Colloid Polym Sci 276:66–71 (1998)

ORIGINAL CONTRIBUTION

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Stabilization of small unilamellar DMPC-liposomes by uncharged polymers

Received: 20 May 1997
Accepted: 03. September 1997

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Abstract Polymer-free and polymer-bearing small unilamellar (SUV) liposomes from dimyristoyl-phosphatidylcholine (DMPC) were prepared under standardized conditions. Polymer-bearing liposomes were formed by incorporating an uncharged polymer [hydrolyzed poly(vinyl alcohol) (PVA), poly(vinyl alcohol-co-vinylacetal) (PVA-Al), poly(vinyl alcohol-co-vinyl propional) (PVA-Prol) poly(vinyl alcohol-co-vinyl butiral) (PVA-Bul) copolymer or poly(vinyl pyrrolidone) (PVP)] into the membrane bilayer of vesicles. The kinetic (long-term) stability of the liposome dispersions stored in distilled water, in physiological NaCl solution and at various pH values, respectively, were studied. The physical stability of vesicles was tested by measuring the size and the zeta potential of liposomes by means of a Malvern Zetasizer 4 apparatus.

It was shown that most of these polymers are effective steric stabilizers for the DMPC-liposomes. Among the polymers, the PVA-Bul and PVA-Prol copolymers and the PVP of high molecular mass exhibited the most efficient stabilizing effect at each pH studied, indicating that the formation of a relatively thick polymer layer around the lipid bilayers ensures an enhanced and prolonged physical stability of liposomes. Also, the butiral or propional side chain in the PVA-based copolymers presumably promotes the anchoring of macromolecules to the vesicles. Using these macromolecules, the colloidal interactions between vesicles can be modified and so the physical stability of liposomes and the kinetic stability of liposome dispersions can also be controlled.

Keywords DMPC-liposomes – size distribution – uncharged polymers – kinetic stability – photon correlation spectroscopy

Introduction

Considerable efforts have been made in pharmaceutical research to develop new drug delivery systems that enhance the efficacy and safety of existing drugs and that underlie optimized drug therapy. Extensive studies show that colloidal carriers provide numerous opportunities for the formulation of controlled release and site-specific drug-delivery systems [1–9]. Biologically active materials can be adsorbed on the surface of particles or incorporated into the carrier.

Liposomes find perhaps the most extensive use as drug carriers. Due to the biphasic nature of liposomes, both lipophilic and hydrophilic ingredients are accommodated depending on their solubility in the liposome

components, consequently, almost any type of drug can be encapsulated.

A considerable shortcoming in the use of conventional liposomes is that owing to their relatively low physical stability, aggregation and fusion of vesicles in time may take place. The control and prediction of liposome stability are therefore important even if they are to be stored for long time after preparation. The kinetic stability of liposome dispersions will largely depend on the physical characteristics of vesicles (lipid composition, type of liposomes, surface charge of vesicles, etc.) and the nature of encapsulated material and its interaction with the liposome components and also, on the composition of the dispersion medium. To ensure prolonged therapeutic activity of active ingredients either bounded or incorporated, reducing the rate of aggregation and fusion of vesicles are therefore, required.

In the present work a way of preparing small unilamellar DMPC-liposomes with well-defined size distribution is described. Also, the aggregation behaviour of vesicles and the long-term (kinetic) stability of the liposome dispersions at various pH values is studied. To this end, the size, the size distributions and the zeta potential of vesicles and also, the change in time of these characteristics have been measured by photon correlation spectroscopy (PCS).

Experimental

Materials

L-α dimyristoylphosphatidylcholine (DMPC) purchased from Sigma Chemical Co. (St. Louis. MO) was used without further purification in the liposome preparations.

Uncharged polymers such as hydrolyzed poly(vinyl alcohol) (PVA), poly(vinyl alcohol-co-vinyl acetal) (PVA-Al), poly(vinyl alcohol-co-vinyl propional) (PVA-Prol) and poly(vinyl alcohol-co-vinyl butiral) (PVA-Bul) copolymers with 5 mol% vinyl acetal, vinyl propional or vinyl butiral content, as well as poly(vinyl pyrrolidone) (PVP) were used for the preparation of polymer-bearing vesicles. The polymers were fractionated samples, prepared from commercial products of Powal 420 poly(vinyl alcohol) and GAF K-30 and K-90 poly(vinyl pyrrolidone), respectively. The hydrolysis of the PVA and the preparation of the copolymers is described elsewhere [10].

The degree of polymerization of the PVA, PVA-Al, PVA-Prol and PVA-Bul copolymers is alike 2450, but that of the K-30 and K-90 poly(vinyl pyrrolidone) is 280 and 8120, respectively.

The pH of the liposome dispersions was adjusted to a physiologically relevant value such as 1.2, 5.4, 7.1 or 8.2, using a mixture of hydrochloric acid and sodium chloride, or mixtures with various ratios of 0.2 M sodium hydrogen phosphate and 0.1 M citric acid, as buffer solution. All the reagents were of analytical grade and were purchased from Reanal (Hungarian Chemical Co.). Aqueous solutions were made with double-distilled water.

Methods

Preparation of liposomes

Vesicles were prepared from pure DMPC using a slightly modified procedure described by Szögyi et al., and Grohmann et al. [11, 12]. The lipid was dissolved in a chloroform/methanol mixture (9:1 volume ratio). Subsequently, the solvent was evaporated by leading nitrogen gas onto the lipid coating and meanwhile the glass was slowly rotated, an even and uniformly dry film was formed. The dried lipid layer was first dispersed by hand shaking in a small amount (≈ 0.5 ml) of distilled water and physiological NaCl solution, respectively, without polymer, or in a polymer solution. The liposomes were formed by twofold ultrasonication. First, the coarse lipid dispersion was sonicated for 20 min at 20 kHz frequency at 25 °C, using a MSE Ultrasonic Disintegrator Model 150 watt (Measuring Sci. Equipment Ltd., Manor Royal, Crawley, Sussex, England). Then, the dispersion was diluted so that the final concentration of DMPC in the dispersion was 2.0 $g\,dm^{-3}$ and the DMPC/polymer ratio, in each polymer-containing dispersion, was 10:1 (w/w). Following a second sonication in the same way as before, the dispersion was ultracentrifuged for 45 min at 19000 rpm by a Beckman ultracentrifuge (Model J2-21, Beckman Inc., USA), in order to remove the possibly formed lipid aggregates or large vesicles.

The liposome dispersions were stored at 5–7 °C in a refrigerator and warmed up to 25 °C before use.

Determination of particle-size distribution and zeta potential of vesicles

Mean vesicle size, size distribution, and polydispersity of the liposomes and also, the change in time of these variables were measured at 25 °C by an advanced technique of PSC using a Malvern Zetasizer 4 apparatus (Malvern Instruments, UK).

The zeta potential of the liposomes was determined from electrophoretic mobility measurements with the Zetasizer 4 at 25 °C.

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Results and discussion

Particle size and size distribution of liposomes

Vesicle size is a relevant feature regarding both the organ distribution and encapsulation or adsorption efficiency of liposomal drug carriers. Based on the measured intensity distribution of scattered light from vesicles by PCS, the corresponding *Z*-average mean size (*Z*, nm), the number (*D*, nm) and mass- or volume-average mean sizes of liposomes can be calculated. Any of these quantities may, in general, be used as adequate characteristic of a dispersion with relatively narrow distribution of vesicle sizes.

The DMPC-liposomes formed in different media are characterized by the mean vesicle sizes and their standard deviation. In Table 1, the number-average and the *Z*-average mean sizes of liposomes, calculated by multimodal analysis from the intensity distribution and also, the zeta potential of vesicles are shown.

Each dispersion exhibited monomodal size distribution soon after the preparation. As the mean sizes demonstrate, small unilamellar vesicles (SUV) were formed from DMPC both in distilled water and the electrolyte solutions, irrespective of the presence or absence of polymer [9, 13].

There are no significant differences in the mean sizes of liposomes of the same composition in these two media, but the liposomes prepared in the NaCl solutions are less polydisperse. Definite increase in the vesicle size can only be observed with liposomes formed in the solution of PVA-Prol and PVA-Bul copolymers, indicating that the dissolved macromolecules are attached to the lipid membrane of vesicles.

Electrokinetic potential of liposomes

The membrane surface potential plays an important role in the rate of aggregation and fusion of vesicles and hence in the physical stability of liposomes. The change in the electrostatic potential profile across the neutral and electrically charged phospholipid membranes, caused by the binding or sorption of dissolved compounds, can be estimated by the direct determination of the zeta potential of lipid vesicles [14]. The zeta potentials (ζ) of the pure DMPC-vesicles and of the polymer-containing liposomes determined by electrophoretic mobility measurements in distilled water and 0.15 M sodium chloride solution, respectively, are also listed in Table 1.

These results show that the vesicles are negatively charged in both media but, due to higher ionic strength, the zeta potential of the pure DMPC-liposomes in the electrolyte solution is lower. Varied reduction in the zeta potential of the vesicles, owing to the presence of different polymers, can be observed.

In principle, the attached macromolecules may alter the distribution of ions in the electrical double layers around the vesicles, as well. As all of the polymers used are uncharged, the approximation may be made that the polymer molecules do not influence the charge distribution in the diffuse part of the double layer. The basis for this approximation is that the volume occupied by neutral polymers in the diffuse double layer is small. Taking this assumption into account, the decrease in the zeta potential may be attributed to the shift of the shear plane outwards with respect to its position in the absence of polymer. The extent of reduction due to the presence of attached macromolecules, as compared with the zeta potential of the bare DMPC-vesicles, may

Table 1 Mean vesicle sizes and zeta potential of bare and polymer-bearing DMPC-liposomes

Liposome	Medium	Mean sizes *D* [nm]	*Z* [nm]	Zeta potential ζ [mV]
DMPC	Water	45 ± 12	81	− 11.2 ± 1.1
	0.15 M NaCl	53 ± 9	73	− 4.5 ± 1.2
DMPC + PVA	Water	50 ± 15	85	− 5.1 ± 1.0
	0.15 M NaCl	55 ± 10	82	—
DMPC + PVA-Al	Water	46 ± 12	83	− 6.3 ± 1.1
	0.15 M NaCl	53 ± 10	68	—
DMPC + PVA-Prol	Water	50 ± 12	95	− 5.2 ± 1.1
	0.15 M NaCl	53 ± 10	80	—
DMPC + PVA-Bul	Water	71 ± 16	118	− 1.5 ± 1.0
	0.15 M NaCl	80 ± 15	128	—
DMPC + PVP K-30	Water	45 ± 11	82	− 7.1 ± 2.0
	0.15 M NaCl	53 ± 10	77	—
DMPC + PVP K-90	Water	48 ± 13	78	− 6.2 ± 1.1
	0.15 M NaCl	53 ± 12	73	—

be considered to a first approximation as a relative measure of the thickness of the polymer layer formed around the vesicles [15, 16].

The formation of a relatively thick layer from the PVA-Bul copolymer and thinner ones from other polymers is indicated by these results. This order is in line with that based on the size measurements. The observed increase in the hydrodynamic radius of particles may be attributed to the adsorption of polymer molecules on vesicle surfaces but incorporation of chain segments in the lipid bilayers may certainly occur. To elucidate the mechanism of attachment in detail and the conformation at vesicle/solution interfaces of these macromolecules, further studies are still needed.

Long-term stability of liposome dispersions

The kinetic stability of liposome dispersions strongly depends on both the physical and chemical stability of vesicles, and can be effectively influenced by the extent of electrical and/or steric repulsive forces operating between the vesicles. Aggregation and fusion of liposomes, which may take place on storage of the dispersions, or when the vesicle stability is lowered, cause alike a shift in the mean size and size distribution towards higher values. From the change in time of size distribution, the aggregation state of vesicles and hence the kinetic stability of liposome dispersions can be estimated.

To illustrate the differences in the kinetic stability of dispersions with polymer-free and polymer-containing vesicles various time periods after their preparation, the number-average mean size (D) of DMPC-liposomes and the standard deviation of D measured in different dispersion media are shown in Tables 2 and 3. For dispersions which exhibited definitely biomodal size distribution after a period of storage, the relative amount in percent of liposomes corresponding to the second peak (II) of the distribution function are also indicated.

These results well demonstrate that the kinetic stability of bare DMPC-liposome dispersions is much lower both in distilled water and physiological NaCl solution than that of the polymer-containing vesicles. In 60 days, the polydispersity of the polymer-free liposomes significantly increased and its monomodal size distribution turned into a bimodal one.

Some increase in the mean size of several polymer-bearing liposomes in both media can also be observed, mainly after longer periods of storage. However, neither the size nor the polydispersity of the DMPC + PVA-Bul liposomes have changed in 120 days, indicating that the PVA-Bul copolymer can prevent the liposomes against aggregation even for longer times. Also, the PVA-Prol copolymer proved to be an effective steric stabilizer for the DMPC-vesicles. The other polymers exerted less stabilizing effect. These findings are consistent with the results of particle size and electrophoretic mobility measurements, as well. It seems very reasonable to assume that the enhanced stability of some polymer-containing liposomes can be ascribed to the formation of a protective macromolecular sheath around vesicles which ensures steric stabilization against the aggregation and fusion of liposomes.

The effect of pH on the stability of liposomes

To illustrate the influence of pH on the long-term stability of liposomes, the Z-average mean size (Z, nm) of vesicles stored for several weeks at pH 1.2 and 8.2, respectively, plotted as a function of the time of storage, are shown in Figs. 1 and 2. Same plots for the liposomes have also been determined at pH 5.4 and 7.1.

At each pH studied, notable differences in the vesicle size between the bare and the polymer-containing

Table 2 Mean vesicle size and polydispersity of bare and polymer-bearing DMPC-liposomes after various times of storage in distilled water

Liposome	Peak	Mean size D [nm]		
		1	60 Days	120
DMPC	I	45 ± 12	82 ± 20	100 ± 52
	II	—	430 ± 128 [3%]	440 ± 120 [10%]
DMPC + PVA	I	47 ± 14	53 ± 16	68 ± 14
	II	—	—	177 ± 48 [2%]
DMPC + PVA-Al	I	44 ± 12	45 ± 11	53 ± 13
DMPC + PVA-Prol	I	48 ± 14	55 ± 16	64 ± 16
DMPC + PVA-Bul	I	70 ± 15	69 ± 16	70 ± 17
DMPC + PVP K-30	I	44 ± 12	70 ± 20	80 ± 21
	II	—	—	560 ± 110 [4%]
DMPC + PVP K-90	I	47 ± 13	60 ± 16	67 ± 17
	II	—	—	480 ± 110 [3%]

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Table 3 Mean vesicle size and polydispersity of bare and polymer-bearing DMPC-liposomes after various times of storage in physiological NaCl solution

Liposome	Peak	Mean size D [nm]		
		1	60 Days	120
DMPC	I	53 ± 16	437 ± 210	540 ± 125
	II	—	1700 ± 500 [29%]	1800 ± 720 [31%]
DMPC + PVA	I	53 ± 14	53 ± 13	53 ± 23
	II	—	—	470 ± 62 [4%]
DMPC + PVA-Al	I	48 ± 11	48 ± 11	51 ± 14
	II	—	—	740 ± 105 [21%]
DMPC + PVA-Prol	I	47 ± 13	50 ± 14	55 ± 12
DMPC + PVA-Bul	I	78 ± 20	76 ± 21	78 ± 21
DMPC + PVP K-30	I	53 ± 10	240 ± 26	470 ± 38
	II	—	440 ± 35 [38%]	800 ± 58 [5%]
DMPC + PVP K-90	I	52 ± 12	54 ± 14	57 ± 15
	II	—	—	480 ± 115 [87%]

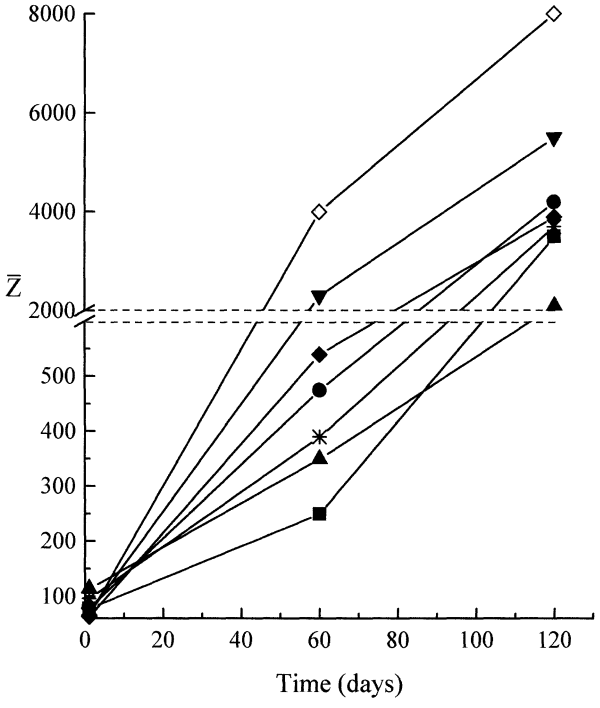

Fig. 1 The Z-average mean size of bare and polymer-bearing DMPC-liposomes stored in buffered medium at pH 1.2, as a function of time: (—◇—) DMPC, (—✳—) DMPC + PVA, (—◆—) DMPC + PVA-Al, (—▼—) DMPC + PVA-Prol, (—▲—) DMPC + PVA-Bul, (—●—) DMPC + PVP K-30, (—■—) DMPC + PVP K-90

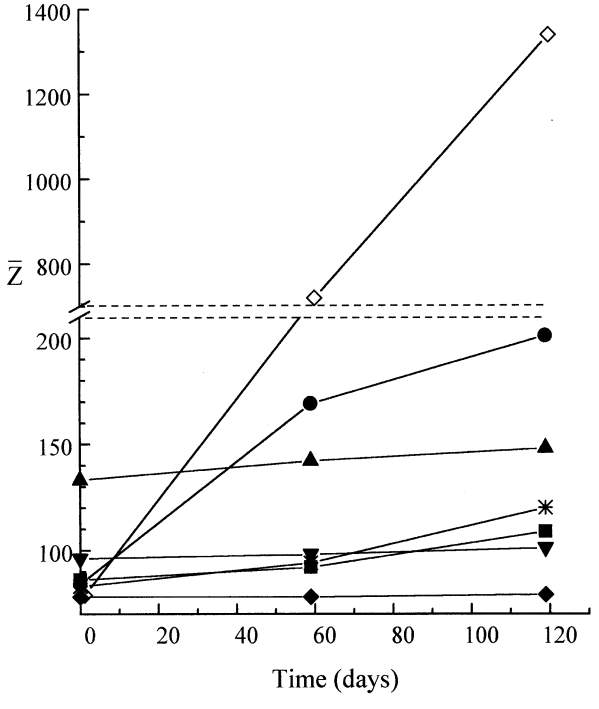

Fig. 2 The Z-average mean size of bare and polymer-bearing DMPC-liposomes stored in buffered medium at pH 8.2, as a function of time: (—◇—) DMPC, (—✳—) DMPC + PVA, (—◆—) DMPC + PVA-Al, (—▼—) DMPC + PVA-Prol, (—▲—) DMPC + PVA-Bul, (—●—) DMPC + PVP K-30, (—■—) DMPC + PVP K-90

DMPC-liposomes was found. Also, the increase in the size of most polymer-bearing liposomes is significantly retarded both in weakly acidic and basic media.

In strongly acidic medium (pH 1.2), the bare DMPC-liposomes are practically unstable (Fig. 1). Some polymers, however, exhibit a weak stabilizing effect for the liposomes, but only for 4–6 weeks. Among the polymers, the PVA-Bul copolymer seems to be the best stabilizer for the DMPC-vesicles at this pH, as well. The DMPC + PVA-Bul vesicles are the only ones the polydispersity index of which has remained below 1,0 after 120 days storage.

In mild environments, all the liposomes are much stable than that kept in the strongly acidic medium, and the polymer-free vesicles exhibit the lowest stability. The stability of the liposomes kept either at the weakly acidic or the weakly basic (Fig. 2). pH values was found to be less than that of stored in neutral medium. Nevertheless, it is worthwhile to point out that the DMPC + PVA-Bul, and the DMPC + PVA-Prol vesicles and also, the DMPC + PVP K-90 liposomes exhibited an enhanced stability in all these dispersion media. These results suggest again that the above polymers may prove to be efficient steric stabilizers for the DMPC-liposomes.

To elucidate the observed alterations in the stability of liposomes in acidic and basic environments, respectively, the changes in the chemical stability of the vesicles at various pH values should certainly be taken into account. In addition, though the uncharged polymers used are not very sensitive to the pH, some decomposition of acetal bonds in the copolymers after long storage in the strongly acidic medium must not be entirely precluded. Most biomolecules, such as lipids may undergo different degradation reactions both in acidic and basic environments [9, 17, 18]. The degradative processes that may take place on storage in phospholipids can affect the chemical stability of vesicles and so the kinetic stability of liposome dispersions, as well. Taking all these into account, it is very reasonable to asssume that the significantly lowest stability of both the bare and polymer-bearing liposomes in strongly acidic medium can be attributed to the more intensive chemical degradation of the phospholipid at pH 1.2 than in milder environments.

Conclusions

The uncharged polymers and copolymers attachable to the phospholipid bilayers can be used as effective steric stabilizers for DMPC-liposome dispersions. Among the polymers used, the PVA-Bul and PVA-Prol copolymers and the PVP of high molecular mass exhibited the most efficient stabilizing effect at each pH studied, indicating that the formation of a relatively thick polymer layer around the lipid bilayers ensures an enhanced and prolonged physical stability of the liposomes. The attachment of the macromolecules to the DMPC-liposomes is well reflected both in the size and the zeta potential of vesicles. Using these macromolecules, the physical stability of liposomes and the kinetic stability of liposome dispersions can be controlled.

Acknowledgement This work was supported by the Hungarian Science Foundation under Grant OTKA T 022923

References

1. Ishiwata H, Vertut-Doi A, Hirose T, Miyagima K (1995) Chem Pharm Bull 43:1005–1011
2. Sadzuka Y, Nakai S, Miyagishima A, Nozawa Y, Hirota S (1995) J Drug Targeting 3:31–37
3. Lasic DD (1996) Nature 380:561–562
4. Hansen CB, Kao GY, Moase EH Zalipsky S, Allen TM (1995) Biochim Biophys Acta1239:133–144
5. Grit M, Crommelin DJA (1992) Chem Phys Lipids 42:113–122
6. Woodle MC, Lasic DD (1992) Biochim Biophys Acta 1113:171–199
7. Plessis J, Ramachandran C, Weiner N, Müller DG (1996) Int J Pharm 127:273–278
8. Müller RH (1991) Colloidal Carriers for Controlled Drug Delivery and Targeting. Wissenschaftliche Verlagsgesellschaft, Stuggart, pp 3–22
9. Crommelin DJA, Schreier H (1994) In: Kreuter J (ed) Colloidal Drug Delivery Systems. Marcel Dekker, New York, pp 73–190
10. Csempesz F, Csáki K, Kovács P, Nagy M (1995) Colloids and Surfaces 101: 113–121
11. Szögyi M, Cserháti T, Tölgyesi F (1993) Lipids 28:847–851
12. Grohmann FL, Csempesz F, Szögyi M (1996) Acta Pharm Hun 66:197–202
13. New RRC (1990) In: New RRC (ed) Liposomes: A Practical Approach. Oxford UniversityPress, Oxford, pp 44–47
14. Carrión FJ, de la Maza A, Parra JL (1994) Colloid Interface Sci 164:78–87
15. Fleer GJ (1971) Thesis, Agricultural University of Wageningen
16. Cohen Stuard MA, Waajen FHWH, Dukhin SS (1984) Colloid Polym Sci 262:423–426
17. New RRC (1990) In: New RRC (ed) Liposomes: A Practical Approach. Oxford University Press, Oxford, pp 100–117
18. Lasic DD (1993) Liposomes: From Physics to Applications. Elsevier, Amsterdam, pp 34–40