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Induction of fusion in aggregated and nonaggregated liposomes bearing cationic detergents

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The addition of polyanionic polymers such as poly(aspartic acid) (PASP), DNA or dextran sulfate to liposomes composed of phosphatidylcholine (PC) and cholesterol (chol), and bearing the quaternary ammonium detergent [[[(1,1,3,3-tetramethylbutyl)cresoxy]ethoxy]ethyl]dimethylbenzylammonium hydroxide (DEBDA[OH]) resulted in liposome aggregation and fusion. Liposome–liposome fusion was studied by using fluorescently labeled liposomes and fluorescence-dequenching (DQ) methods. Addition of monoanions, such as aspartate or acetate, to liposomes bearing DEBDA[OH] caused neither their aggregation nor liposome–liposome fusion. Aggregation of liposomes bearing DEBDA[OH] by the binding pair avidin-biotin did not result in their fusion. Fusion in such aggregated liposomes was observed by the addition of chaotropic anions, such as nitrate or thiocyanate, or by PASP. A variety of other quaternary ammonium detergents behaved similarly to DEBDA[OH] in their ability to confer fusogenic properties upon PC/chol liposomes. The relevance of these findings to the mechanism of liposome–liposome fusion is discussed.

Introduction

It has been well-established that negatively charged liposomes such as those composed of phosphatidylserine are extensively fused in the presence of calcium or polycations such as polylysine [1–4]. Two separate functions have been attributed to the calcium or to the polycations in the process of liposome–liposome fusion. On the one hand, they cause aggregation of the liposomes, thus bringing their bilayers into close proximity, and, on the other hand, destabilization of the attached bilayers. Such destabilization is due to the ability of Ca^{2+} ions to cause dehydration of the phospholipid headgroups [5]. Aggregation of negatively charged liposomes, by itself, does not result in membrane fusion. Magnesium ions, for example, at relatively low concentrations, promote the aggregation of such liposomes without inducing their fusion [6].

In a previous work [7], we have shown that positively charged liposomes are also amenable to fusion processes. Such positively charged liposomes were constructed by the insertion of the cationic detergent, DEBDA[OH], into vesicles composed of neutral lipids, namely PC and chol. Aggregation and fusion of these liposomes was promoted by the addition of the negatively-charged polymer, PASP [7]. Similarly to Ca^{2+}-induced fusion of negatively charged liposomes, also with positively charged liposomes, a close attachment between the liposomes caused destabilization of the lipid bilayer and eventually promotion of liposome–liposome fusion. Fusion between liposomes was monitored by DQ measurements as well as by microscopic observations [7].

In the present work we have extended our previous studies [7] and have investigated in a more detailed manner the mechanism of membrane fusion in positively charged liposomes. Specifically, an attempt was made to study the question whether, in addition to close proximity between the liposome bilayers, neutralization of the DEBDA[OH] positive charges is required to allow liposome–liposome fusion.

Abbreviations: chol, cholesterol; PC, phosphatidylcholine; DEBDA [OH], [[[(1,1,3,3-tetramethylbutyl)cresoxy]ethoxy]ethyl]dimethylbenzylammonium hydroxide; DEBDA[Cl], [[[(1,1,3,3-tetramethylbutyl) cresoxy]ethoxy]ethyl]dimethylbenzylammonium chloride; PASP, poly (aspartic acid); REV, reverse-phase evaporated vesicles; DQ, fluorescence dequenching; *N*-Biot-PE, *N*-biotinylphosphatidylethanolamine; *N*-NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine; N-Rho-PE, *N*-(lissamine rhodamine B sofonyl)phosphatidylethanolamine; liposome-DEBDA[OH], liposomes bearing DEBDA[OH]; BSA, bovine serum albumin.

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Materials and Methods

Chemicals. PC from egg yolk, chol, DEBDA[OH], cetyl pyridinium chloride, benzalkonium chloride, benzyl dimethylhexadecyl ammonium chloride, avidin, DNA (herring sperm), PASP and polylysine were purchased from Sigma. [^{14}C]PC was purchased from Amersham. *N*-Biot-PE, *N*-NBD-PE and *N*-Rho-PE were purchased from Avanti Biochemicals (Birmingham, AL). DEBDA [Cl] and hexadecyl trimethyl ammonium bromide were from Fluka. SM-2 Bio-Beads were from Bio-Rad. Dextran sulfate was from Pharmacia. BSA was succinylated according to Gibbons et al. [8]. All other chemicals used were of analytical grade.

Preparation of REV. REV bearing the fluorescent molecules *N*-NBD-PE and *N*-Rho-PE were prepared as described earlier [7]. In short, chloroformic solutions of PC, chol, *N*-NBD-PE and *N*-Rho-PE were mixed. The mixture was evaporated and the thin layer obtained was dissolved in ether followed by the addition of acetate buffer (150 mM sodium acetate (pH 7.4)). The liposomes obtained were suspended to give 3 mg/ml of PC (PC : chol, w/w, 1 : 0.5 in acetate buffer) and kept at 4°C until used. Non-fluorescently labeled liposomes were prepared in the same way except that the fluorescent lipids were omitted.

Liposomes carrying biotin were prepared by the addition of *N*-Biot-PE (in chloroform) to the above chloroformic solution of PC and chol, to give a weight ratio of 0.1 : 1 : 0.5, respectively. Whenever needed, [^{14}C]PC or fluorescent probes was added to this lipid solution. The amount of liposomes is given in the present work by their PC content; however, in all the experiments described, the liposomes used were composed of PC and chol at a ratio (w/w) of 1 : 0.5.

Preparation of liposomes bearing DEBDA[OH]. Liposome-DEBDA[OH] were prepared essentially as described earlier [7]. Briefly, DEBDA[OH] was dried from its methanolic solution, dissolved in toluene, and kept at room temperature until use. Before use, the toluene was evaporated and the dry layer obtained was resuspended in acetate buffer. The DEBDA[OH] suspension was then added with continuous vortexing to a liposome preparation. Following incubation for 15 min with gentle shaking at 37°C, SM-2 Bio-Beads were added and the resulting suspension was further incubated for 20–30 min with vigorous shaking. Liposome-DEBDA[OH] were also prepared simply by the addition of a measured amount of DEBDA[OH] to liposome preparations.

Fluorescence measurements. All fluorescence measurements were performed in a Perkin-Elmer LS-5 spectrofluorometer at room temperature as described earlier [7]. NBD fluorescence was measured at 471 nm (excitation) and 527 nm (emission).

Determination of fluorescence dequenching. Percentage of DQ was determined according to the following equation [9]:

$$DQ = 100[1 - (F - F_1)/(F - F_0)]$$

where F (total fluorescence) is the fluorescence obtained after solubilization of the liposomes with 0.2% Triton X-100 and corrected for the quenching effect of Triton X-100 (×1.5) [7]; F_1 is the measured fluorescence of the liposomes at different times of incubation and F_0 is the fluorescence obtained before the incubation period.

Analytical methods. PC was estimated by the Stewart method [10]. For a quantitative estimation of fluorescently labeled liposomes, [^{14}C]PC was used as a marker (100 dpm/μg PC). DEBDA[OH] was determined according to Bradford [11], using DEBDA[Cl] as a standard. DEBDA[OH] content in a liposome suspension was determined by its absorption at 274 nm, following solubilization in 90% methanol [7].

Results

Induction of fusion in liposome-DEBDA[OH]: requirement for anionic polymers

Fusion between liposomes was monitored in the present work as previously reported [7], by estimating the increase in the fluorescence (DQ) obtained following incubation of fluorescently labeled and non-labeled liposomes. The results in Table I confirm previous observations [7] showing that the addition of the anionic polymer, PASP, to the above mixture of liposomes carrying the cationic detergent, DEBDA[OH], induced a high degree of DQ. In contrast, very little, if any DQ was observed following the addition of aspartic acid (Table I). A low degree of DQ was also obtained in the presence of other negatively charged, low-molecular weight substances, such as citric acid or ATP. As can be seen in Table I, the ability of BSA to promote DQ was greatly stimulated following insertion of negative charges to its polypeptide chain by succinylation. The view that polymers with a high density of negative charges are required for effective DQ, is further strengthened by the results showing that incubation with DNA or dextran sulfate also resulted in a high degree of DQ (Table I, Fig. 1). However, in the presence of relatively high concentrations of the latter polymers (> 20 μg/system), a decrease in the DQ was noted (Fig. 1).

Fusion can be induced only between liposomes bearing DEBDA[OH]

The results in Fig. 2 show that when non-fluorescent liposomes bearing DEBDA[OH] were mixed with fluorescently labeled liposomes lacking DEBDA[OH] at a weight ratio of 2 : 1 respectively, no DQ was obtained upon the addition of PASP. Under the same conditions,

TABLE I

Induction of DQ in liposomes bearing DEBDA[OH]: effect of various negatively charged molecules

Fluorescently labeled (6 μg) and non-labeled (60 μg) liposomes both of which contained DEBDA[OH]. (DEBDA[OH]: PC, w/w = 0.55) were mixed in a total vol of 0.6 ml of acetate buffer as described in Materials and Methods. Following determination of fluorescence (F_0), the various anions in acetate buffer were added and the extent of fluorescence (F_t) was determined again after 15 min of incubation at 26° C. The degree of DQ was calculated as described in Materials and Methods.

Anion added	(μg/ml)	DQ (%)
–	–	2
PASP	30	58
DNA	30	64
Dextran sulfate	30	64
BSA	8	9
	400	18
Succinylated-BSA	8	26
	80	47
	400	45
Aspartic acid	800	4
Citric acid	80	2
	800	8
	1500	10
ATP	1.5	3
	30	8
	300	18

DQ was observed, however, when DEBDA[OH] was present in the entire population of the liposomes, namely, in the non-fluorescent as well as in the fluorescent liposomes. From these results, it seems that only

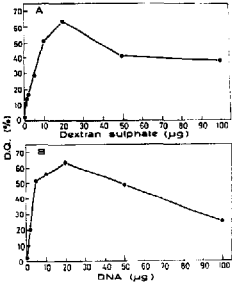

Fig. 1. Induction of DQ in liposomes bearing DEBDA[OH] by dextran sulfate and DNA: effect of concentration. Fluorescently labelled (6 μg) and non-fluorescent (120 μg) liposomes both bearing DEBDA[OH] ((DEBDA[OH]: PC, w/w = 0.5), were mixed as described in Table I. Various amounts of dextran sulfate (A) and DNA (B) were added to give a total vol of 0.6 ml (in acetate buffer). The fluorescence was determined following 15 min incubation at 26° C and the DQ was calculated as in Materials and Methods.

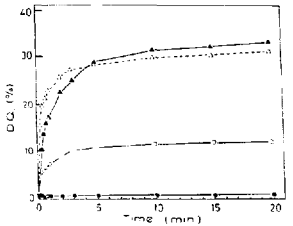

Fig. 2. Induction of DQ in mixtures containing liposomes lacking and bearing DEBDA[OH]. Fluorescently labeled liposomes (6 μg) lacking (●, ▼) and bearing DEBDA[OH] (○, △) were mixed with non-labeled liposomes bearing DEBDA[OH] (DEBDA[OH]: PC, w/w, 0.4). The w/w ratio between non-labeled and labeled liposomes was either 2:1 (○, ●) or 5:1 (△, ▲). Following 5 min of preincubation, PASP (20 μg) was added. Fluorescence was monitored and the DQ was calculated as described in Materials and Methods and previously [7]. The absolute degree of DQ in the present system is dependent on the w/w ratio between the non-fluorescent and the fluorescently labeled liposomes.

liposomes bearing DEBDA[OH] possess fusogenic properties. Fusion with liposomes lacking DEBDA-[OH] was observed, however, when such liposomes were mixed with relatively large amounts of liposomes-DE-BDA[OH]. As can be seen (Fig. 2), about 30% DQ was observed upon addition of PASP to a suspension containing liposomes bearing and lacking DEBDA[OH] at a w/w ratio of 5:1, respectively. These results may be explained by assuming redistribution of DEBDA[OH] between the entire population of the liposomes.

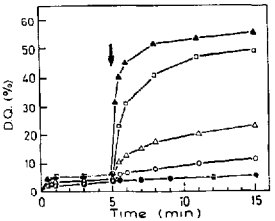

Fig. 3. The effect of externally added DEBDA[OH] on PASP-induced DQ in a liposome suspension. Fluorescently labeled (5 μg) and non-labeled liposomes (100 μg) were suspended in 0.6 ml of acetate buffer. A concentrated suspension of DEBDA[OH] (3 mg/ml) in acetate buffer was added to give a final concentration of 8 (●), 13 (▽), 18 (△), 26 (□), or 40 μg (▲) DEBDA[OH] per reaction mixture. After 5 min of incubation at 26° C (arrow), PASP (20 μg) was added, and the extent of fluorescence was monitored. All other experimental conditions were as described in Fig. 2 and in Materials and Methods.

The results in Fig. 3 show that addition of PASP to liposomes which had been preincubated with DEBDA[OH] for 5 min only, caused an immediate increase in the degree of DQ. Based on these observations, it should be inferred that PC/chol liposomes were converted into fusogenic liposomes simply by external addition of DEBDA[OH]. Unless otherwise stated, such liposomes were used throughout the present work.

The ability of various lypophilic cations to confer fusogenic properties upon PC/chol liposomes

The results in Fig. 4 show that, in addition to DEBDA[OH], five other cationic detergents also confer fusogenic properties upon PC/chol liposomes. This was inferred from the results showing that addition of PASP to mixtures of fluorescently labeled and non-labeled liposomes, bearing the various cationic detergents, induced a relatively high degree of DQ (Fig. 4). Only the addition of the detergent benzylhexadecyldimethylammonium chloride induced relatively high degree of DQ in the absence of non-labeled liposomes (Figs. 4, 6C) or even in the absence of PASP (Figs. 4, 6B). Evidently, in this case, the DQ observed is due to solubilization of the liposomes by the detergent rather than to liposome-liposome fusion.

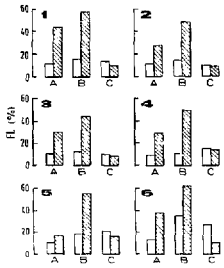

Fig. 4. Effect of various quaternary amines on the fusogenic ability of PC/chol liposomes. Mixtures of fluorescently labeled (5 μg) and non-labeled (50 μg) liposomes (A and B) or preparations of fluorescently labeled liposomes only (50 μg) (C) were suspended in 0.6 ml of acetate buffer. The following quaternary amines were added to the liposomes suspension: (1) DEBDA[OH]; (2) DEBDA[Cl]; (3) benzalkonium chloride; (4) hexadecyltrimethylammonium bromide; (5) cetylpyridinium chloride; and (6) benzylhexadecyldimethylammonium chloride, at 20 μg (A); and 50 μg (B) and (C). Following 5 min of incubation, the fluorescence was recorded (①), and then 20 μg of PASP were added. After an additional 5 min of incubation, the fluorescence was recorded again (②). Total fluorescence (100% fluorescence) is the fluorescence obtained after solubilization of the liposome suspension with 0.2% Triton X-100. The intrinsic fluorescence of the various liposome suspensions was about 7%.

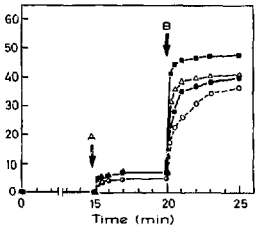

Fig. 5. Induction of DQ in liposomes preaggregated by the avidin-biotin binding pair. *N*-Biot-PE was inserted into liposomes composed of PC and chol as described in Materials and Methods. Fluorescently labeled (5 μg) and non-labeled (50 μg) of biotinylated liposomes were mixed and avidin was added to the suspension at the following amounts (μg) 0 (○), 1 (●), 5 (△) and 25 (■). To induce aggregation, the liposome mixtures were incubated (with gentle shaking) for 15 min at 37°C. At the end of the incubation period (arrow A), DEBDA[OH] (30 μg) was added and following 5 min of incubation at 26°C (Arrow B), PASP (20 μg) was added. All other experimental conditions were as described in Materials and Methods and the legend to Fig. 3.

Induction of fusion between liposomes preaggregated by the avidin-biotin binding pair

Induction of fusion by the addition of PASP to liposome-DEBDA[OH] is always accompanied by the formation of large agglutinates [7]. Evidently, the question arises whether aggregation of liposome-DEBDA[OH] by itself is sufficient to induce the fusion process. Aggregation of neutral liposomes can be promoted by using the interaction between the binding pair, avidin and biotin [12]. In our experiments, we have inserted *N*-Biot-PE into liposomes composed of PC and chol, thus obtaining biotinylated liposomes (see Materials and Methods and Fig. 5). Addition of avidin to such liposomes induced strong aggregation, as was observed by phase microscopy and followed by turbidity measurements (data not shown). The results in Fig. 5 show that incubation of a mixture of fluorescently labeled and non-labeled biotinylated liposomes in the presence of avidin did not induce any DQ. Furthermore, even the addition of DEBDA[OH] (arrow A in Fig. 5) to these aggregated liposomes did not cause any significant DQ. From these results it should be inferred that aggregation of liposome-DEBDA[OH], by itself, did not result in membrane fusion. A rapid and marked increase in fluorescence was observed only after addition of PASP (arrow B in Fig. 5) to the aggregated liposomes. The rate, as well as the final degree of DQ, were dependent upon the amounts of avidin added to the suspension of the biotinylated liposomes. Stimulation of liposome fusion was more effective at low amounts than at higher amounts of avidin (Fig. 5). The requirement for PASP

to induce DQ even in aggregated liposomes may suggest its direct involvement in the fusion step itself.

The function of PASP in the fusion of aggregated liposomes can be fulfilled by nitrate or thiocyanate salts, as is shown in the experiments summarized in Figs 6 and 7. The addition of potassium nitrate to avidin-biotin-aggregated liposomes (bearing DEBDA[OH]), caused a fast and significant increase in the fluorescence intensity (Fig. 6). Incubation of a mixture containing only fluorescently labeled liposomes with potassium nitrate did not result in increase of fluorescence (Fig. 6), thus supporting again the view that the DQ observed was not due to solubilization of the lipo.omes. Very little DQ was also observed upon the addition of potassium nitrate to non-aggregated liposomes (Fig. 6). As expected, the addition of PASP to these non-aggregated liposomes resulted in a fast and high DQ.

The results in Fig. 7 show that thiocyanate salts behaved very similar to potassium nitrate, namely, their addition to aggregated liposomes induced a rapid and substantial increase in the fluorescence. Thiocyanate (Fig. 7) and nitrate induced fusion only in liposome-DEBDA[OH]. No DQ was observed following the addition of thiocyanate to aggregated liposomes lacking DEBDA[OH] (Fig. 7). When DEBDA[OH] was added after the addition of thiocyanate to aggregated liposomes, it caused a slow and gradual increase in fluorescence. Subsequent addition of PASP to this system (Arrow B, Fig. 7 – caused a further increase in the degree of DQ. It is noteworthy that acetate (300 mM) was unable to substitute potassium nitrate or thiocyanate

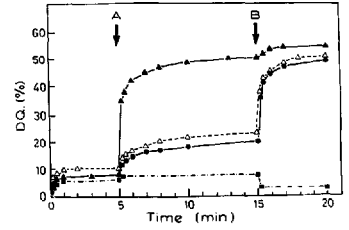

Fig. 6. Induction of DQ by nitrate salt in DEBDA-containing aggregated liposomes. Fluorescently labeled (5 μg) and non-labeled (50 μg) biotinylated liposomes were incubated for 15 min at 37°C in the absence (△) or in the presence (▲) of avidin (1 μg) as described in Fig. 5. At the end of the incubation period (0 - time of incubation), DEBDA[OH] (30 μg) was added and after 5 (arrow A) and 15 min (arrow B) of incubation (at 26°C), potassium nitrate (100 mM final concentration) and PASP (20 μg) were added, respectively. ■, suspension containing only fluorescently labeled biotinylated liposomes (50 μg) and avidin (1 μg). ●, a mixture of fluorescently labeled (5 μg) and non-labeled (50 μg) liposomes with avidin (1 μg).

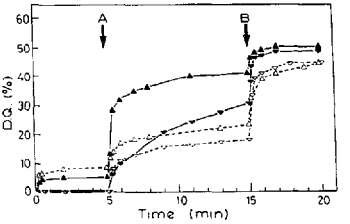

Fig. 7. Induction of DQ in aggregated liposomes: effect of thiocyanate. Fluorescently labeled and non-labeled biotinylated liposomes were mixed and incubated with (▲, ▼) or without (△, ▽) avidin as described in Fig. 6 (▲, △). DEBDA[OH] (30 μg) and thiocyanate (20 mM final concentration) were added at 0 and 5 min (arrow A) of incubation, respectively (▼, ▽); ammonium thiocyanate and DEBDA[OH] were added at 0 and 5 min (arrow A), respectively. PASP (20 μg) was added (arrow B) following 15 min incubation.

in their ability to induce DQ of aggregated liposomes (data not shown).

Discussion

The results of the present work show that several anionic polymers, such as PASP, dextran sulfate or DNA, promote the aggregation and fusion of PC/chol liposomes bearing the quaternary ammonium detergent, DEBDA[OH]. It has been suggested earlier [7] that the anionic polymer PASP serves as a bridge between the positively charged liposomes, bringing them to close proximity and eventually leading to their fusion.

Our present studies also show that the monomer, aspartic acid, neither promotes aggregation nor fusion of liposome-DEBDA[OH]. Moreover, even molecules carrying more than one negative charge, such as ATP, induced very little fusion. BSA, which is also negatively charged at the pH of the reaction, practically failed to induce fusion unless additional charges were introduced to its chain. Thus, it appears that in order to induce aggregation and subsequently fusion, the inducer should possess a high density of negative charges. In this regard, it should be added that a requirement for a polymer with high density of positive charges was claimed as a requisite for induction of fusion in negatively charged liposomes [4].

No clear distinction could be made between the aggregation and the fusion steps in the present system. Once aggregation was promoted, a process of membrane–membrane fusion followed. In order to differentiate between these two steps, we have constructed liposomes bearing the ligand biotin. Such liposomes could be aggregated by the addition of avidin. Fluores-

cence energy transfer measurements have clearly shown that aggregation of the biotinylated liposomes did not result in liposome–liposome fusion. Moreover, no significant DQ was observed following the addition of DEBDA[OH] to these aggregated liposomes, showing that aggregation by itself is not sufficient to allow fusion [7]. On the other hand, addition of PASP to preaggregated liposomes induced their fusion. Evidently, when added to preaggregated liposomes, PASP is not required as bridging agent. This may suggest that in addition to its function as an aggregating agent PASP also plays an active role in the fusion step itself.

Using this system, we have shown that certain anions could replace PASP in its role in the fusion step. Addition of relatively low concentration of either nitrate or thiocyanate salts to aggregated liposomes bearing DEBDA[OH], promptly induced liposome fusion, as was inferred from the DQ studies. Nitrate and thiocyanate could not bridge between liposomes and therefore did not cause fusion of unaggregated liposomes. Also these anions did not have any effect on the fluorescence of liposomes lacking DEBDA[OH]. This is in spite of their being chaotropic agents and being known to destabilize proteins and biological membranes [13]. Nitrate and thiocyanate are also known to lower the surface charge density in micelles of Hyamine 1622 (an analog of DEBDA[OH]) [14]. It is conceivable that in the present system these anions act in a similar way, namely by lowering the surface charge density of liposomes bearing DEBDA[OH], and thus removing the last barrier that have prevented their fusion. Acetate, which does not have any effect on the surface charge density of hyamine micelles [14], did not induce fusion in aggregated liposome-DEBDA[OH] even at concentrations of 0.5 M (data not shown). Therefore, it can be concluded that binding of polyanions also such as PASP and DNA, to the positively charged liposomes resulted in reduction of the liposome surface charge density and consequently led to membrane fusion.

Our observation on PASP-induced fusion of aggregated liposome-DEBDA[OH] clearly demonstrate that the binding pair avidin-biotin did not block the fusion process. Inhibition of fusion processes by high-molecular weight binding molecules has been demonstrated before in other systems [3,15]. In the present system, however, a partial inhibition of fusion was observed at high concentrations of DNA, dextran sulfate and avidin.

Several years ago, Martin and MacDonald [16] introduced the positively charged molecule stearylamine into PC liposomes. These liposomes were able to attach to negatively charged membranes, such as human erythrocytes. Liposome–membrane fusion was induced however only with liposomes which in addition to stearylamine contained lysolecithin. It is noteworthy that similar to lysolecithin, DEBDA[OH] possesses detergent activity and is able to cause destabilization of phospholipid bilayers and to increase their permeability [7]. Thus, in the fusion process described in the present work, DEBDA[OH] fulfils the function of both stearlyamine and lysolecithin in the system described earlier [16]. In the aggregation step, it functions as a hook for the added polyanions, and in the fusion step it serves as a detergent which destabilizes the lipid bilayer and renders them susceptible to the fusion process.

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