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ORIGINAL CONTRIBUTION

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# Adsorption of neutral polymers on negatively charged liposomes. A novel quantitative method to measure the rate of polymer adsorption on the liposomal surface

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**Abstract** We studied the adsorption of two neutral polymers [poly(vinyl pyrrolidone) and poly(vinyl alcohol) (PVA)] on negatively charged liposomes composed of 25:2:3 (molar ratio) soy lecithin/dicetyl phosphate/cholesterol.The liposomes were prepared in buffered solution at pH 7.4 and were mixed with the solution of the polymers in the desired polymer/lipid ratios. Adsorption was measured by determination of the equilibrium bulk concentration of the polymer. Protamine hydrochloride was used to aggregate the liposomes with polymers adsorbed on their surface and to facilitate their separation from the equilibrium bulk solution. In the case of PVA, quantitative adsorption measurements with a specific reagent were possible. Adsorption isotherms were recorded at 25 ± 0.2 °C. It was concluded that adsorbed and non-adsorbed PVA molecules are in equilibrium even at low polymer/lipide ratios. The results were confirmed by dynamic laser light scattering, X-ray diffraction and thermal activity monitoring experiments.

**Key words** Liposomes · Polymer adsorption · Microcalorimetry · Particle size determination · Adsorption layer structure

## Introduction

Liposomes are ideal drug carriers in the field of drug delivery systems, and many previous studies have demonstrated the enhanced efficacy of encapsulated drugs and the reduction of the side effects of drugs so entrapped in this manner [1–6]. Conventional liposomes which are not specifically modified to provide them with a long circulating ability, however, are of limited use, since they tend to be trapped by the mononuclear phagocyting system (MPS). 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 [6–14]. 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 the liposomes to macrophages, as a result of their conformational flexibility and water-binding ability [13, 14]. Until recently only a few studies have dealt with other polymers physically adsorbed on the surface of liposomes.

Poly(vinyl alcohol) (PVA) and a PVA modified with a hydrophobic moiety (PVA-R) were examined as a coating material for liposomes to be loaded with an anticancer drug, doxorubicin [15, 16]. Besides experiments on animals (pharmacokinetic and distribution studies) the authors performed dynamic laser light scattering (DLS) measurements and determined the coating amounts of the polymers. It was shown that PVA-R binds to liposomes with higher affinity than PVA does.

The kinetic (long-term) stability of dimyristoyl phosphatidyl choline liposomes was examined by Grohmann

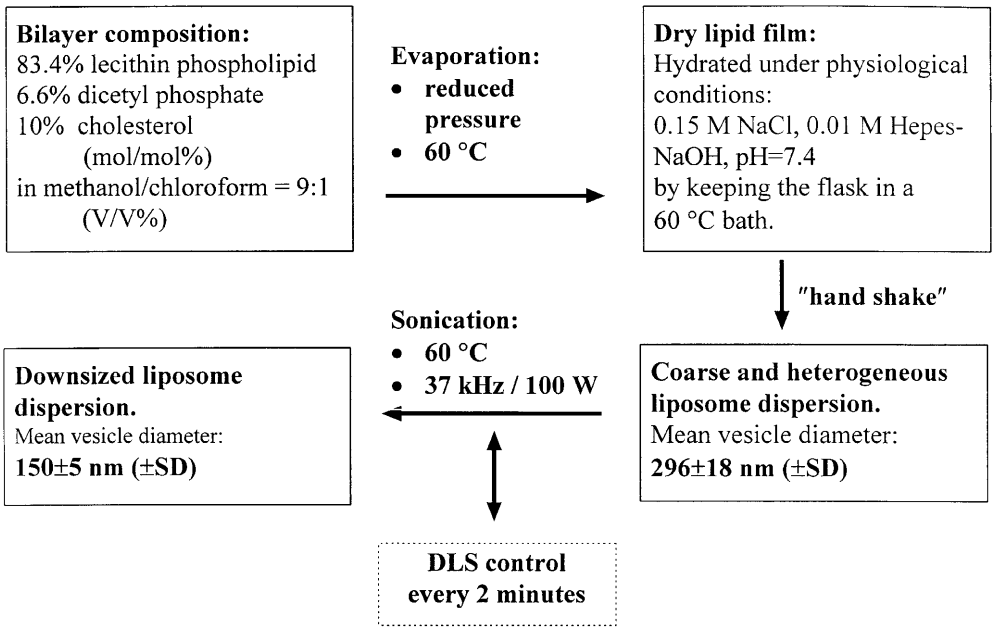



**Fig. 1** Preparation of liposomes

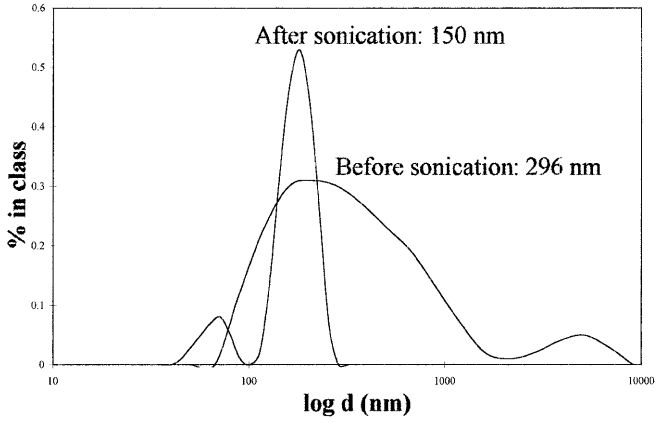



**Fig. 2** Effect of treatment with a bath sonicator on the average vesicle size and the size distribution (by dynamic light scattering)

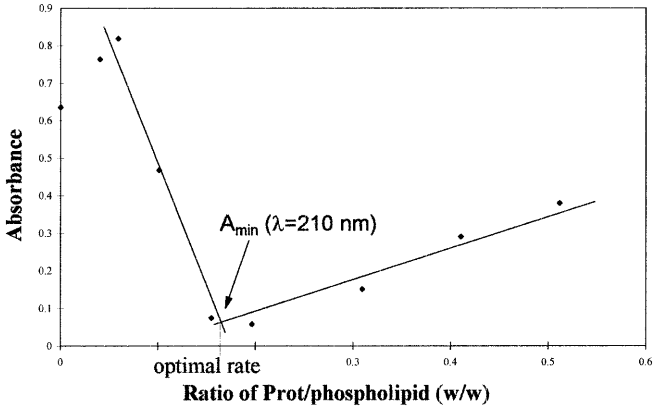



**Fig. 3** Determination of the optimal protamine hydrochloride (*Prot*)/phospholipide ratio as the minimum of $A_{min}$ in order to minimize the disturbing effect of nonprecipitated liposomes and Prot in the measurement of nonadsorbed poly(vinyl pyrrolidone) (*PVP*)

et al. [17], who incorporated PVA and poly(vinyl pyrrolidone) (PVP) derivatives into the bilayer membrane of the vesicles. These experiments were carried out at different pH values, and the enhancement of the average vesicle diameter was checked by DLS after certain time intervals for 3 months. It was concluded that the rate of the diameter increase can be significantly diminished by stabilizing the dispersions with PVP or PVA and its co-vinyl acetal, propional or butyral copolymers. In another approach differential scanning calorimetry was used to examine the polymer–liposome interactions [18].

Our primary aim was a detailed investigation of the interactions of PVA and PVP and the liposomal bilayer by DLS, X-ray diffraction (XRD) and thermal activity monitoring (TAM). We introduced a novel method, protamine hydrochloride (Prot)-induced vesicle aggregation, for a quantitative evaluation of the adsorption process.

## Experimental

### Materials

Soy lecithin containing about 85% w/w phospholipids was produced by Lucas Mayer., Germany. Dicetyl phosphate and cholesterol were purchased from Sigma, St. Louis, Mo., USA. PVP K30, $M_r \approx 40{,}000$, and 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (Hepes) were products of Fluka Chemie, Switzerland. Prot was obtained from F. Hoffmann-La Roche, Switzerland. All other substances, PVA, $M_r \approx 72000$, sodium

chloride, iodine, potassium iodide, hexadecylpyridinium chloride (HDPCl) and boric acid were purchased from Reanal, Budapest, Hungary, and were of analytical grade purity.

## Methods

### *Preparation of liposomes*

It was necessary to determine the exact phospholipid ratio in the soy lecithin used. This was performed according to the Rouser assay [19]. Liposomes consisting of 25:2:3 (molar ratio) soy lecithin phospholipid/dicetyl phosphate/cholesterol were prepared by the classic Bangham method [20] with some modifications (Fig. 1). The lipids were dissolved in a mixture of 9:1 (v/v) chloroform/methanol. A lipid film was prepared on the wall of a round-bottomed flask with a Rotadest 2118 vacuum evaporator. After the removal of all traces of organic solvent the film was hydrated with a buffer solution (pH 7.4) containing 0.15 M sodium chloride, 0.01 M Hepes and 0.005 M sodium hydroxide. The desired average vesicle diameter of 150 ± 5 nm was achieved by sonicating the dispersion with a Realsonic RS-16F ultrasonic bath. The process was checked by DLS every 2 min during this step (Fig. 2).

Different amounts of polymer were added to the dispersions of the liposomes at polymer/liposome ratios of 0.024–0.61 w/w. The liposome dispersions obtained in this way were mixed by vortexing them before they were incubated at 25 ± 0.5 °C for 2 h.

### *Determination of the amounts of polymer adsorbed*

This step was carried out according to the Prot aggregation method described in Ref. [21]. Samples (200 $\mu$l) were taken from each dispersion and an equal volume of Prot solution was added in order to aggregate the vesicles. These aggregated systems were diluted with the buffer solution in which the original liposomes were prepared before they were centrifuged at 2000*g* for 20 min.

In the case of PVA the amount of nonadsorbed polymer was determined from the supernatant with a specific reagent containing boric acid, potassium iodide and iodine [22] which forms a green compound with PVA. The optical density of this green colour was measured spectrophotometrically at 670 nm.

This measurement is more complicated in the case of PVP: in the absence of a specific chromophore-producing reagent we were forced to measure in the UV. In order to circumvent this problem we minimized the disturbing effect of unbound liposomes and Prot. We added increasing amounts of Prot to dispersions containing only liposomes and after centrifuging them we searched for the absorbance minimum at 210 nm. Figure 3 reveals the optimal ratio of Prot/phospholipide where PVP displays an absorbance maximum in 9:1 (v/v) ethanol:water. We added Prot in this Prot/phospholipid ratio to the liposome–PVP systems to be examined. This was followed by centrifugation, dilution of an aliquot of the supernatant with 96% ethanol and measurement of the concentration of nonadsorbed (equilibrium) PVP at 210 nm.

For the systems containing PVA these experiments were performed with another separation method employing a Beckmann TL 100 ultracentrifuge. In this case the vesicles were exposed to 100000*g* instead of Prot and centrifugation at 2000*g*.

We used a Kontron Uvikon 930 spectrophotometer for the measurements. The adsorption was expressed via the adsorption isotherms at 25 ± 0.2 °C by the following equation.

$$n^{\mathrm{s}}_{\mathrm{polymer}} = \frac{V}{100}\frac{c_0 - c_{\mathrm{e}}}{m} \quad ,$$

where $n^{\mathrm{s}}_{\mathrm{polymer}}$ is the amount of polymer in milligrams adsorbed on 1 g liposome, $c_0$ is the initial concentration of polymer (milligrams per 100 cm$^3$), $c_{\mathrm{e}}$ is the measured equilibrium concentration of polymer (milligrams per 100 cm$^3$), $V$ is the volume of sample (cubic

**Table 1** The results of dynamic light scattering experiments for the particle diameter and the adsorbed layer thickness

| Polymer/liposome (w/w) | 0 | 0.0125 | 0.025 | 0.041 | 0.061 | 0.122 | 0.305 | 0.61 |
|---|---|---|---|---|---|---|---|---|
| Average vesicle diameter ± standard deviation (nm) | | | | | | | | |
| Poly(vinyl alcohol) | 154.1 ± 0.85 | 164.3 ± 4.01 | 168.6 ± 0.90 | 172.7 ± 0.55 | 172.5 ± 2.56 | 170.7 ± 1.65 | 172.5 ± 0.66 | 177.7 ± 1.34 |
| Poly(vinyl pyrrolidone) | 150.1 ± 1.75 | 155.2 ± 1.96 | 151.9 ± 0.72 | 156.2 ± 1.89 | 155.0 ± 1.25 | 156.9 ± 0.70 | 157.6 ± 0.90 | 159.0 ± 1.53 |
| Thickness of the layer adsorbed ± standard deviation (nm) | | | | | | | | |
| Poly(vinyl alcohol) | 0 | 10.2 ± 0.24 | 14.5 ± 0.08 | 18.6 ± 0.06 | 18.4 ± 0.27 | 16.6 ± 0.16 | 18.4 ± 0.07 | 23.6 ± 0.17 |
| Poly(vinyl pyrrolidone) | 0 | 5.1 ± 0.06 | 1.8 ± 0.01 | 6.1 ± 0.07 | 4.9 ± 0.04 | 6.8 ± 0.0.03 | 7.5 ± 0.04 | 8.9 ± 0.09 |

**Table 2** Microcalorimetric data: total enthalpies, enthalpies of dilutions and adsorption enthalpies

| Polymer/ liposome (w/w) | $\Delta H_{tot}$ (mJ) | $\Delta H_{dil}$ of liposomes (with buffer) (mJ) | $\Delta H_{dil}$ of poly (vinyl alcohol) (added to buffer) (mJ) | $\sum\Delta H_{dil}$ of poly(vinyl alcohol) and liposomes (mJ) | $\Delta H_{ads}$ (mJ) |
|---|---|---|---|---|---|
| 0.04 | 2.16 | 4.88 | 0.56 | 5.44 | −3.28 |
| 0.08 | −0.78 | 3.43 | −1.10 | 2.33 | −3.11 |
| 0.12 | −2.99 | 1.23 | −2.77 | −1.54 | −1.45 |
| 0.16 | −4.69 | −1.94 | −3.69 | −5.63 | 0.95 |
| 0.20 | −6.16 | −4.43 | −4.66 | −9.09 | 2.93 |
| 0.24 | −7.32 | −5.90 | −5.00 | −10.90 | 3.58 |
| 0.28 | −8.46 | −7.70 | −4.87 | −12.57 | 4.11 |
| 0.32 | −9.46 | −9.45 | −4.34 | −13.79 | 4.33 |
| 0.36 | −7.99 | −9.73 | −2.76 | −12.49 | 4.50 |
| 0.40 | −3.81 | −4.70 | −3.44 | −8.14 | 4.33 |

centimetres) and $m$ is the mass of the liposomes in the sample (grams).

*DLS measurements*

The DLS experiments were carried out with SEMA Tech SEM-633 equipment at a measuring angle of 90° for 100 s. The samples to be examined were diluted 15-fold with the buffer solution. The existence of adsorption was concluded on the basis of increasing average vesicle diameters (Table 1).

*XRD studies*

The aim of the XRD measurements was to detect the structural changes brought about by polymer adsorption in the liposome membrane. The samples were first dried in a heat box and then exposed to water vapour at 60 °C for 12 h. They were measured in both dry and water-vapour-exposed conditions over angles from 1° to 8° (Philips PW1820 diffractometer Cu K$\alpha$, 40 kV, 35 mA). The accuracy of the identity period ($d_L$) was $\pm 0.01$ nm.

*Determination of surface charge density*

We used a Mütek PCD 02 particle charge detector (PCD). The original liposome dispersion and two diluted samples (3, 1.5 and 0.6% liposome, grams per 100 $cm^3$) were titrated with 0.1 M HDPCl. The endpoint of this process was indicated by a PCD signal of 0 mV. HDPCl was used at the exact concentration and with a high grade of purity. This process was performed in the Teflon cell (10 ml) of the PCD instrument. The surface charge density is calculated by

$$Z = V^0_{charge} c\mathrm{HDP}^{+/m} \ ,$$

where $Z$ is the surface charge density (milliequivalents per gram), $V^0_{charge}$ is the volume (cubic decimetres) of 0.1 M HDPCl at zero charge and $m$ is the mass of the liposomes (grams).

*Calorimetric measurements*

The data were obtained with a thermometric thermal activity monitor (TAM Type 2277) titration microcalorimeter. A portion of the original liposome dispersion (0.333 $cm^3$) was diluted to 2.00 $cm^3$ with the buffer solution, and the total enthalpies (Table 2) were determined when 800 $\mu$l of 0.5% PVA was added in ten steps. The PVA/liposome ratio (weight/weight) was set from 0.04 to 0.4. The total duration of this measurement was 17 h (2 h for the baseline and 1.5 h for each step).

We also measured the dilution enthalpies of both liposomes and PVA under the same conditions as mentioned earlier. Measurements on PVP are not reported here because of the failure to achieve reproducibility of the small heat effects.

## Results and discussion

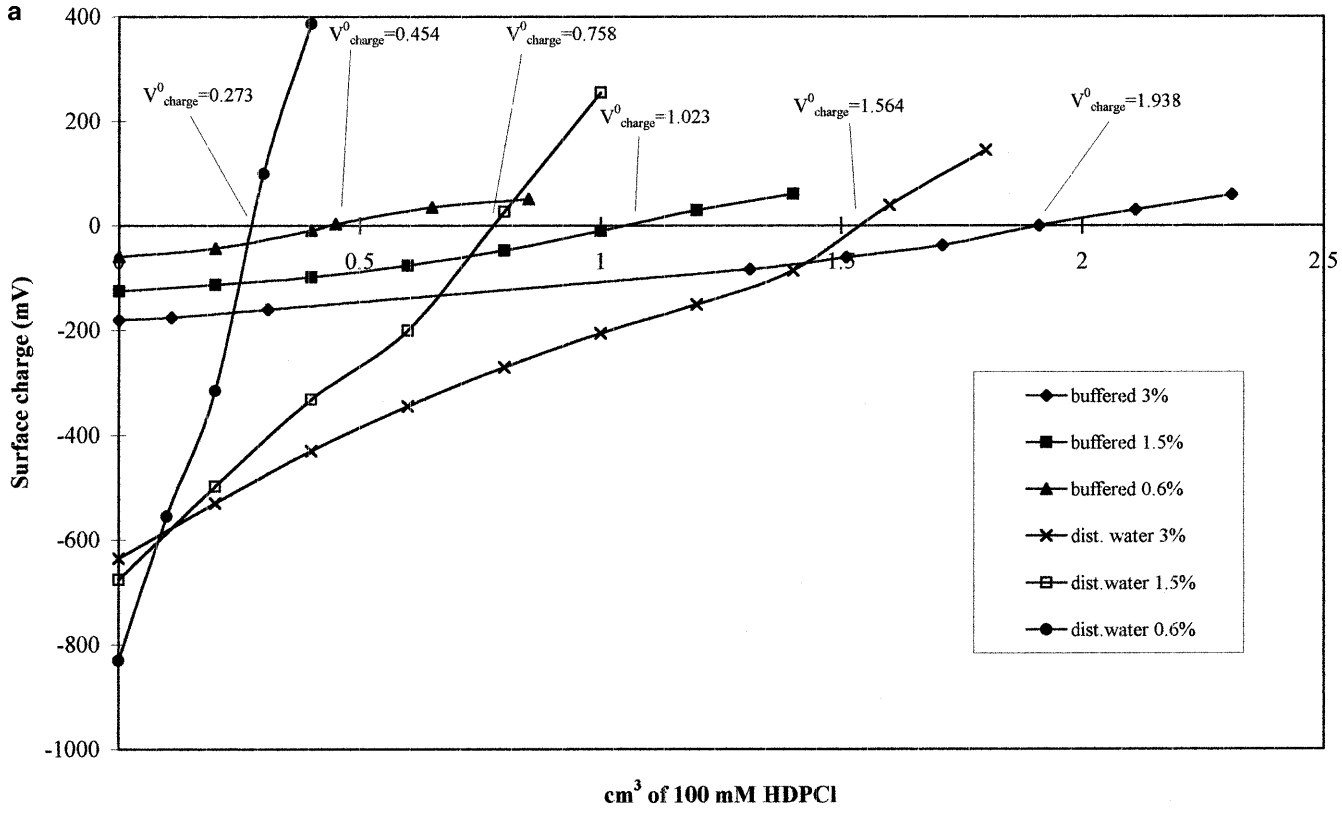
### Preparation and size control

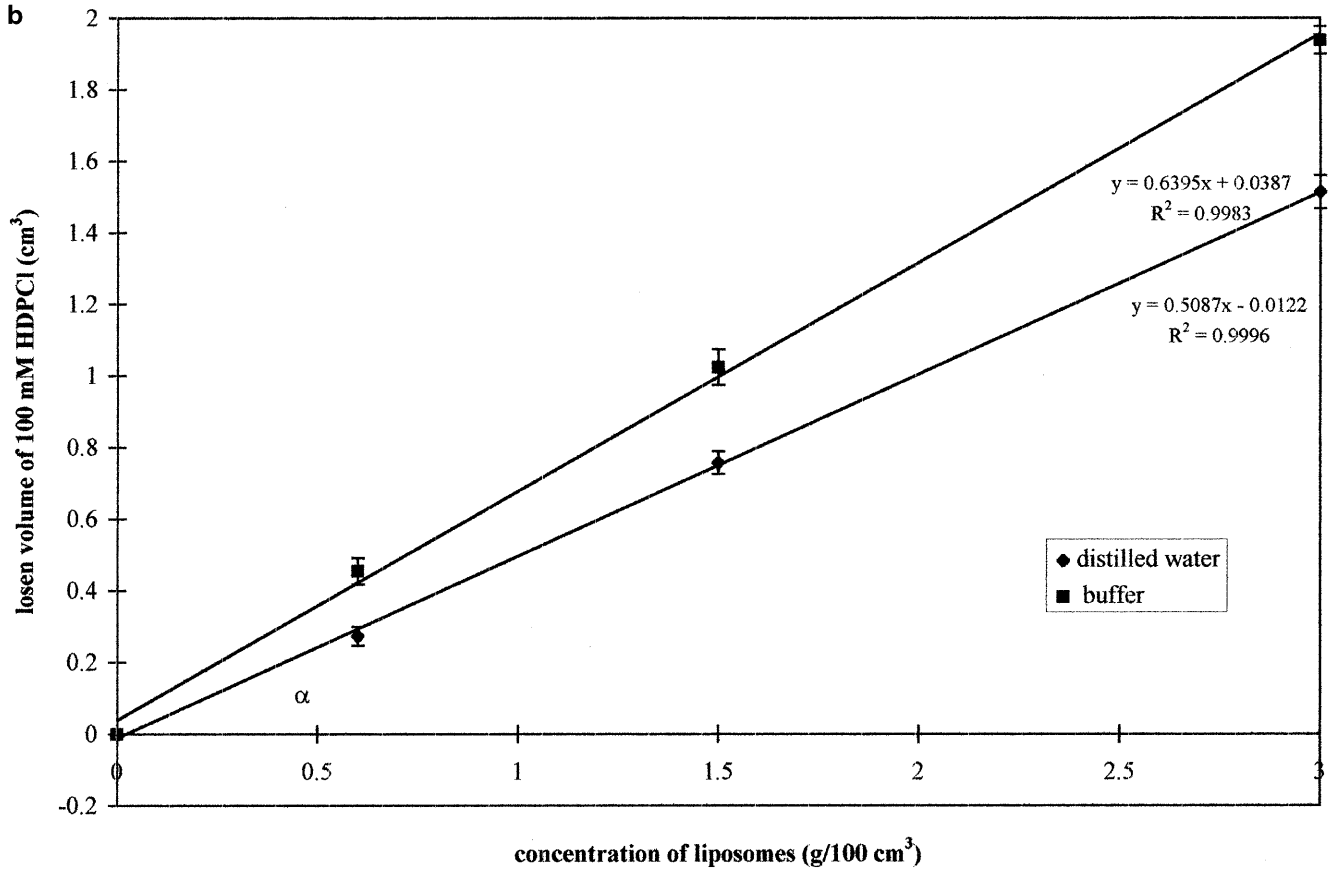
Downsized liposomes were used for our further experiments prepared according to the method shown in Fig. 1. The size distribution resulting from the DLS control revealed that the average liposome diameter decreased to 150 ± 5 nm. This relation shows that the sample treated by sonication is monodisperse, while the previous one is polydisperse containing large particles (4.5–5.5 $\mu$m) which are disaggregated by the bath sonicator (Fig. 2).

### Determination of particle charge density

A simple and quick method to determine the surface charge of liposomes is as follows. Ionic polymers adsorb on oppositely charged surfaces with high affinity. In the case of negatively charged liposomes cationic polymers also adsorb well, but they cause aggregation of the dispersion by neutralizing the negative surface charges [23].

The principle of our method is that negatively charged liposomes are titrated with a cationic tenside (HDPCl) which neutralizes their surface charges. The surface charge of liposomes dispersed either in water or in buffer is negative (about 200 and 600 mV) and decreases on addition of HDPCl (Fig. 4a). A certain amount of HDPCl ($V^0_{charge}$) causes a zero streaming potential and further addition leads to liposomes with an opposite charge. As a consequence the stability of the liposome dispersion is lost, and the system coagulates. When such experiments were carried out on liposome dispersions,


a
400
200
0
-200
-400
-600
-800
-1000
0.5
1
1.5
2
2.5
$V^0_{charge}$=0.273
$V^0_{charge}$=0.454
$V^0_{charge}$=0.758
$V^0_{charge}$=1.023
$V^0_{charge}$=1.564
$V^0_{charge}$=1.938
Surface charge (mV)
cm³ of 100 mM HDPCl
buffered 3%
buffered 1.5%
buffered 0.6%
dist. water 3%
dist.water 1.5%
dist.water 0.6%


b
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
0
0.5
1
1.5
2
2.5
3
losen volume of 100 mM HDPCl (cm³)
concentration of liposomes (g/100 cm³)
y = 0.6395x + 0.0387
R² = 0.9983
y = 0.5087x - 0.0122
R² = 0.9996
distilled water
buffer
α

$V^0_{charge}$ increased with the concentration. Plots of $V^0_{charge}$ against liposome concentrations are illustrated in Fig. 4b. From the slopes of the lines presented in this figure the surface charge density ($Z = \tan\alpha$) is obtained: 0.50 mEq/g for liposomes hydrated in water and 0.63 mEq/g in 0.01 M Hepes–NaOH buffer of pH 7.4.

## Polymer adsorption

The absorbance values are shown as a function of the Prot/liposome (weight/weight) ratio in Fig. 4. From these data the optimal ratio (about 17% w/w) was chosen at which minimal absorbance ($A_{min}$ 0.05) was obtained. This ratio provides the possibility to measure PVP at 210 nm.

Adsorption isotherms of the polymers are seen in Fig. 5. For PVA we compare two analytical methods. Measurement of PVA adsorption with ultracentrifugation or Prot aggregation as a separating method yielded very similar isotherms. PVA binds much better than PVP to liposomes because its hydroxyl groups bond by hydrogen bonds to the surface of the liposomes, which are rich in hydroxyl and oxo groups.

Although PVP also has a hydrogen-bonding oxo group in the pyrrolidone ring, the interaction with liposomes is weaker. The adsorption isotherm of PVP has already reached saturation at 50 mg/g.

## DLS experiments

DLS measurements revealed that the adsorption of PVA and PVP enlarged the average vesicle diameter. The extent of this enlargement was 15–23 nm for PVA and 5–9 nm for PVP. Thus, the stronger adsorption of PVA can also be proved by the thickness of the adsorption layer. PVA has a higher molecular mass than PVP (72,000 relative to 40,000) and, therefore, PVA spreads more extensively and is in a well-hydrated condition in the adsorption layer. This causes the observed excellent stability for the liposome dispersions (Fig. 6, Table 1).

## TAM measurements

The heat production/absorption during titration of liposomes with PVA is illustrated in Fig. 7. The first

**Fig. 4** **a** Titration of liposomes (3, 1.5, and 0.6 g liposome/100 $cm^3$) with hexadecylpyridinium cations in buffer or in water. $V^0_{charge}$ was calculated from the intersection points. **b** Correlation between liposome concentration and the consumed volume of the titrating agent (100 mM hexadecylpyridinium chloride, *HDPCl*). The slopes of the regression lines (tanα) show the surface charge density in milliequivalents per gram (three parallel measurements)

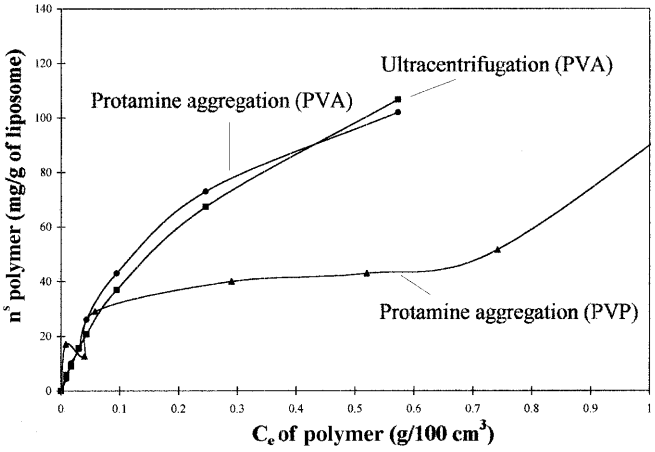



**Fig. 5** Isotherms of adsorption of poly(vinyl alcohol) (*PVA*) and PVP on liposomes. Comparison of Prot aggregation and ultracentrifugation as separating methods in the determination of nonadsorbed PVA

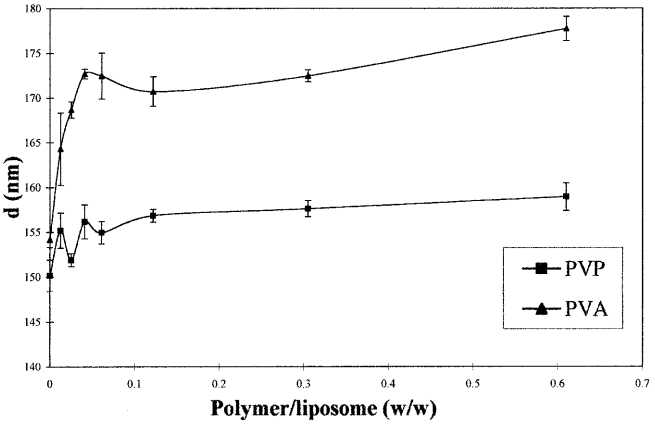



**Fig. 6** Average vesicle diameter as a function of the polymer/liposome (weight/weight) (*w*/*w*) ratio (three parallel measurements)

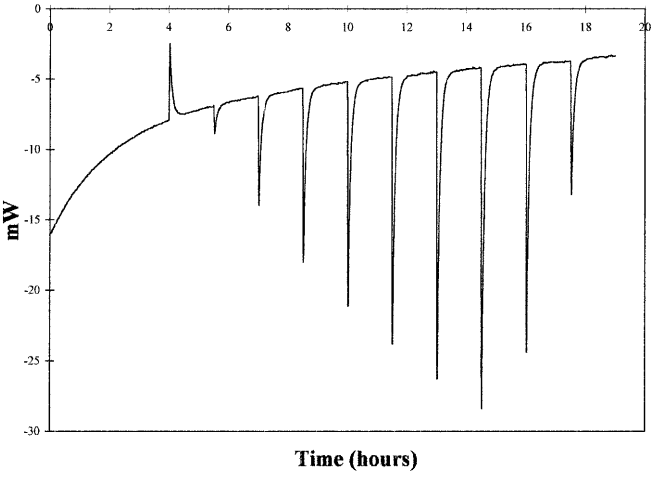



**Fig. 7** Titration microcalorimetric enthalpogram of PVA/liposome 0.04–0.4 (w/w) systems from which the $\Delta H_{tot}$ values were calculated by integration of the areas under peaks

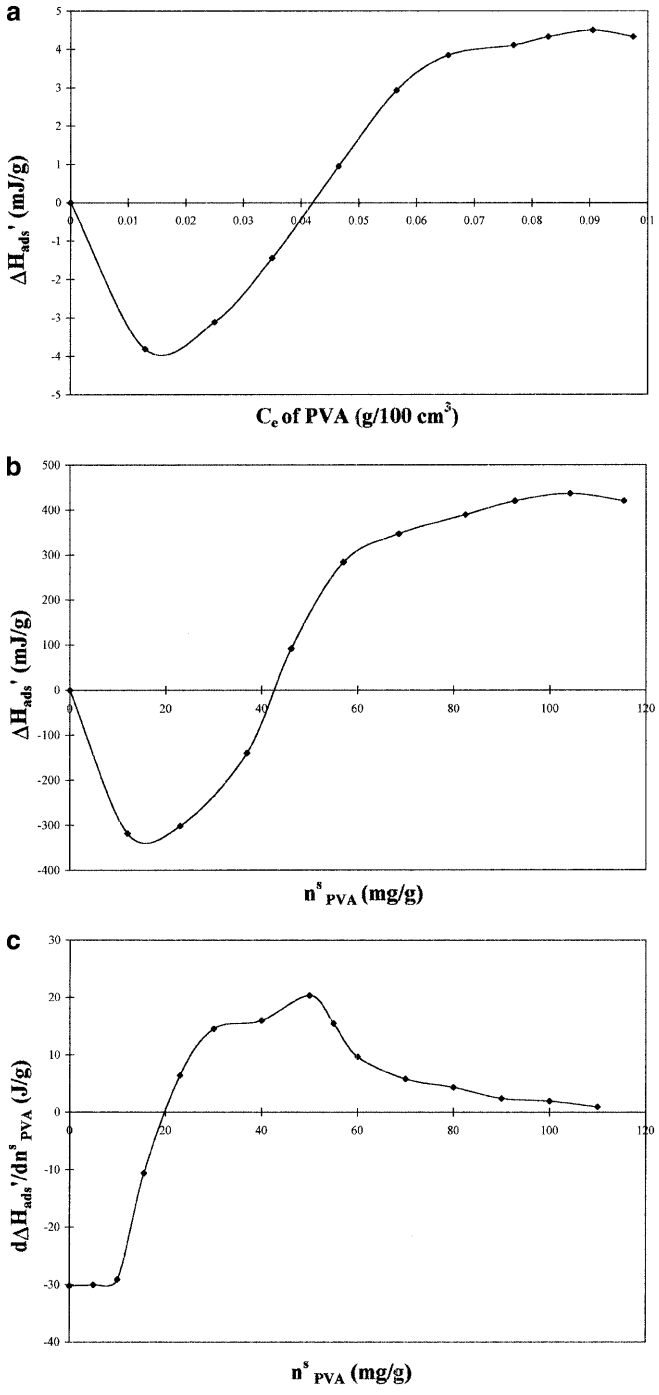



**Fig. 8** **a** $\Delta H_{ads}$ shows exothermic interaction up to $c_e = 0.04$ g/100 cm$^3$. The $c_e$ values were calculated from the adsorption isotherm (Fig. 5, Prot aggregated system). **b** $\Delta H_{ads}$ per gram of liposome as a function of the amount of PVA adsorbed per gram of liposome ($n^s_{PVA}$). **c** Derived form of Fig. 8b. Calculation of the amount of energy liberated by the adsorption of 1 g PVA

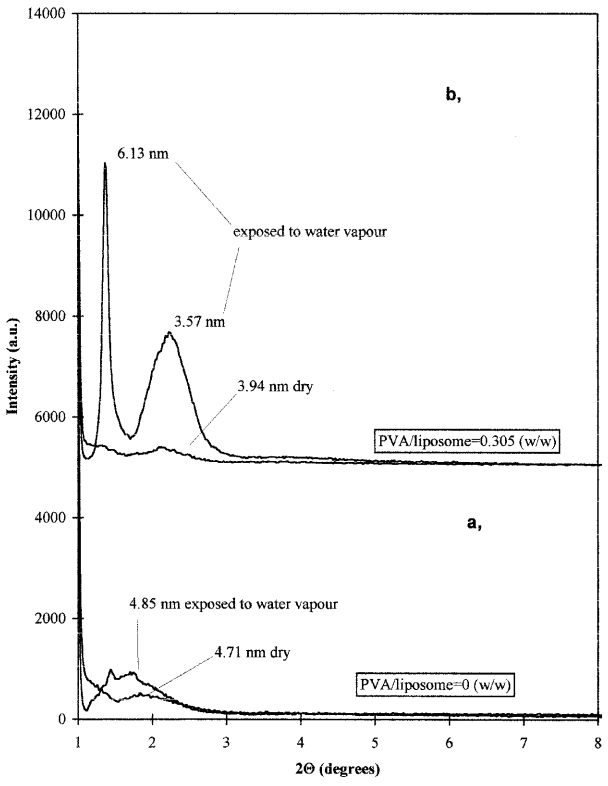



**Fig. 9** X-ray diffraction (*XRD*) diffractograms of PVA–liposomes

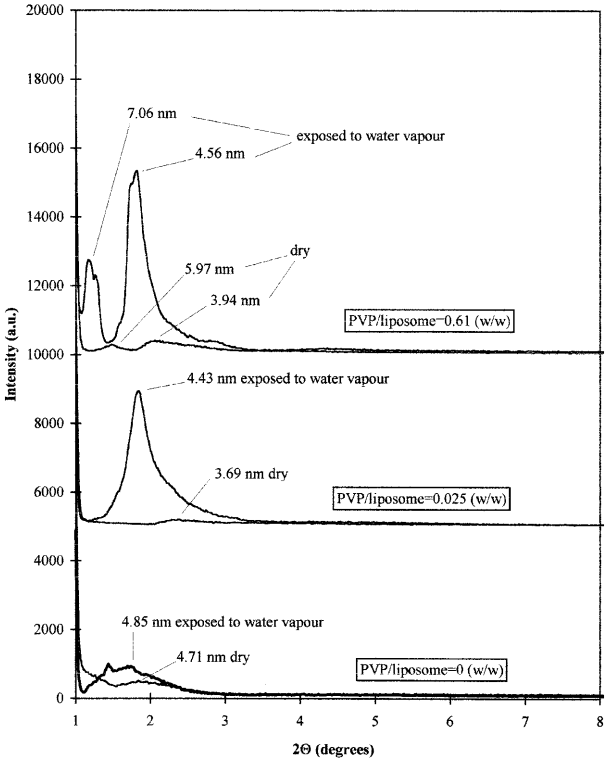



**Fig. 10** XRD diffractograms of PVP–liposomes

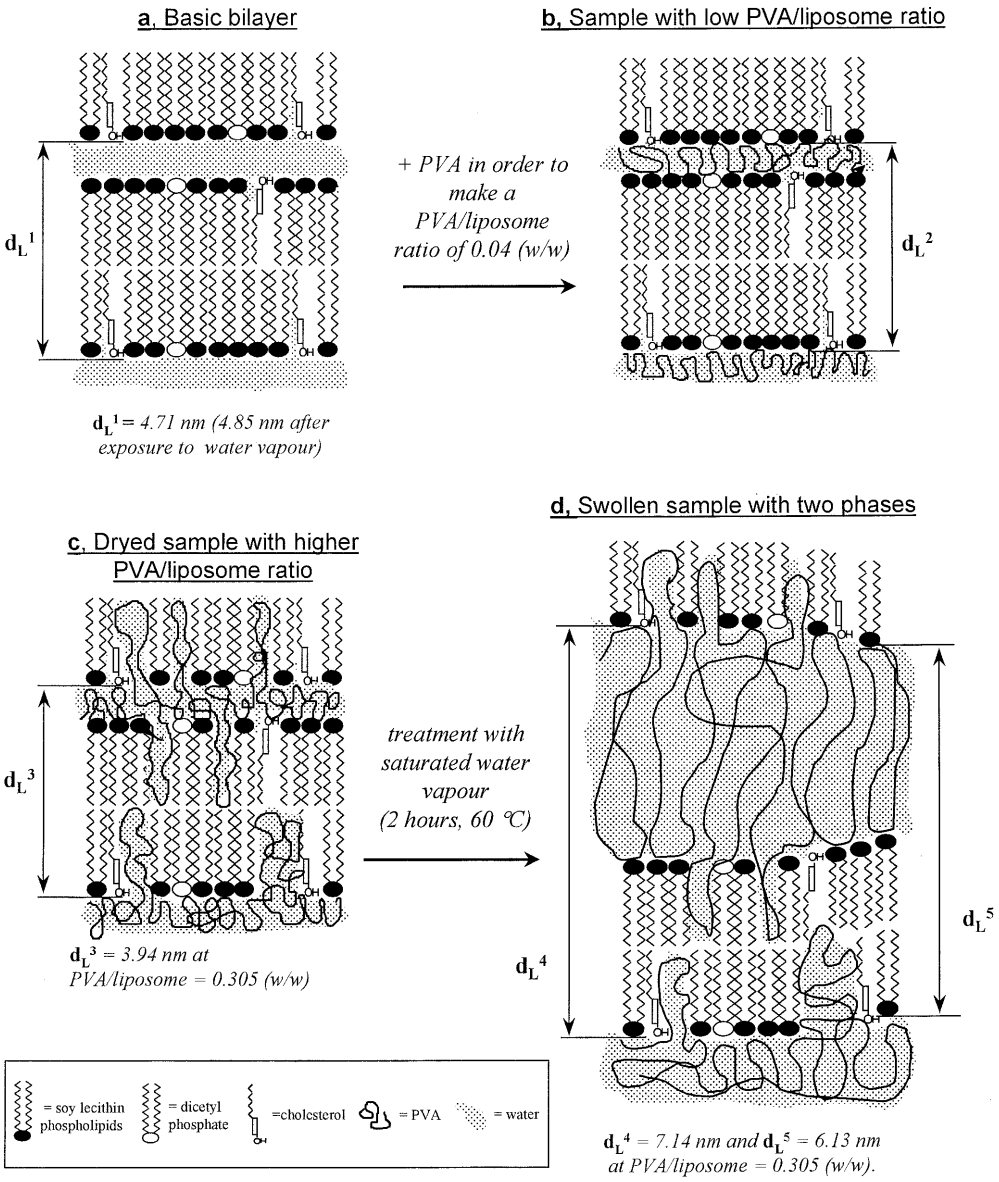



**Fig. 11a–d** Schematic figure of PVA–liposome. **a** Without PVA and with **b** a small and **c** a high amount of PVA. **d** The sample in **c** after swelling in water vapour

peak reflects an exothermic process and the others represent endothermic effects. The enthalpy values required to obtain the adsorption enthalpy are listed in Table 2. This enthalpy is obtained from the relation

$$\Delta H_{\text{ads}} = \Delta H_{\text{tot}} - (\Delta H_{\text{dil(PVA)}} + \Delta H_{\text{dil(liposomes)}}) \ ,$$

where $\Delta H_{\text{ads}}$ is the adsorption enthalpy and $\Delta H_{\text{tot}}$ is the total enthalpy and $\Delta H_{\text{dil}}$ is the dilution enthalpy of the components (PVA and liposomes).

The adsorption enthalpy is plotted against the equilibrium concentration of PVA (Fig. 8a). The interaction is exothermic up to a PVA concentration of 0.04 g/100 $cm^3$ but is endothermic at higher concentration. An analogous representation may be seen in Fig. 8b, where the adsorption enthalpy is related to 1 g liposome ($\Delta H_{\text{ads}}'$) as a function of the amount of PVA adsorbed. This relation shows that in the initial stage of the isotherm, up to an amount of 40 mg/g adsorbed, exothermic effects are recorded. The differential adsorption enthalpy in Fig. 8c shows the adsorption enthalpy related to the unit mass of PVA.

These data clearly demonstrate a strong exothermic interaction between the liposome membranes and the segments of the PVA molecules in the initial state of the adsorption. The interaction may primarily involve hydrogen bonding (see later). At higher PVA concentrations endothermic interactions occur, which indicates that the polymer is incorporated between the alkyl chains of the bilayer. This process decreases in the structural order of the bilayers, the disorientation

of the alkyl chains being controlled by entropy. It must also be considered that there are cholesterol and dicetyl phosphate molecules between the lecithin phospholipids and the hydroxyl groups of cholesterol might favour the penetration of PVA chains into the lipid layer.

### XRD results

The changes in the liposomal bilayer can also be detected by small-angle scattering (Figs. 9, 10). In the PVA–liposome systems a lamellar distance ($d_L$) of 4.71 nm was found. After storage in saturated water vapour a small degree of swelling was observed with $d_L = 4.85$ nm. These distances are almost the same as those reported in Refs. [24, 25], where $d_L$ was reported to be 4.80 nm for dipalmitoyl phosphatidyl choline bilayers in the gel ($L_{\beta'}$) phase. The layer thickness was related to the structure of the hydrated lipid membrane (Figs. 9, spectra a, 11a). At a ratio of 0.305 PVA/liposome $d_L$ in the dry condition was 3.94 nm (Fig. 9, spectra b). This reveals that the adsorbed PVA compresses the bilayer by connecting molecules in the membrane (Fig. 11c). When this sample was exposed to water vapour, two peaks appeared at $d_L = 6.13$ nm and 3.57 nm, which are probably the (00l) and (002) reflections of a highly expanded bilayer with a spacing of about 7 nm (Fig. 11d). The nonintegrality ($d_{001}$ is not equal to $2d_{002}$) indicates a certain interstratification of somewhat differently expanded interlayer spaces, i.e. the distances from interlayer to interlayer of the expanded phase are not exactly the same. The XRD records of PVP–liposomes reveal essentially the same effects. In dry conditions PVP compresses the bilayer, while saturated water vapour expands the membrane to $d_L = 4.43$ nm. This expansion is maintained after further addition of PVP but another peak appears at 7.06 nm which is characteristic of completely expanded bilayers.

A comparison of the XRD results for the two neutral polymers with liposomes suggests that, as already proved in connection with adsorption isotherms, PVP binds more weakly on the surface of the bilayer membrane than PVA at PVP/liposome (weight/weight) ratios of 0.025 and 0.61. The expanded structure shows $d_L = 4.43$ nm and 4.56 nm. The amount of the highly expanded phase with $d_L = 7.06$ nm seems to be distinctly smaller than for PVA.

## Conclusions

The interaction of neutral polymers (PVA and PVP) with the liposomal bilayer were examined. PVA is adsorbed by liposomes more strongly than PVP as a consequence of stronger hydrogen bonds. These interactions were detected by several measuring techniques. DLS demonstrated that the diameter of the liposomes was higher after stabilization with PVA; thus, the adsorption layer is thicker (10.2–23.6 nm) than in the case of PVP (5.1–8.9 nm). Microcalorimetric titration measurements revealed exothermic interactions in the initial stages of the adsorption of PVA. XRD examination of films of liposomal bilayers revealed swelling by water vapour and the influence of stabilizing polymers on the membrane structure. The results indicate that PVA binding is stronger because it allows a lower extent of expansion for the bilayer by water vapour.

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