LIPOSOMES AS DRUG CARRIERS AND THEIR CHARACTERIZATION USING DIFFERENT ANALYTICAL METHODS

Guráň R.[1, 2], Komínková M.[2], Kopel P.[2, 3], Chudobová D.[2], Zítka O.[2, 3], Adam V.[2, 3], Kizek R.[2, 3]

[1]Department of Chemistry, Faculty of Science, Masaryk University, Kotlarska 2, 611 37 Brno, Czech Republic

[2]Department of Chemistry and Biochemistry, Faculty of Agronomy, Mendel University in Brno, Zemedelska 1, 613 00 Brno, Czech Republic

[3]Central European Institute of Technology, Brno University of Technology, Technicka 3058/10, 616 00 Brno, Czech Republic

E-mail: r.guran@email.cz, kizek@sci.muni.cz

ABSTRACT

The physicochemical properties of liposomes are significantly affected by the composition of phospholipid bilayer; differences in composition allow the use of liposomes for analytical purposes and for therapeutic purposes. One of the most used components of phospholipid bilayer is cholesterol. Its concentration plays a significant role in the behaviour of liposomes.

This study points to changes in the properties of liposomes and its influence on encapsulated doxorubicin according to the content of cholesterol in the phospholipid bilayer. The influence of SDS addition to liposomal variants was also evaluated. Three variants of liposomes differing in various concentrations of cholesterol were assessed.

Firstly, the toxicity of all types of liposomal doxorubicin was evaluated and it was found that the content of cholesterol increases the IC50 values of encapsulated doxorubicin in liposome. The highest concentration of cholesterol in liposome increased the IC50 value even four times compared to liposomes without cholesterol.

Secondly, the new approach to compare the influence of different variants of liposomes on detection of carried doxorubicin was used using the electrochemical detection with construction of differential hydrodynamic voltammograms.

Key words: liposome, cholesterol, doxorubicin, sodium dodecyl sulphate

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INTRODUCTION

Since the discovery in the 1960s [1], the phospholipidic structures have been studied for their potential to serve as the carriers for drug transportation. Liposomes are particles with lipid bilayer enclosing a vesicular space, wearing a number of attractive properties including the ability to encapsulate aqueous solutions within the liposome core, segregate lipophilic compounds within the bilayer and support tailored surface chemistries of the liposomes for targeted delivery [2]. Nowadays, pegylated liposomes with immobilized polyethylene glycol on their surface are the most common drug carriers [3]. By using liposomes it is possible to achieve prolonged persistence of the drug in the body and to reduce the degradation of drug in the liver; this leads to reduction of negative effects of drug while the anticancer efficacy is maintained [4]. The ability of liposomes to aggregate, open and release the drug at the targeted place is crucial for their use in therapy, but the mechanism hasn't been completely resolved yet [5]. The release of the drug from liposomes is usually based on the fusion with the membrane, but other options can be used, e.g. sonication [6]. The cholesterol present in the structure of liposomes supports their stability and enables the control of permeability and solubility of the liposome membrane; it also gives them a similarity to natural cell membranes [7]. We have focused on different properties of synthesized liposomes, which differed in the content of cholesterol in the lipid bilayer, and on the possibilities of opening these liposomes after the addition of sodium dodecyl sulphate (SDS).

MATERIAL AND METHODS

Preparation of liposomes

LIP-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. LIP-9: was prepared in the same way as LIP-8, but with 50 mg of cholesterol and 3.75 ml of chloroform.

LIP-10: was prepared as LIP-9 but without the cholesterol.

Preparation of encapsulated doxorubicin

Solutions containing 25, 12.5, 6.25 and 0 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). Samples were then washed several times with Britton-Robinson buffer (pH = 10) on Amicon 3k (Millipore). Final volume of samples was 0.5 ml.

The opening of liposome

Aliquots of prepared liposomes with doxorubicin were mixed with 30 mM SDS in volume ratio 1:1 and vortexed for several seconds.

Growth curves of doxorubicin in liposomes

The antimicrobial activity of doxorubicin encapsulated in liposomes was determined by the evaluation of antimicrobial effect of tested compounds on bacterial culture of Staphylococcus aureus and it was performed on Multiskan EX (Thermo Fisher Scientific, Germany). The subsequent evaluation in the form of so-called growth curves was made. 24-hour grown bacterial culture was diluted with LB medium in spectrophotometer Specord 210 (Analytik Jena, Germany) at a wavelength of 600 nm to the absorbance of 0.1 AU. 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). Measurements were carried out at starting time 0, then at each half-hour intervals for 24 hours, at 37 °C and at wavelength of 620 nm. The achieved values were evaluated in a graphic form of growth curves for each variant individually.

FIA-ED analysis

FIA 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 III (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}.

RESULTS AND DISCUSSION

Growth curves and IC_{50} determination

Characterization of doxorubicin encapsulated in liposome included growth curves of *Staphylococcus aureus* culture and determination of IC_{50} (*Fig. 1*). The impact 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 used bacterial culture. The highest toxicity for bacterial cells had liposome 10 – for all evaluated times (6, 12, 18 and 24 hours), the IC_{50} was in the range 2.5 – 3.3 µM. Therefore, the concentration of encapsulated doxorubicin didn't affect IC_{50} significantly. On the contrary, the toxicity of both variants of liposomes with cholesterol was dependent on the concentration of cholesterol. For liposome 8, the IC_{50} at 24 hours was 13.3 µM, and for liposome 9, it was 8.6 µM. This is in correlation with behaviour of cholesterol in phospholipid bilayer; it was described that cholesterol strengthens the bilayer and decreases bilayer's permeability [7]. Increased IC_{50} can be also explained by the positive influence of cholesterol on the growth of *Staphylococcus aureus*. Stimulation effect of lower cholesterol concentrations on microorganisms' growth was described in literature [8].

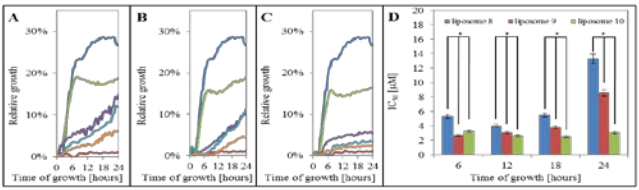

Fig. 1: *Growth curves. Measurements were carried out at 30 minutes intervals at 37 °C and at 620 nm. All graphs contain growth curves for doxorubicin (100 µg.ml^{-1}, red curve) and Staphylococcus aureus (S.a.) (dark blue curve). (**A**) Growth curves of doxorubicin encapsulated in liposome 8. (**B**) Growth curves of doxorubicin encapsulated in liposome 9. (**C**) Growth curves of 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) µg.ml^{-1} – it's 0, 23, 46 and 92 µM after conversion. (*) Differences between measured values are statistically significant (at the significance level α = 0.05).*

Optimization of FIA-ED conditions

Flow injection analysis with electrochemical detection (FIA-ED) was used for analysis of doxorubicin and subsequently for analysis of its release from liposomes. The effect of different buffers on detection of doxorubicin itself was evaluated before the carrying out this kind of analysis. Standard solution of doxorubicin was always diluted to 50 $\mu g.ml^{-1}$ concentration using 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). The largest peak area was achieved using the highest pH – a Britton-Robinson buffer at pH 10 (*Fig. 2D*). With decreasing pH the peak area of doxorubicin was also decreasing. This effect of pH was surprising because with other types of electrochemical detection the lower pH is usually used [9]. For similar types of detection (in HPLC), the lower pH is also used. Often a phosphate buffer with addition of triethylamine is used at pH lower than 5 [10].

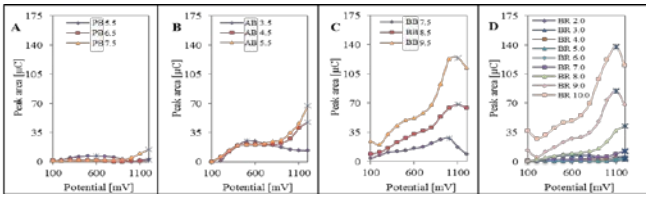

Fig. 2: *FIA-ED analysis of doxorubicin (50 mg.ml^{-1}) in different buffers. The buffer used for dilution of doxorubicin's aliquot was also used as a mobile phase. The potential range was 100-1200 mV with 100-mV step. Blue marks in graphs represent maximal measured values.* ***(A)*** *Phosphate buffer (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.*

Electrochemical monitoring of doxorubicin releasing

The electrochemical characterization of liposomes was performed under optimized conditions according to the results obtained above. Differential HDVs of all samples are shown in *Fig. 3*. Differential HDV's curves were obtained by subtracting the peak areas of blank samples (liposomes without doxorubicin) from the peak areas of doxorubicin encapsulated in liposomes. In *Fig. 3D, 3H* are shown the maximal differences of peak areas at 900 mV potential, which provided the highest response of detector. In *Fig. 3A, 3B, 3C and 3D* the biggest differences showed liposome 9 with all concentrations of doxorubicin. It's interesting, that liposome 8 with higher concentration of cholesterol showed smaller difference of peak areas than liposome 9. Cholesterol has probably a role in the improvement of electrochemical detection of encapsulated doxorubicin, but this improvement has a limitation factor in the concentration of cholesterol (critical concentration). It's possible, that cholesterol enhances the electron transfer at the applied conditions, but further experiments are necessary to prove it. In *Fig. 3E, 3F, 3G and 3H* 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 μC in maximal difference of peak areas occurred 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.

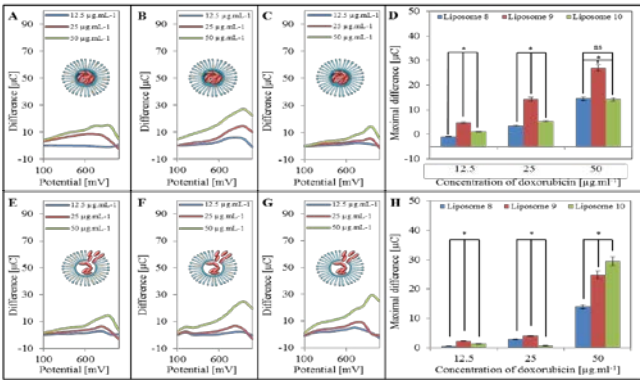

Fig. 3: *Differential hydrodynamic voltammograms (HDV) of doxorubicin encapsulated in liposomes and after the addition of SDS. The analysis was performed on FIA-ED. All measurements were in the range 100-1000 mV with 100 mV steps. Samples were prepared in Britton-Robinson buffer with pH 10.0.* ***(A)-(D)*** *Concentrations of doxorubicin in liposomes were 12.5, 25 and 50 μg.ml*$^{-1}$*.* ***(E)-(H)*** *30 mM SDS has been added to the liposomes with doxorubicin in volume ratio 1:1, so 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 α = 0.05).* ***(ns)*** *Not significant – differences between measured values are statistically insignificant (at the significance level α = 0.05).*

CONCLUSION

We have characterized the toxicity and electrochemical properties of doxorubicin encapsulated in various types of liposomes differing in the content of cholesterol in their phospholipid bilayer. It was found that the toxicity of liposomal doxorubicin is very dependent on the concentration of cholesterol in liposomes' bilayers – the IC_{50} values at 24 hours were increased even nearly four times when comparing liposome 8 (has the highest amount of cholesterol) with liposome 10 (without cholesterol). Cholesterol also influenced the electrochemical properties of liposomes in the way that it probably enhanced the electron transfer in phospholipid bilayer, but this enhancement has a limitation factor in concentration of cholesterol – a liposome 9, with two times lower concentration of cholesterol than was in a liposome 8, showed the highest difference of peak areas in hydrodynamic voltammograms. After opening of liposomes with sodium dodecyl sulphate, the increased maximal differences of peak areas in hydrodynamic voltammograms occurred at liposomes with the highest amount of doxorubicin. Liposomes with cholesterol showed decreased signal. These findings are important for our future research of liposomes as drug carriers.

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