mM SDS was added to the liposomes with doxorubicin in volume ratio 1:1, thus the final concentrations were 12.5, 25 and 50 µg.ml^{-1}. (**A**), (**E**) Liposome 8. (**B**), (**F**) Liposome 9. (**C**), (**G**) Liposome 10. (**D**), (**H**) The comparison of maximal differences from differential HDVs. (*) Differences between measured values are statistically significant (at the significance level $\alpha = 0.05$). (ns) Not significant differences between measured values are statistically insignificant (at the significance level $\alpha = 0.05$).

3.3. Fluorescence monitoring of doxorubicin releasing

In another part of our study we focused on the effect of liposomes on optical properties of doxorubicin. Firstly, the absorption spectra were determined (insets in Fig. 6A, B, C, E, F and G). It clearly follows from the results obtained that absorption spectra of doxorubicin encapsulated in liposomes were minimally affected. In contrast, the fluorescence spectra (λ_{ex} = 480 nm) show that with the increasing concentration of doxorubicin the fluorescence of each liposome was also increasing (Fig. 6D).

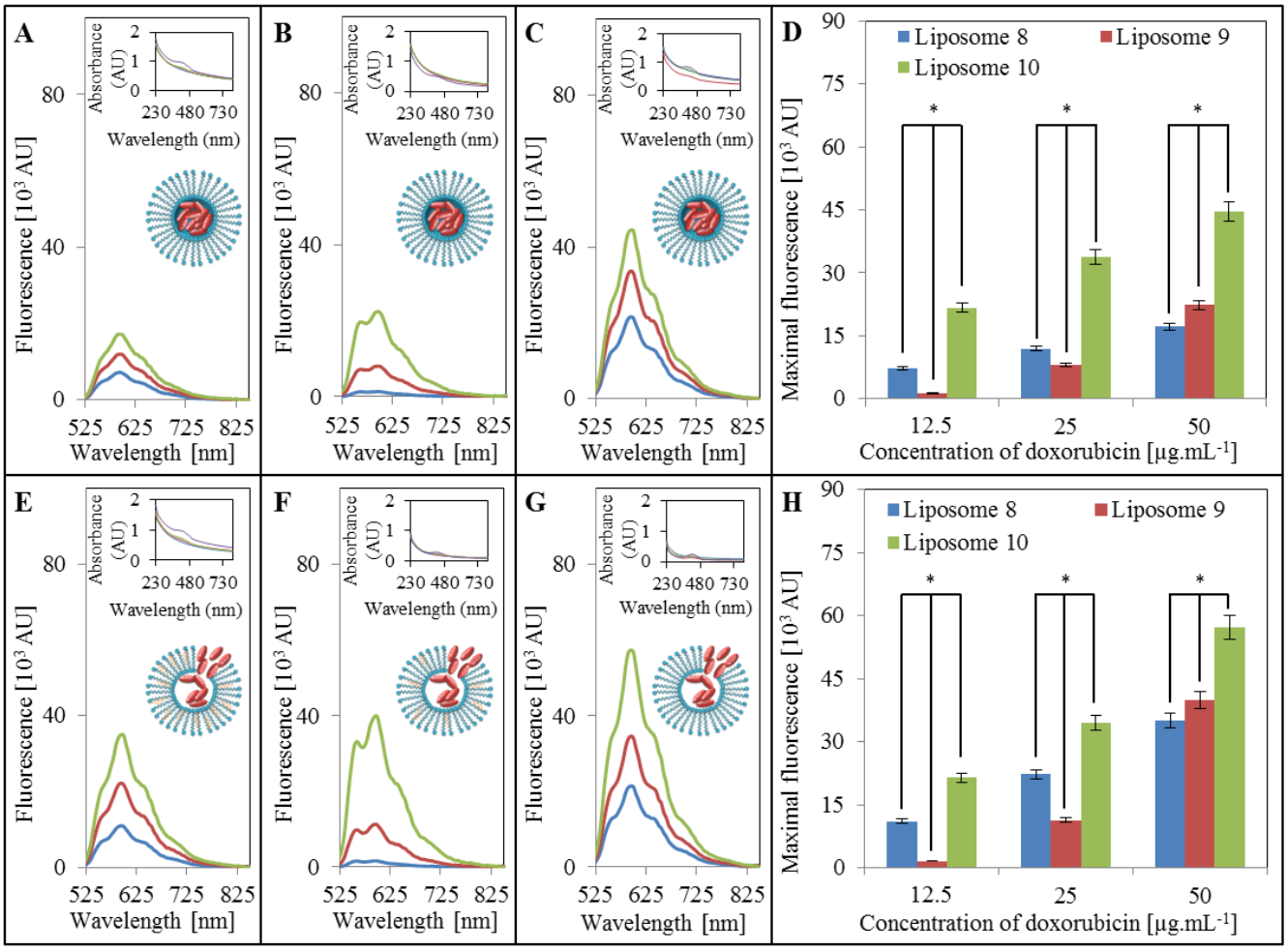

Figure 6. Differential fluorescence spectra (λ_{ex} = 480 nm) and absorption spectra (inserted graphs) of liposomes 8, 9 and 10 with doxorubicin measured on Tecan Infinite 200 PRO. Differential spectra were obtained by subtracting the values of blank samples from the values of samples with doxorubicin. Samples were pipetted into the UV-transparent 96-well plate with a flat bottom (CoStar; Corning, USA). 50 µl of sample was always pipetted. Fluorescence spectra were measured within the range of 510-850 nm with 5 nm steps. Absorption spectra were measured in the range of 230-800 nm. (**A**)-(**D**) The samples of liposomes were diluted with ACS water in volume ratio 1:1. (**E**)-(**H**) 30 mM SDS was added to the samples of liposomes in volume ratio 1:1 in order to unfold liposomes. (**A**), (**E**) Liposome 8. (**B**), (**F**) Liposome 9. (**C**), (**G**) Liposome 10. Concentrations of doxorubicin in the samples after the dilution were 12.5 (blue curve), 25 (red curve) and 50 (olive green curve) µg.ml^{-1}. (**D**), (**H**) Graphs show a maximal fluorescence for the samples after the subtraction of blank samples. (*) Differences between measured values are statistically significant (at the significance level $\alpha = 0.05$).

The increased fluorescence of all liposomes with addition of 30 mM SDS confirms that liposomes were opened and doxorubicin was released into the solution. It confirms the function of SDS as the substance that is commonly used for releasing encapsulated substance from liposome [11,32,33]. The highest fluorescence from all liposomes was observed in liposome 10 either with encapsulated doxorubicin or with doxorubicin released into the solution (Fig. 6H). Liposomes themselves show negligible fluorescence, thus, it is obvious that these results show changes of

fluorescence on the basis of a composition of phospholipid bilayer, especially on the concentration of cholesterol, eventually of its distraction.

4. CONCLUSIONS

The composition of phospholipid bilayer significantly affects the physicochemical properties of a liposome and allows the use of liposomes for therapeutic purposes as for analytical purposes. One of the most used components of phospholipid bilayer is cholesterol; especially its concentration can significantly affect the properties of liposomes. In this study, we attempted to characterize the toxicity, electrochemical properties and fluorescence of doxorubicin encapsulated in liposomes with different concentrations of cholesterol in phospholipid bilayer. We also studied the influence of sodium dodecyl sulphate (SDS) addition to the liposomes, because SDS served to open liposomes and release the drug into the solution and found that various composition of liposome strongly influenced the releasing of doxorubicin, which is of high importance for further drug delivery studies.

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References

1. A. D. Bangham and R. W. Horne, *J. Mol. Biol.*, 8 (1964) 660.
2. R. R. Hood, C. R. Shao, D. M. Omiatek, W. N. Vreeland and D. L. DeVoe, *Pharm. Res.*, 30 (2013) 1597.
3. K. Maruyama, *Adv. Drug Deliv. Rev.*, 63 (2011) 161.
4. K. Hayashi, T. Tatsui, T. Shimanouchi and H. Umakoshi, *Colloid Surf. B-Biointerfaces*, 106 (2013) 258.
5. T. Nguyen, J. Ostergaard, S. Sturup and B. Gammelgaard, *Int. J. Pharm.*, 449 (2013) 95.
6. T. L. Andresen, S. S. Jensen and K. Jorgensen, *Prog. Lipid Res.*, 44 (2005) 68.
7. Y. Komizu, Y. Matsumoto and R. Ueoka, *Bioorg. Med. Chem. Lett.*, 16 (2006) 6131.
8. T. J. Evjen, S. Hupfeld, S. Barnert, S. Fossheim, R. Schubert and M. Brandl, *J. Pharm. Biomed. Anal.*, 78-79 (2013) 118.
9. Y. Omokawa, T. Miyazaki, P. Walde, K. Akiyama, T. Sugahara, S. Masuda, A. Inada, Y. Ohnishi, T. Saeki and K. Kato, *Int. J. Pharm.*, 389 (2010) 157.
10. T. M. Allen and C. Hansen, *Biochim. Biophys. Acta*, 1068 (1991) 133.
11. H. Ohvo-Rekila, B. Ramstedt, P. Leppimaki and J. P. Slotte, *Prog. Lipid Res.*, 41 (2002) 66.
12. Y. Kuang, J. Liu, Z. L. Liu and R. X. Zhuo, *Biomaterials*, 33 (2012) 1596.
13. L. Y. Qiu, M. F. Yao, M. H. Gao and Q. H. Zhao, *J. Liposome Res.*, 22 (2012) 245.
14. L. Hosta-Rigau, Y. Zhang, B. M. Teo, A. Postma and B. Stadler, *Nanoscale*, 5 (2013) 89.
15. C. Zhang, J. Li, L. Xu and Z. G. Shi, *J. Chromatogr. A*, 1233 (2012) 78.
16. S. W. Wang, C. Wang, X. Zhao, S. L. Mao, Y. T. Wu and G. R. Fan, *Anal. Chim. Acta*, 713 (2012) 121.
17. J. Mei, Y. P. Tian, W. He, Y. X. Xiao, J. A. Wei and Y. Q. Feng, *J. Chromatogr. A*, 1217 (2010) 6979.
18. D. Corradini, G. Mancini and C. Bello, *Chromatographia*, 60 (2004) S125.

19. K. P. McNamara and Z. Rosenzweig, *Anal. Chem.*, 70 (1998) 4853.
20. A. Gomez-Hens and J. M. Fernandez-Romero, *TRAC-Trends Anal. Chem.*, 24 (2005) 9.
21. Y. Kumada, K. Tomioka and S. Katoh, *Kag. Kog. Ronbunshu*, 28 (2002) 77.
22. Y. Kumada, K. Tomioka and S. Katoh, *J. Chem. Eng. Jpn.*, 34 (2001) 943.
23. W. E. Shine, R. Silvany and J. P. McCulley, *Invest. Ophthalmol. Vis. Sci.*, 34 (1993) 2291.
24. D. T. Clark and M. Soory, *Steroids*, 71 (2006) 352.
25. M. A. M. Rodrigo, O. Zitka, M. Kominkova, V. Adam, M. Beklova and R. Kizek, *Int. J. Electrochem. Sci.*, 8 (2013) 4409.
26. D. Hynek, L. Krejcova, O. Zitka, V. Adam, L. Trnkova, J. Sochor, M. Stiborova, T. Eckschlager, J. Hubalek and R. Kizek, *Int. J. Electrochem. Sci.*, 7 (2012) 13.
27. Y. J. Guo, Y. H. Chen, Q. Zhao, S. M. Shuang and C. Dong, *Electroanalysis*, 23 (2011) 2400.
28. D. Nieciecka and P. Krysinski, *Langmuir*, 27 (2011) 1100.
29. Z. Jemelkova, J. Zima and J. Barek, *Collect. Czech. Chem. Commun.*, 74 (2009) 1503.
30. P. M. Loadman and C. R. Calabrese, *J. Chromatogr. B*, 764 (2001) 193.
31. G. Nicholls, B. J. Clark and J. E. Brown, *J. Pharm. Biomed. Anal.*, 10 (1992) 949.
32. M. Cocera, O. Lopez, L. Coderch, J. L. Parra and A. de la Maza, *Colloid Surf. A-Physicochem. Eng. Asp.*, 221 (2003) 9.
33. A. M. Tan, A. Ziegler, B. Steinbauer and J. Seelig, *Biophys. J.*, 83 (2002) 1547.