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Use of a dialyzable short-chain phospholipid for efficient solubilization and reconstitution of influenza virus envelopes

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Abstract

Virosomes are reconstituted viral envelopes that can serve as vaccines and as vehicles for cellular delivery of various macromolecules. To further advance the use of virosomes, we developed a novel dialysis procedure for the reconstitution of influenza virus membranes that is easily applicable to industrial production and compatible with encapsulation of a variety of compounds. This procedure relies on the use of 1,2-dicaproyl-*sn*-glycero-3-phosphocholine (DCPC) as a solubilizing agent. DCPC is a short-chain lecithin with detergent-like properties and with a critical micelle concentration of 14 mM. DCPC effectively dissolved the influenza virus membranes after which the nucleocapsids could be removed by ultracentrifugation. The solubilized membrane components were reconstituted either by removal of DCPC by dialysis or by a procedure involving initial dilution of the solubilized membrane components followed by dialysis. Both protocols resulted in removal of 99.9% of DCPC and simultaneous formation of virosomes. Analysis of the virosome preparations by equilibrium sucrose density gradient centrifugation revealed co-migration of phospholipid and protein for virosomes produced by either method. Moreover, both virosome preparations showed morphological and fusogenic characteristics similar to native influenza virus. Size, homogeneity and spike density of the virosomes varied with the two different reconstitution procedures employed. The recovery of viral membrane proteins and phospholipids in the virosomes was found to be higher for the dilution/dialysis procedure than for the simple dialysis protocol. This novel procedure for the production of virosomes is straightforward and robust and allows further exploitation of virosomes as vaccines or as drug delivery vehicles not only in academia, but also in industrial settings.

Keywords: Short-chain lecithin; Dialysis; Reconstitution; Influenza virus; Virosome

1. Introduction

Virosomes are reconstituted viral envelopes composed of membrane lipids and viral spike glycoproteins but devoid of viral DNA or RNA. The external surface of virosomes resembles that of virus particles with the spike proteins protruding from the membrane while the lumen of virosomes is empty. Virosomes were first described by Almeida et al. by insertion of purified spike proteins of the influenza virus into pre-formed liposomes [1]. Thereafter, reconstitution of a range of viral envelopes has been achieved including those of Sendai virus [2–4], Semliki Forest virus (SFV, [5,6]), vesicular stomatitis virus (VSV, [7,8]) and Sindbis virus [9]. Since virosomes are virus-like particles, which display the viral envelope glycoproteins and thus the most important viral antigens for humoral immune responses in a native configuration, they are highly suitable for use as vaccines [10–12]. Moreover, the receptor binding and membrane fusion properties of the viral envelope glycoprotein can be preserved which allows the use of virosomes as transport vehicles for cellular delivery of biologically active macromolecules (reviewed in: [13–16]).

Influenza virus is one of the viruses most commonly used for virosome production. It possesses two envelope glycoproteins; hemagglutinin (HA) and neuraminidase (NA). HA, the major

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spike protein, is involved in receptor binding and membrane fusion, while NA is involved in virus budding. HA is a class I fusion protein and associates into trimers. HA0, the precursor of HA, is cleaved into HA1 and HA2, which are linked by two disulfide bonds. When influenza encounters a target cell, HA1 binds to sialic acid residues on the surface of the cell, after which, the virus enters the endocytic pathway. The mildly acidic pH in the endosome induces a conformational change in HA. Thereby, the N-terminal fusion peptide of HA2, previously buried in the stem of the HA trimer, is exposed and inserted into the endosomal membrane. Subsequently, the protein refolds into a hairpin structure, which brings the viral membrane and the target membrane into close proximity thereby inducing fusion. Through the merging of the two membranes, the viral nucleocapsid containing the genetic material is released into the cytoplasm [17–21].

A prerequisite for successful use of virosomes as vaccines or as delivery vehicles is reconstitution of the membrane proteins such that their immunogenic properties and receptor binding and membrane fusion activities are retained. Previously, we have developed a method for the functional reconstitution of influenza virus membranes [22]. This method is based on solubilization of the viral membrane by the detergent octaethyleneglycol mono(*n*-dodecyl)ether ($C_{12}E_8$). After the solubilization step, the nucleocapsid of the virus is removed by ultracentrifugation and reconstitution of the viral membranes is accomplished by removal of $C_{12}E_8$ through adsorption onto a hydrophobic resin (Bio-Beads SM-2). Virosomes produced by this method fuse in a pH-dependent manner similar to native influenza virus [22]. Successful delivery of virosome-encapsulated proteins to target cells in vitro was demonstrated for gelonin [23], subunit A of diphtheria toxin [24] and ovalbumin [25]. Moreover, when cationic lipids were added prior to membrane reconstitution, virosomes proved suitable for binding/encapsulation of DNA [26] or siRNA [27] and subsequent delivery of these nucleic acids. In all cases, inhibition of HA-mediated fusion completely abolished delivery, indicating that virosomes indeed exploit the viral fusion mechanisms to transport their cargo to the target cell cytosol.

Virosomes are also suitable for delivery of encapsulated macromolecules in vivo as was demonstrated by immunisation of mice with antigen-containing virosomes. Administration of virosomes containing peptides derived from the influenza nucleoprotein or intact ovalbumin induced strong cytotoxic T lymphocyte responses against the respective antigens indicating that the encapsulated peptides and proteins gained access to the cytosol and the MHC class I presentation route of antigen-presenting cells [28,29].

Although the $C_{12}E_8$ method is very useful for the production of virosomes, it suffers from some inherent drawbacks. Production according to this method, involves batch processes, often in open systems. This is a challenging situation for industrial processing and requires special attention to obtain and maintain sterility. Moreover, in case virosomes are used as transport vehicles, compounds to be encapsulated could be adsorbed to or inactivated by the hydrophobic resin [24]. Application of dialysis as a means of detergent removal circumvents the above mentioned complications, since it would avoid the use of Bio-Beads while dialysis is also up-scalable and applicable in a closed continuous process, for example, by the use of ultrafiltration.

Efficient detergent removal by dialysis requires the use of a detergent with a relatively high critical micelle concentration (c.m.c.). Detergents commonly used for reconstitution of membrane proteins like $C_{12}E_8$ and Triton X-100 have low c.m.c.s (<1 mM) and are, therefore, not suitable. A detergent with a high c.m.c. that has been applied frequently is octylglucoside (OG), which has a c.m.c. of approximately 25 mM. Previous attempts to reconstitute the influenza virus envelope using OG, however, have failed [22]. Similar efforts with 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), cholate and deoxycholate did not result in the formation of fusion-active virosomes either.

Short-chain lecithins represent a class of amphiphilic molecules that exhibit detergent-like properties ([30], reviewed in: [31]). The dispersing power of short-chain lecithins was first observed by Gabriel and colleagues in 1984 [32]. Later, the short-chain lecithin 1,2-diheptanoyl-*sn*-glycero-3-phosphocholine (DHPC) was used in a series of studies on the reconstitution of a variety of integral membrane proteins, such as the $_D$-glucose cotransporter, sucrase-isomaltase, Ca2+-ATPase and a cholesterol transporter into proteoliposomes [33–36]. Kessi et al. [33] and Shivanna and Rowe [34] have shown that DHPC is superior to conventional detergents in preserving enzyme activity. Furthermore, activity seemed unimpaired over a broad range of DHPC concentrations. However, since DHPC has a relatively low c.m.c. (2 mM), it is not easily removed by dialysis. As the c.m.c. increases with decreasing chain length [30], we hypothesized that the short-chain lecithin 1,2-dicaproyl-*sn*-glycero-3-phosphocholine (DCPC), with a chain length of six carbon atoms and a c.m.c. of 14 mM, would be appropriate for the reconstitution of membrane proteins by dialysis.

In this report, we demonstrate that DCPC is suitable for the solubilization of influenza virus membranes and that it is almost completely removed by dialysis. Two reconstitution procedures are presented in which the rate of DCPC removal is varied. Both methods result in the generation of virosomes with morphological characteristics and fusogenic properties similar to those of influenza virus. Yet, the removal rate influences size, homogeneity and spike density of the virosomes. Moreover, upon reconstitution using the fast detergent removal protocol, we observed higher protein and phospholipid recoveries than after reconstitution by a slow detergent removal procedure. Reconstitution of the influenza virus envelope from DCPC-solubilized viral membranes by means of dialysis is efficacious and compatible with virosome production on industrial scale. This new procedure may facilitate the production of virosomal vaccines and stimulate the use of virosomes for cellular delivery purposes.

2. Materials and methods

2.1. Materials

DCPC was obtained from Avanti Polar Lipids, Inc (Alabaster, Alabama, USA). 1-hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphocholine

(pyrPC) was purchased from Molecular Probes (Eugene, OR, USA). Slide-A-Lyzers, all with a molecular weight cut off (MWCO) of 10 kDa, were obtained from Pierce (Rockford, Illinois, USA). Influenza virus (A/Panama/2007/99 (H3N2)) was kindly provided by Solvay Pharmaceuticals B.V. (Weesp, The Netherlands).

2.2. Virus inactivation

Where indicated influenza virus was inactivated by β-propiolactone (BPL 98%; Acros Organics, Geel, Belgium) treatment prior to virosome preparation. For this purpose, BPL was added to the virus suspension (1.78 μmol/mL viral phospholipids in 125 mM Na-citrate, 35–47% sucrose (w/w), pH 7.3–8.0) at a final dilution of 1:1000 (v/v). The virus suspension was incubated for 24 h with gentle mixing in the dark at room temperature. Residual BPL was removed by dialysis against HEPES-buffered saline (HBS; 5 mM HEPES, 0.15 M NaCl, pH 7.4) overnight at 4 °C. Alternatively, influenza virus was inactivated by treatment with formaldehyde (0.02% (w/v) final concentration) overnight at 4 °C.

2.3. Reconstitution of virosomes

Influenza virus (1.5 μmol membrane phospholipid) was sedimented by ultracentrifugation (100,000×*g* for 1 h at 4 °C) and the virus pellet was resuspended in 375 μl HBS with a 1 ml syringe equipped with a 25-gauge needle. The viral membrane was dissolved by addition of 375 μl 200 mM DCPC in HBS. The suspension was incubated on ice for 30 min and the nucleocapsid was pelleted by ultracentrifugation (100,000×*g* for 30 min at 4 °C). Reconstitution of the virus membrane was accomplished by removal of DCPC by means of dialysis. Two reconstitution procedures were applied, varying in the rate of detergent removal. In the course of the first procedure, the supernatant containing the viral lipids and membrane proteins was dialyzed against 2 L of HBS overnight at 4 °C in a 0.5–3 ml Slide-A-Lyzer. The buffer was exchanged once and the supernatant was dialyzed for another 4 h. This method is referred to as 'slow reconstitution'. In the second procedure, the supernatant containing the viral lipids and membrane proteins was instantly diluted 5 times with HBS to reach a DCPC concentration of 20 mM (c.m.c. of DCPC: 14 mM). The diluted mixture was then dialyzed as described above. This procedure is referred to as 'fast reconstitution'.

After dialysis the preparations (termed 'crude virosome preparation') were applied to a discontinuous sucrose gradient (10%/50% w/v sucrose in HBS) and centrifuged for 1.5 h (100,000×*g* at 4 °C) to separate non-incorporated material from the reconstituted virus membranes. The final preparations (termed 'purified virosome preparation') were recovered from the interface of the two sucrose layers, transferred to a 0.1–0.5 ml Slide-A-Lyzer and dialyzed against 2 L HBS overnight at 4 °C to remove the sucrose.

2.4. Biochemical analyses

Virosomes were analyzed for protein content by a micro Lowry assay [37] and subjected to SDS-PAGE followed by Coomassie blue staining. Phospholipid content was determined by a phosphate assay [38]. Residual DCPC in the virosomes was determined as follows: Virosomes and virus (100 nmol phospholipid) were subjected to lipid extraction [39]. The extracted lipids were applied onto a silica gel plate for thin-layer chromatography (TLC plate, RP-8 F254s, Merck, Darmstadt, Germany) and eluted with a solvent mixture of methanol:chloroform:water (10:5:4, by vol.). Phospholipids were stained using a phosphorus spray containing 40 ml reagent 1 (20.5 g of molybdic anhydride (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) in 500 ml concentrated sulfuric acid), 40 ml of reagent 2 (0.89 g of molybdenum metal (Sigma-Aldrich) in 250 ml reagent 1) and 120 ml water [40]. TLC plates were sprayed with a mixture of the phosphorus spray:water:methanol (1:1:2, by vol.), resulting in a better color development than using the phosphorus spray alone. The TLC plates were scanned using a flatbed scanner and the DCPC spots were quantified using image analysis software (Zero-Dscan, version 1.0). The DCPC content of the virosomes was determined by comparison with DCPC calibrants.

2.5. Analysis by equilibrium density centrifugation

Virosomes were analyzed for co-migration of phospholipids and protein on a linear sucrose density gradient (10–60% sucrose (w/v) in HBS). The sucrose gradients were centrifuged until equilibrium at 300,000×*g* for 65 h at 4 °C. Fractions were analyzed for protein and phosphate content as described above.

2.6. Membrane fusion

For determination of their fusogenic properties, virosomes were labeled with 10 mol% pyrPC relative to viral phospholipids. PyrPC was dissolved in chloroform and the required amount was dried under a stream of nitrogen gas and kept under vacuum for 2 h to remove remaining traces of chloroform. The supernatant containing the viral membrane lipids and proteins was added to the dried pyrPC and incubated for 30 min at room temperature prior to reconstitution. To study the fusion characteristics of the virosomes, fusion assays and fusion inactivation were performed as described [25,41]. The initial rate of fusion was determined from the slope of the fusion curve during the first 8 s after initiation of fusion.

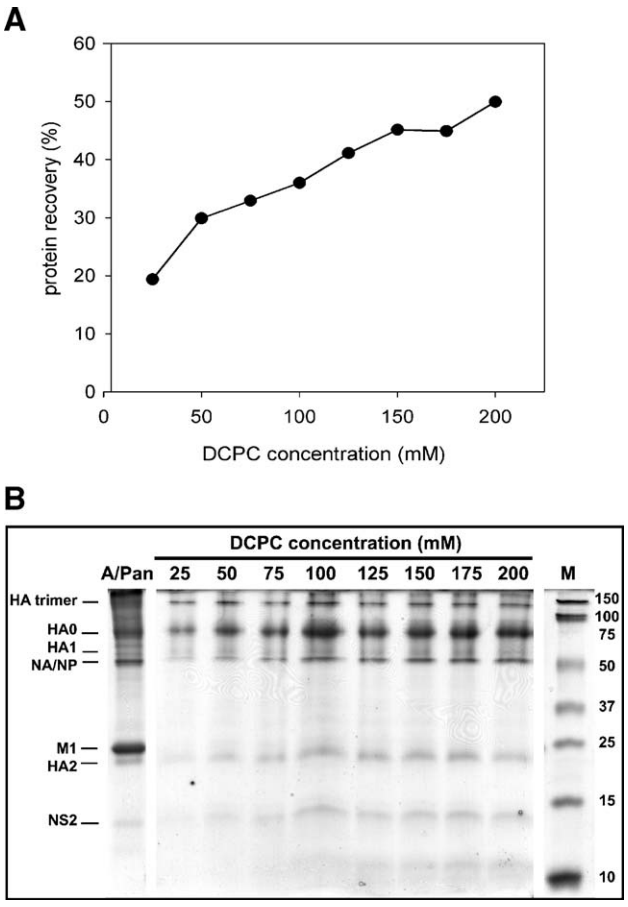

Fig. 1. Efficiency of virus solubilization by DCPC. Influenza virus (2 mM viral lipid) was treated with increasing concentrations of DCPC. Non-solubilized material was removed by ultracentrifugation and the dissolved virus components (supernatant) were subjected to analysis. (A) Protein content (% of total viral protein) of the supernatant after solubilization at the indicated concentrations of DCPC. (B) SDS-PAGE analysis of the solubilized proteins. Under the non-reducing conditions primarily HA is visible, however, traces of HA1 and HA2 were also detected. A/Pan, A/Panama virus; M, protein marker, figures indicate molecular weight in kDa.

2.7. Electron microscopy

Virus or virosomes were dialyzed against ammonium acetate buffer (75 mM ammonium acetate, 2.5 mM HEPES, pH 7.4) overnight at 4 °C. The virosome suspensions were applied to glow-discharged 200 mesh grids covered with a Formvar film. Virosomes or virus were stained with freshly prepared 3% ammonium molybdaat, pH 7.2 and analyzed with a Philips CM 12 electron microscope.

3. Results

3.1. Conditions for virus solubilization by DCPC

In an attempt to develop a novel method for the production of virosomes as an alternative for the $C_{12}E_8$ method, with its limitations for manufacturing and applications, we studied the use of DCPC as a dialyzable detergent-like agent. In order to determine optimal conditions for the solubilization of the viral membrane, influenza virus (2 μmol of viral lipid in 1 mL) was dissolved in buffers with increasing concentrations of DCPC. After 30 min incubation at 4 °C, the non-solubilized material was removed by ultracentrifugation and the supernatants were analyzed for protein content. The protein detected in the supernatants increased with increasing DCPC concentrations (Fig. 1A). At DCPC concentrations of 150 mM and higher the amount of solubilized viral protein exceeded the amount of total membrane proteins present in the influenza virus, which is approximately 40% of the total viral protein [42]. This excess indicates that proteins other than membrane proteins were dissolved under these conditions.

SDS-PAGE analysis of the supernatants revealed that under all conditions tested HA and a protein migrating at the molecular weight of NA/NP, most likely NA, were the major proteins solubilized (Fig. 1B). The matrix protein M1, the protein that is most prominent in intact virus particles, was not detected in the supernatant fractions. Up to DCPC concentrations of 100 mM the amount of solubilized HA and NA increased. Beyond this concentration, no significant increase in HA/NA recovery was observed and increasing amounts of other proteins such as the non-structural protein 2 (NS2) were detected. Therefore, the 100 mM DCPC concentration was chosen for the solubilization of the influenza virus membranes in the subsequent experiments.

For the determination of the maximal amount of virus that can be efficiently solubilized, increasing amounts of virus were dissolved in a fixed volume of 100 mM DCPC buffer (0.5 mL) and the supernatants were analyzed for protein content. At virus concentrations of 1 and 2 mM viral lipid, 35% of viral protein was solubilized. A further increase in virus concentration resulted in lower protein recovery (data not shown). Thus, 2 mM viral lipid was the highest virus concentration at which maximal recovery of membrane protein was obtained and this concentration was therefore used in the subsequent experiments.

3.2. Biochemical and biophysical characterization of crude virosome preparations

Reconstitution of the virus membrane was achieved by removal of DCPC by dialysis from the dissolved viral membrane components. The rate of DCPC removal was varied by applying two different reconstitution methods. In the first protocol, the supernatant containing the solubilized membrane components was directly dialyzed, starting from a DCPC concentration of 100 mM. In this way, DCPC is gradually removed from the mixed micelles. This method will be referred to as 'slow reconstitution'. In the second protocol, the supernatant containing the membrane components was diluted 5 times with buffer prior to dialysis. This procedure reduces the DCPC concentration instantaneously from 100 mM to 20 mM, the latter being close to the c.m.c. of DCPC (14 mM). Consequently, fast removal of DCPC from mixed micelles is

Table 1
Protein and phospholipid recoveries, protein-to-phospholipid ratios and densities of virosomes during different stages of preparation

	Viral membrane protein recovery (%)		Viral phospholipid recovery (%)		Protein/phospholipid		Average density (g/mL)	
	Slow reconstitution	Fast reconstitution	Slow reconstitution	Fast reconstitution	Slow reconstitution	Fast reconstitution	Slow reconstitution	Fast reconstitution
Virus	100[a]	100[a]	100	100	2.15[a]	2.15[a]		
Crude virosome preparation	81	90	84	90				
Crude virosomes peak fractions	34 (42)	52 (58)	57 (68)	64 (71)	0.84	1,11	1,136	1,149
Purified virosome preparation	38	43	41[b]	60[b]				
Purified virosome peak fraction	29 (77)	38 (89)	36 (87)	54 (90)	1,07	1,16	1,140	1,145

Data refer to crude and purified virosome preparations and virosome peak fractions of sucrose density gradients. Peak fractions 7,8 and 9 of gradients shown in Fig. 3A and B containing the crude virosome preparations were pooled for calculations. For the gradients containing the purified virosome preparations, peak fractions 5–8 (Fig. 3C) and 6–8 (Fig. 3D) for the slow and fast reconstitution, respectively, were pooled for calculations. The data were obtained from a typical experiment. Numbers between brackets indicate percentage recovery with respect to phospholipid or protein in the virosome preparation applied to the sucrose density gradient.

[a] Based on membrane proteins (~40% of total viral protein).

[b] not correct for residual DCPC.

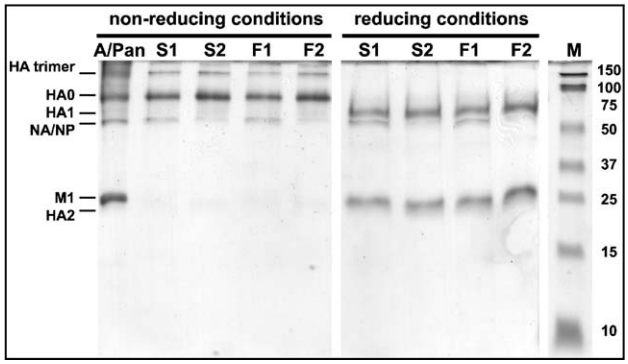

Fig. 2. SDS-PAGE analysis of virosomes. Virosome preparations obtained by the slow reconstitution procedure (S) and the fast reconstitution procedure (F) were analyzed before (S1, F1) and after (S2, F2) purification on a discontinuous sucrose gradient. Analysis was performed under reducing and non-reducing conditions. A/Pan, A/Panama virus; M, protein marker, figures indicate molecular weight in kDa.

achieved during the dilution step. This method will be referred to as 'fast reconstitution'.

After reconstitution of the membrane components by the slow or the fast procedure, the 'crude' virosome preparations obtained, contained approximately 80–90% of the viral membrane proteins and viral phospholipids (Table 1). The proteins consisted primarily of HA and small amounts of NA, as was shown by SDS-PAGE analysis (Fig. 2, samples S1, F1).

To determine the efficiency of the two different reconstitution procedures, the crude virosome preparations were analyzed by equilibrium centrifugation on linear sucrose density gradients. Analysis of the preparation obtained by the slow reconstitution procedure revealed co-migration of proteins and phospholipids in fractions 7, 8 and 9 indicating that these fractions contained vesicles with proteins incorporated in their membranes (Fig. 3A). Fractions 10 and 11 contained mainly proteins and little phospholipid. It is likely that these proteins formed aggregates with few phospholipid molecules incorporated. Analysis of the preparation obtained by the fast reconstitution procedure revealed overlapping protein and phospholipid peaks again in fractions 7, 8 and 9 (Fig. 3B). Small amounts of protein aggregates with little phospholipid were detected in fractions 10 and 11. The recovery of proteins in the virosome fractions 7, 8 and 9 was higher for the fast reconstitution procedure (approximately 60%) than for the slow reconstitution procedure (approximately 40%), while the

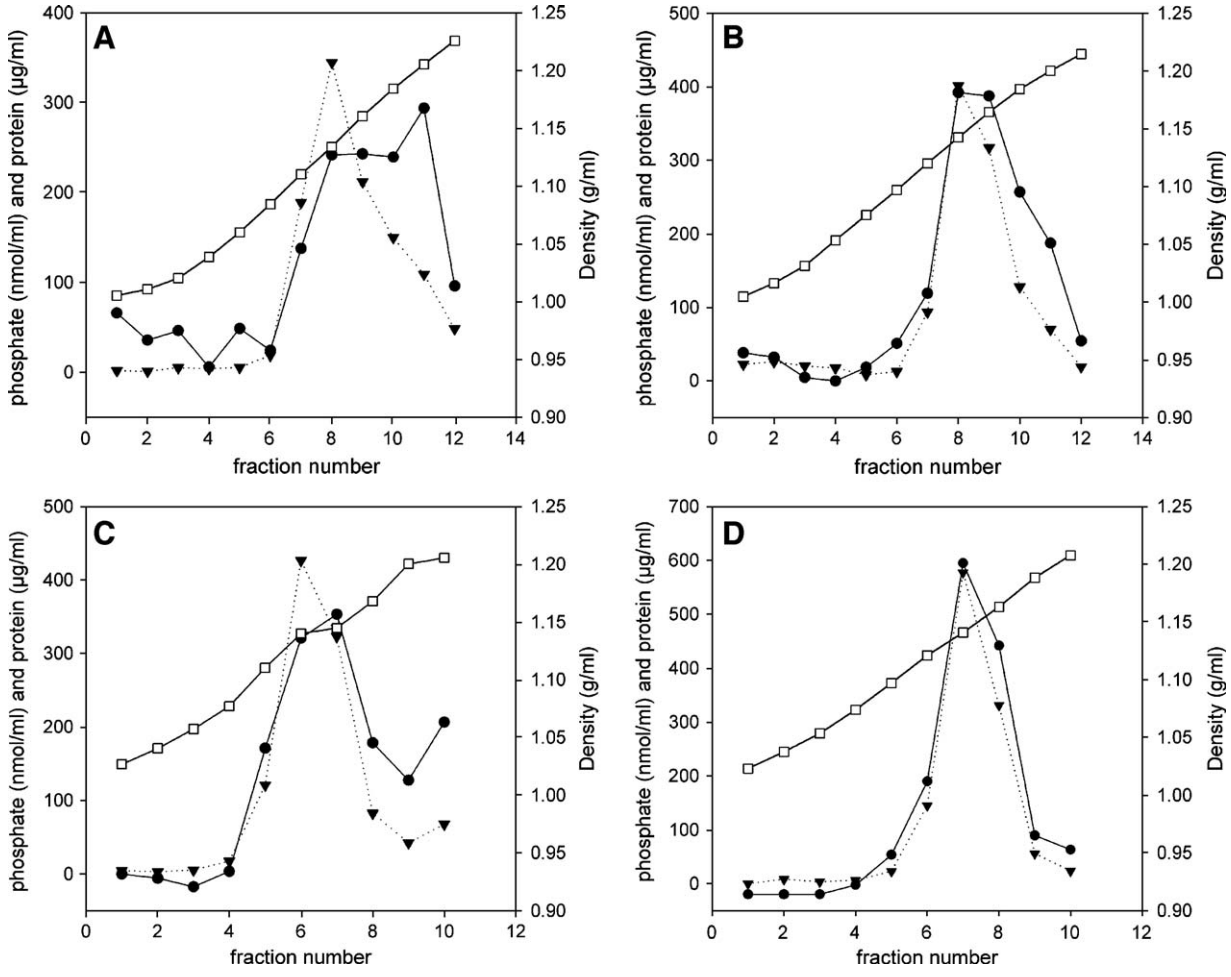

Fig. 3. Analysis by equilibrium density centrifugation. Slow reconstitution virosomes (A, C) and fast reconstitution virosomes (B, D) were analyzed directly after reconstitution (crude virosomes: A, B) or after purification on a discontinuous sucrose gradient (purified virosomes: C, D). Filled triangles represent phospholipid, filled circles represent protein and open squares represent the density.

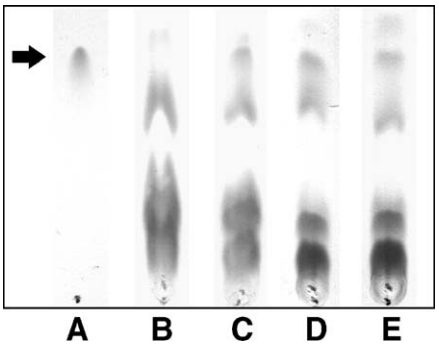

Fig. 4. Phospholipid analysis by TLC to determine residual DCPC in virosomes. (A) 10 nmol DCPC; (B) 100 nmol A/Panama viral phospholipids; (C) 100 nmol A/Panama viral phospholipids spiked with 10 nmol DCPC; (D) 100 nmol phospholipids of slow reconstitution virosomes; (E) 100 nmol phospholipids of fast reconstitution virosomes. Before elution, lipids were extracted from the virosome or virus suspensions by the Bligh and Dyer method. Phospholipids were visualized by a phosphate stain. Arrow indicates DCPC spots.

phospholipid recovery was approximately 70% for both methods (Table 1).

3.3. Biochemical and biophysical characterization of purified virosomes

The crude virosome preparations were purified on a discontinuous sucrose gradient to remove non-incorporated proteins and lipids. In a typical experiment the resulting purified virosomes contained approximately 40% of the initial membrane proteins for both methods and approximately 40% and 60% of viral phospholipids for the slow and fast reconstitution procedures, respectively (Table 1). SDS-PAGE analysis showed that the amount of NA in the purified virosomes declined compared to the crude preparations, the purified virosomes containing mainly HA (Fig. 2, S2 vs. S1 and F2 vs. F1). Therefore, it would appear that NA is incorporated less efficiently than HA. SDS-PAGE revealed no differences in the protein profiles of virosomes prepared by the two different reconstitution methods.

The purified virosomes were further characterized by equilibrium density gradient centrifugation. Generally, the phospholipid and protein migration patterns of virosomes produced by the slow reconstitution procedure revealed some variability. The majority of protein and phospholipid of a typical preparation was detected in fractions 5–8 (Fig. 3C). It appeared that the protein aggregates, present in the crude virosome preparation, had been removed to a large extent by the purification step, however, traces of non-incorporated protein were still found in fraction 10. The pooled virosome fractions (5–8) contained vesicles with a mean density of 1.140 g/mL and a protein-to-phospholipid ratio of 1.07 mg/μmol (Table 1). Routinely, virosomes produced by the fast reconstitution revealed highly reproducible phospholipid and protein migration patterns on a linear sucrose density gradient. A typical virosome preparation showed prominent protein and phosphate peaks in fractions 6, 7 and 8 (Fig. 3D). When pooled, these fractions contained vesicles with a density of 1.145 g/mL and a protein-to-phospholipid ratio of 1.16 mg/μmol (Table 1). Thus, virosomes produced by the fast reconstitution method are more densely packed with HA.

3.4. Determination of residual DCPC

To evaluate whether DCPC was removed efficiently by the dialysis procedure, the DCPC content of the purified virosomes was determined by thin layer chromatography (TLC). Using an eluent consisting of methanol:chloroform:water (10:5:4 by vol.) DCPC could be clearly separated from the more hydrophobic viral lipids (Fig. 4A, B and C). Densitometric analysis of the DCPC spots showed that slow reconstitution virosomes contained approximately 8 nmol DCPC per 100 nmol virosomal phospholipids and virosomes prepared by the fast reconstitution procedure contained approximately 6 nmol DCPC (Fig. 4D and E). Thus, residual DCPC is less than 10% of the virosomal

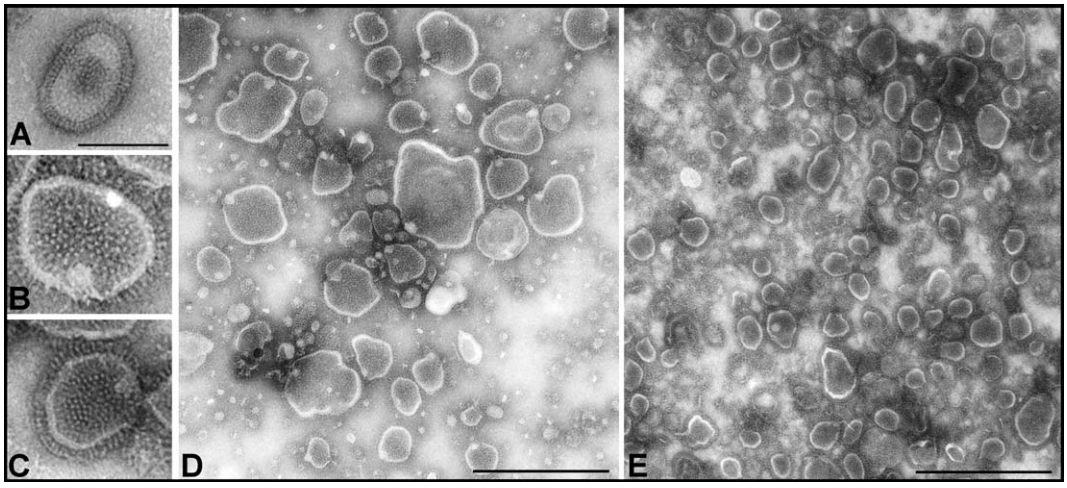

Fig. 5. Analysis of virus and virosome preparations by negative stain transmission electron microscopy. (A) A/Panama virus; (B) slow reconstitution virosomes; (C) fast reconstitution virosomes; (D) overview of slow reconstitution virosomes and (E) overview of fast reconstitution virosomes. Bar in A, B and C = 100 nm, bar in D and E = 500 nm.

phospholipids for both reconstitution procedures. In either case the dialysis protocol effectively removed more than 99.9% of the initial amount of DCPC used for solubilization of the viral membrane.

3.5. Electron microscopy

Virosomes produced by both reconstitution methods resemble native virus, as revealed by negative stain electron microscopy (Fig. 5A–C). The preparations contained predominantly vesicles densely covered with spikes protruding from their membranes. Virosomes obtained by the slow reconstitution method and purified on a discontinuous sucrose gradient had an average diameter of 146±64 nm, which was calculated from 98 representative virosome particles (Fig. 5D). This diameter is larger than the average diameter of native virus, which is 100 nm (±20) [42]. Additionally, small protein aggregates (rosettes) were observed likely representing the non-incorporated proteins that were detected in the bottom fraction of the linear sucrose density gradient (Fig. 3C). Virosomes prepared by the fast reconstitution method resulted in smaller and more homogenous vesicles compared to virosomes prepared by the slow reconstitution method (Fig. 5E). The average diameter was calculated to be 113±20 nm (n=116) with a size distribution narrower than that of the slow reconstitution virosomes. Moreover, these preparations were devoid of protein aggregates.

3.6. Fusion activity

Retention of biologically relevant fusion activity is a prerequisite for application of virosomes as a cellular delivery system. To measure fusion activity, a pyrene-labeled lipid was incorporated in the virosomal membrane during the reconstitution process. When incorporated, pyrene-labeled lipids form dimers that exhibit excited-dimer (or "excimer") fluorescence. This excimer fluorescence intensity is dependent on the concentration of the pyrene-labeled lipids in the membrane. After fusion of a pyrene-labeled virosome with a substantially larger target membrane, the excimer fluorescence decreases due to dilution of the pyrene probe into the target membrane, which is a direct measure for fusion. In this fusion assay, erythrocyte ghosts were used as target membranes. When exposed to pH 5.5, virosomes prepared by the slow and fast reconstitution procedure showed rapid fusion with erythrocyte ghosts (Fig. 6A). Maximal fusion was reached within 60 s and fusion was strictly pH-dependent, since no fusion was observed at neutral pH. When HA was inactivated by pre-exposure of the virosomes to low pH in the absence of target membranes, fusion activity was abolished completely. These findings demonstrate that fusion of virosomes is dependent on the conformational change of HA induced by a decrease in pH, as described for native virus (reviewed in: [17]). Like influenza virus [22], the virosomes exhibited a pH optimum for fusion (Fig. 6B and C). Maximal fusion was achieved at pH 5.6 and the fastest initial rate of fusion was reached at pH 5.2. The difference in pH optimum for maximal fusion and fastest initial rate of fusion is explained by the fact that fusion is a balance between activation and inactivation of HA induced by low pH. At lower pH values, both fusion activation and HA inactivation increase, which may result in a higher initial rate, but a lower final extent of fusion [43]. Thus, virosomes produced by both reconstitution methods fuse with the same kinetics and to approximately the same final extent (Fig. 6B and C). Moreover, the fusion characteristics of

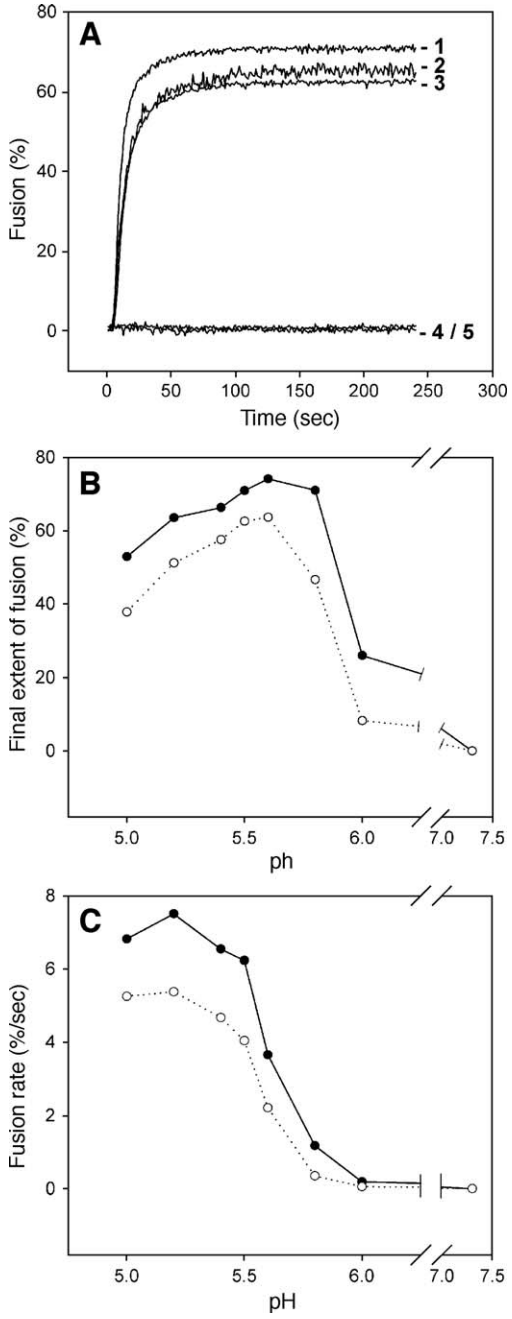

Fig. 6. Hemagglutinin-dependent fusion of virosomes with erythrocyte ghosts. (A) Fusion curves of slow reconstitution virosomes (1), $C_{12}E_8$ virosomes (2) and fast reconstitution virosomes (3) after exposure to pH 5.5. Fusion curves of virosomes at neutral pH (4) and after inactivation by exposure to low pH without target membranes (5). (B) Maximal fusion at different pH values for slow reconstitution virosomes (filled circles) and fast reconstitution virosomes (open circles). (C) Initial fusion rates of slow reconstitution virosomes (filled circles) and fast reconstitution virosomes (open circles).

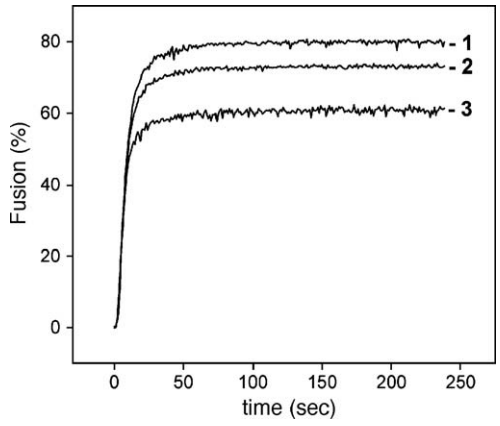

Fig. 7. Influence of virus inactivation on fusion activity of virosomes. Fusion at pH 5.5 of slow reconstitution virosomes prepared from active virus (1), prepared from BPL-inactivated virus (2) and prepared from FA-inactivated virus (3).

virosomes produced by the dialysis procedure are similar to those produced by the $C_{12}E_8$ method (Fig. 6A).

3.7. Effect of virus inactivation on virosome characteristics

While all the above experiments were carried out with virosomes prepared from active virus, the use of inactivated virus is a prerequisite when virosomes are to be used for medical applications. Treatment with β-propiolactone (BPL) or formaldehyde (FA) are two commonly used inactivation procedures for viruses applied in vaccine production [44,45]. Virus was inactivated by either method and subsequently virosomes were prepared from the inactivated virus by the DCPC dialysis protocol. The reconstitution procedure resulted in virosomes that accumulated on the interface of a discontinuous sucrose gradient with protein and phospholipid recoveries comparable to the recoveries of virosomes prepared from active virus (data not shown). Pre-treatment of virus with BPL or FA did not affect the fusion kinetics of virosomes generated from the inactivated virus (Fig. 7). Moreover, virus inactivation with BPL hardly affected the final extent of fusion of the virosomes, whereas virus inactivation with FA reduced the final extent with approximately 25%. Thus, inactivation of virus for the production of virosomes does not interfere with virosome yield and has a limited effect on the fusogenic properties of virosomal HA.

4. Discussion

In this paper we report on a new procedure for the generation of virosomes based on the use of a short-chain phospholipid with detergent-like properties. The short-chain lecithin DCPC has a high c.m.c. of 14 mM and is therefore suitable for removal by dialysis. Upon solubilization of influenza virus membranes with DCPC high amounts of HA were extracted from the virus. Removal of DCPC by dialysis resulted in reconstituted membrane vesicles with the envelope glycoproteins inserted in the lipid bilayer. These virosomes contained less than 0.1% of the initial amount of DCPC, which shows that DCPC removal by dialysis is very efficient. The size and homogeneity of the virosomes varied according to the reconstitution procedure applied, namely, standard dialysis of the dissolved viral membrane components or an initial dilution step followed by dialysis.

Importantly, the cell-binding and membrane-fusion properties of HA were preserved in influenza virosomes prepared from DCPC-solubilized viral membranes even when excess concentrations of 200 mM DCPC were applied (data not shown). The described method was also found suitable for the preparation of fusion-active virosomes from Semliki Forest virus, the fusion protein of which is structurally very different from HA (P. Schoen, unpublished observations). These observations are in line with findings of others that short-chain lecithins are suitable for the solubilization of a range of different biological membranes and preserve the functional integrity of proteins over a broad concentration range [33,34]. In contrast, attempts to functionally reconstitute viral membranes using OG, a frequently used dialyzable detergent, were often unsuccessful [7,22]. If reconstitution of viral membranes to fusion-active virosomes was achieved, low concentrations of OG needed to be used and conditions of solubilization had to be carefully controlled [46].

Reconstitution of the influenza virus membrane was achieved by either slow removal of detergent from the mixed micelles, accomplished by gradual dialysis (slow reconstitution method) or by fast detergent removal, accomplished by a dilution step prior to dialysis (fast reconstitution method). Interestingly, virosome preparations obtained by the two procedures differed with respect to average size and size distribution (113±20 nm vs. 146±64 nm), as well as efficiency of HA incorporation into the lipid bilayers.

An effect of detergent removal rate on vesicle size was earlier reported for the preparation of liposomes. As found for the virosomes, larger liposomes were obtained when detergent removal was slow [47–49]. According to current hypotheses, the process of vesicle formation involves so-called disc-like structures that are obtained when mixed micelles aggregate upon detergent removal [47,49]. These discs slowly gain in diameter through collision and subsequent fusion with other discs. When the detergent becomes limiting, disc-curvature is induced, which finally leads to vesiculation [50–53]. A fast detergent removal rate may leave little time for the discs to fuse. Consequently, the discs have a small surface area at the stage of vesicle closure, which could explain the formation of small vesicles [47]. On the other hand, when detergent is removed slowly there is more time for collisions and fusions to take place, resulting in larger and more heterogeneously sized discs and consequently a more heterogeneous population of vesicles [47,49]. Our data imply that the influence of the rate of detergent removal on vesicle size and size distribution holds true not only for liposomes but also for virosomes, which are much more complex systems.

With respect to the incorporation efficiency of HA into lipid bilayers we observed that the amounts of protein and phospholipid reconstituted to proper virosomes (the virosome fractions of the linear sucrose density gradients of the ‘crude’ virosome preparation) were higher for the fast reconstitution than for the slow reconstitution method (Fig. 3A,B). Additionally, fast reconstitution resulted in virosomes with higher protein to phosphate ratios (Fig. 3C,D). Thus, fast detergent extraction from mixed micelles obviously results in a more efficient

incorporation of protein into the lipid bilayers. This was confirmed by electron microscopy, which revealed that the fast reconstitution virosome preparation consists almost entirely of vesicles, while in the slow reconstitution preparations substantial amounts of small protein rosettes were found (Fig. 5D,E).

Reconstitution of proteins into lipid membranes is a rather complex process. According to the model for proteoliposome formation as proposed by Rigaud and colleagues [54], reconstitution of solubilized membrane proteins starts from a solution containing mixed lipid–detergent and lipid–protein–detergent micelles. For membrane proteins with a tendency to aggregate, like HA, the model suggests that the lipid–protein–detergent micelles are less stable than the lipid–detergent micelles and are therefore the first to coalesce upon slow removal of detergent. This initially results in the formation of protein-rich aggregates and more or less protein-free liposomes into which proteins can post-insert in a later stadium. The result would be a heterogenous population of protein aggregates and proteoliposomes with varying amounts of membrane-inserted proteins. In contrast, fast detergent removal is suggested to lead to simultaneous coalescence of lipid–detergent and lipid–protein–detergent micelles resulting in direct co-reconstitution of lipids and proteins into rather homogenous proteoliposomes. Our observations on the amount of HA incorporated into lipid bilayers and the homogeneity of the virosome preparation upon slow and fast detergent removal, respectively, are well in line with this model.

Influenza virosomes are currently commercially used as influenza vaccines (Inflexal V®, Berna Biotech; Invivac®, Solvay Pharmaceuticals) and as a carrier in a Hepatitis A vaccine (Epaxal®, Berna Biotech). Techniques for the use of virosomes for the delivery of drugs, proteins, plasmid DNA and siRNA are being developed and commercial application of virosomes for these purposes are being investigated. Considering these applications, the production of virosomes by detergent dialysis has several advantages as compared to the production by detergent removal by adsorption to a hydrophobic resin. Detergent dialysis is upscalable and can be performed reproducibly by applying 'controlled dialysis' or ultrafiltration [48,55,56]. Ultrafiltration can be performed as a continuous process, which is convenient for industrial production. By applying dialysis, the use of substances like Bio-Beads that have to be added during the production process and withdrawn from the final product is avoided. This simplifies the process and prevents undesired interactions between the hydrophobic resin and the compounds to be encapsulated. Thus, combining the advantages of DCPC as a solubilizing agent with the gentle removal by dialysis, a novel approach to reconstitute viral membranes could be developed. Compared to the previously reported $C_{12}E_8$ method [22], this approach is a significant improvement for the production of virosomes, which will further advance the clinical use of virosomes as vaccines on the one hand and as drug delivery vehicles on the other hand.

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