Summary of PhD thesis titled: "Examination of the structural and permeability properties of liposomes stabilized by neutral polymers"

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In the recent decades liposomes became popular among different colloidal drug carriers and many studies prove that therapy carried on with liposome-associated drugs involves higher efficacy with reduced side effects. One of the most intensively examined systems used for medical applications are the liposomes containing anticancer agents which usually placed into the organism by the form of injection or infusion. The usage of conventional liposomes is limited for this purpose because these vesicles are removed from the bloodstream by the mononuclear phagocyting system (MPS) in short time after injected intravenously. Many attempts have been made to avoid the MPS-trapping of liposomes and to achieve longer half-lives in the bloodstream by modification of the liposomal surface. Successful results have been obtained by the modification of liposomes with poly(ethylene glycol) (PEG) derivatives. These molecules were covalently grafted onto the surface of the liposomes, and provided higher stability and a prolonged duration of circulation time for the vesicles examined. The PEG chains are believed to prevent or diminish the adsorption of opsonizing proteins which direct liposomes to macrophages, as a result of their conformational flexibility and water-binding ability.

Apart from the PEG-derivatives there are only a few polymer compounds known which were attached covalently or similarly bounded by physical adsorption to the liposomal surface. It can be explained by two major reasons why we need for such researches.

a, Primarily; however PEG protects liposomes from MPS it may also inhibit their interaction with the biological milieu at the site of action. For example: if we covered liposomes with biodegradable polymers adsorbed physically it might be easier to transport active agents into cells in pathological tissues.

b, Secondly; this is a more simple and cheap way to prepare steric stabilized vesicles compared to the production of peggylated phospholipids.

In the knowledge of these preliminaries my aim was the detailed investigation of the liposome-polymer interactions in model systems composed of three liposome types: **1.** soy lecithin phospholipid (SLPL) / Chol / dicetyl phosphate (DCP) = 25 : 3 : 2, **2.** DPPC / Chol / DCP = 4 : 1 : 1, **3.** DPPC / Chol = 2 : 1, and neutral polymers: poly (vinyl alcohol) (PVA), poly (vinyl pyrrolidone) (PVP), dextrane.

Results obtained by different measurement techniques are summarized as follows.

1. Description of the system composed of SLPL / Chol / DCP

1.1. Liposomal systems were prepared by lipid film-hydration (Bangham method). SLPL / Chol / DCP liposomes were downsized by sonication in bath sonicator. The checking of the average vesicle size diameter of the dispersions was carried out by dynamic laser light scattering (DLS) measurements. Average vesicle size diameters changed between 148 ± 10 and 150 ± 14 nm in the dispersions prepared by different buffer systems with the pH value of 7.4 (phosphate, 4-(2-hydroxyethyl) piperazine-1- ethanesulfonic acid (Hepes)) after preparations. This value was 164 ± 13 nm at liposomes dispersed in 0.1 M ammonium sulphate and intended to model-drug release experiments.

1.2. After preparation and downsizing I determined the surface charge density of the vesicles by the measurement of the streaming potential which is particular to the kinetic stability of the dispersions but influences the adsorption of polymers too. It is 0.42 mEq / g for the SLPL / Chol / DCP and this relatively big negative charge derives partly from the DCP (6.7 mol/mol %) and partly from the components of soy lecithin: phospholipids (85 w/w %), glycolipids, sterols, tocopherols, and carbohydrates which wear negative charge in unknown rate.

1.3. Results of the interactions of SLPL / Chol / DCP liposomes and PVA are as follows:

- The increasing of the vesicle diameter (max. 23.5 nm) is well-detected by DLS, because the adsorption layer formed on the surface of the vesicles is the thickest at this system.
- At the determination of the adsorption isotherms it was also experienced that the rate of adsorption is the biggest here with the maximum value of 97.5 mg / g.
  The primary reason of the phenomena that SLPLs which mostly contain unsaturated fatty acid esters form liquid crystalline phased ($L_{\alpha'}$) membrane at the temperature of the measurement (25 ± 0.1 °C). In one hand SLPL-liposomes fix PVA on the surface with hydrogen bridges and in the other hand the polymer has the possibility to penetrate into the membrane because of its loose structure.
- This theory was justified by the changes in the adsorption enthalpies obtained by titration microcalorimetry (25 ± 0.001 °C): at the beginning stage of adsorption the interaction is exothermic which can be explained by the PVA-liposome hydrogen bonds formed on the surface. If the concentration of polymer is increased endothermic heat effects are obtained which reveal that PVA penetrates between the alkyl chains of the membrane composing lipids.
- Films prepared from PVA / liposome systems with different mass ratio were also examined with X-ray diffraction (XRD). I was able to prove the membrane structure modifying effect of the polymer by swelling the samples with water vapour.

- During the densimetric studies based on high precision measurement of frequency the compression of the membrane structure was experienced. Molar excess volume values (V_m^e) were calculated from the density data and showed decreasing tendency with the increasing of the polymer concentration. This indicates a structural change which reveals to the compression of the membrane. The value of V_m^e reached –4.47 cm^3 / mol in the systems containing PVA.

1.4. Interaction of SLPL liposomes with PVP is much weaker than as it was experienced in the case of PVA.

According to the DLS measurements the increasing of the average vesicle diameter is only 8.8 nm, and at the adsorption isotherms the maximum amount of polymer adsorbed is also only 40.68 mg / g. Probably the higher hydrogen bridge forming ability of the hydroxyl groups in the PVA and dextrane compared to the oxo groups of the PVP can be the reason of this difference.

- At the evaluation of the adsorption isotherms it is also spectacular that at the beginning stage of adsorption (up to PVP / liposome = 0.12 w/w) PVP binds better than PVA and dextrane apart from the facts described previously. I suppose that electrostatic interaction may exist between the very weakly basic nitrogen in the 2-pyrrolidone heterocycle and the negatively charged liposome surface.
- XRD results also prove the weaker interaction with PVP and this is supported by the smallest V_m^e value (-3,40 cm^3 / mol) obtained from densimetry which shows that PVP also compresses the membrane.

1.5. The strength of the interaction with dextrane is similar as detected at PVA but the maximum values are lower considered both the increasing of the average vesicle diameter (21.5 nm) measured with DLS and the amount of polymer adsorbed (82.73 mg / g). According to my concept there are two main reasons of the differences: in one hand dextrane has smaller average molecular weight compared to PVA (M_w (PVA) = 72000, M_w (dextrane) = 40000), and in the other hand dextrane has ramified chain in contrast to PVA which is a linear polymer. It is also important to note that while at dextrane there are three hydroxyl groups per one monomer unit, there is only one in the case of PVA. On the basis of all the facts mentioned before I suppose that dextrane has a more compact adsorption layer than PVA. The PVA layer with looser structure is more bulky and gives chance for further PVA molecules to bind as the mass ratio of PVA / liposome increases.

- The compactness of the adsorption layer of dextrane is proved by the lowest V_m^e value measured with densimetry: -5.71 cm^3 / mol.

2. Description of the system DPPC / Chol / DCP

2.1. Preparation of liposomes containing DPPC was also done with lipid film-hydration, however downsizing was carried out with the more effective membrane-extrusion in the face of sonication. The final liposome diameters changed between 110.4 ± 5.3 and 111.7 ± 5.4 nm in the buffer solutions (pH = 7.4). The surface of the liposome membrane which has negative charge (0.136 mEq / g) at pH = 7.4 turns into neutral if the pH is 5.0 because of the dissociation-recession of phosphatidyl head groups. This effect and the fairly rigid bilayer membrane contained saturated phospholipids as major component cause that DPPC / Chol / DCP system hydrated in ammonium sulphate stays an easily sedimentable, coarse dispersion with the average liposome diameter of 1642 ± 132 nm.

2.2. The interacting ability of DPPC / Chol / DCP system with polymers is weaker than SLPL / Chol / DCP does. At the temperature of measurements DPPC forms gel phased ($L_{\beta'}$) membrane which is more rigid than SLPL liposomes have. The decreased membrane fluidity allows less molecular interactions, therefore the amounts of polymers adsorbed are diminished at all the three polymers with the maximum values of 35.41-73.69 mg / g. The increasing of the liposome diameters are also smaller rated (max. 9.6-16.3 nm).
Regarding to the adsorption of PVP similarity can be seen to the previous system. (see. 1.4.!).

3. Description of the DPPC / Chol system

3.1. These liposomes were effectively downsized with the membrane-extrusion method both at physiologic pH (114 ± 6.2 - 116 ± 7.3 nm) and in ammonium sulphate (151 ± 8.1 nm) because the fluidity of the neutral membrane (0.013 mEq /g) is not influenced by pH.

3.2. I detected the weakest interaction at this system because in addition it has also $L_{\beta'}$ phased membrane, it has no surface charge and contains cholesterol in 33.3 mol %. Cholesterol decreases the fluidity and this way permeability of the biological membranes through which polymers have even smaller chance to bind to the liposome surface. The maximum amount of polymers adsorbed on 1 g of liposome is 15.6-35 mg / g and the growth of liposomes caused by adsorption is also only 5.1-11.8 nm.

4. Optimisation of entrapment of the model drug: acridine orange

In order to investigate the liposome-polymer interaction indirectly on the basis of the change in the permeability of lipid membrane I entrapped a test material, acridine orange (AO) into the SLPL / Chol / DCP and DPPC / Chol liposomes dispersed in ammonium sulphate. The weakly basic dye was brought into the inner place of liposomes by the pH-gradient (or remote loading method). The process was optimised on the SLPL-system in such a way that I changed the molar ratio of AO / phospholipid (PL) between 0.02-0.30 and the quality of the buffer systems (phosphate, Hepes, Tris(hydroxymethyl) aminomethane (Tris)) which maintained the pH-gradient between the outer (pH = 7.4) and the inner (pH = 5.0) areas of the liposomes. During these experiments I was searching for that system at which the biggest entrapment efficiency with the smallest rated aggregation (DLS exams) can be experienced. I found that the AO / PL = 0.1 system equilibrated with Tris buffer is the most suitable for further exams.

5. Effect of polymer adsorption on AO release

Liposomes filled with AO were separated with gel permeation from the non-entrapped dye fraction and then PVA or dextrane was added to the dispersions in the polymer / liposome mass ratio of 0.1-0.8. Systems made ready this way were placed into the donor phase of the self-developed membrane diffusion cell thermostated at 37 °C and the amount of AO leaked out from liposomes was measured in the stirred acceptor phase at certain sampling times spectrophotometrically.

The polymer free SLPL / Chol / DCP dispersions released 7.51 % of the originally entrapped AO amount but at samples containing PVA or dextrane I could obviously show the inhibition of diffusion through the adsorption of polymers. This can be explained with the compression of the membrane structure shown by densimetry and the polymer barrier formed on the vesicle-surface. Whereas at the DEX / liposome systems mostly the membrane compression of DEX, at the systems containing PVA rather the thickening of the polymer layer has influence on the efflux of AO. On the basis of the measurements the amount of AO released during 24 hours decreased more determined to 5.2 % at the DEX / liposome = 0.1 (w/w) systems than at the systems containing PVA in the same mass ratio (6.15 %).

At DPPC / Chol liposomes no AO release was observed. The reason of this phenomena is obviously based on the facts mentioned at 3.2. with the supplement that the membrane of these liposomes is still in the orderly transitional phase ($P_{\beta'}$) at 37 °C.