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Incorporation of bacterial membrane proteins into liposomes: factors influencing protein reconstitution

Manjeet M. Parmar [a], Katarina Edwards [b], Thomas D. Madden [a,*]

[a] *Department of Pharmacology and Therapeutics, University of British Columbia, 2176 Health Sciences Mall, Vancouver, BC V6T 1Z3, Canada*

[b] *Department of Physical Chemistry, Uppsala University, Uppsala, Sweden*

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Abstract

Meningococcal and gonococcal outer membrane proteins were reconstituted into liposomes using detergent-mediated dialysis. The detergents octyl glucopyranoside (OGP), sodium cholate and Empigen BB were compared with respect to efficiency of detergent removal and protein incorporation. The rate of OGP removal was greater than for cholate during dialysis. Isopycnic density gradient centrifugation studies showed that liposomes were not formed and hence no protein incorporation occurred during dialysis from an Empigen BB containing reconstitution mixture. Cholate-mediated reconstitution yielded proteoliposomes with only 75% of the protein associated with the vesicles whereas all of the protein was reconstituted into the lipid bilayer during OGP-mediated reconstitution. Essentially complete protein incorporation was achieved with an initial protein-to-lipid ratio of 0.01:1 (w/w) in the reconstitution mixture; however, at higher initial protein-to-lipid ratios (0.02:1) only 75% protein incorporation was achieved. Reconstituted proteoliposomes were observed as large (>300 nm), multilamellar structures using cryo-electron microscopy. Size reduction of these proteoliposomes by extrusion did not result in significant loss of protein or lipid. Extruded proteoliposomes were unilamellar vesicles with mean diameter of about 100 nm.

Keywords: Reconstitution; Detergent; Adjuvant; Proteoliposome; Subunit vaccine

1. Introduction

Although chemotherapy is one form of fighting infectious diseases, vaccination would be an ideal alternative to chemotherapy. However, conventional vaccines using attenuated or killed whole organisms may cause mild to severe reactions [1–6]. This problem has prompted the development of subunit and peptide vaccines. Unfortunately, these vaccines are often poorly immunogenic in the absence of an adjuvant [7–11]. Alum (aluminum hydroxide) is the only adjuvant licensed for use in humans; however, it is less than ideal as it tends to elicit a high rate of mild to moderate reactions at injection sites [12,13]. This fact has led to the search for alternative adjuvant candidates.

Liposomes have long been used as models for lipid membranes [14]. They have been useful in studying membrane receptors, channel proteins, and membrane-bound enzymes [15–20]. Recent studies have examined the use of liposomes as potential immu-

* Corresponding author. Fax: +1-604-822-4843;
E-mail: tmadden@unixg.ubc.ca

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noadjuvants [21–27]. There are several advantages of liposomes: they are biodegradable and non-toxic and can be prepared quite easily with varying compositions to obtain the most efficacious antigen-presenting liposome formulation. In addition, liposomes have the ability to elicit both a cellular mediated immune response [28] and a humoral immune response [21–25,29]. Studies have shown liposomes to be effective immunopotentiators in hepatitis A [12,13] and influenza vaccines [30,31]. In addition, liposomes have adjuvant activity in vaccines against protozoan [32–34] and bacterial organisms [35–37].

Two organisms for which vaccines are currently being sought are *Neisseria meningitidis* and *Neisseria gonorrhoeae*. *Neisseria meningitidis* (meningococcal) disease arises from various serogroups and varies from country to country. A large proportion of this disease is caused by strains A, B, C, Y and W135. Highly effective capsular polysaccharide vaccines against strains A, C, Y and W135 have been developed. Unfortunately, group B capsular polysaccharide vaccines are found to be essentially non-immunogenic. The fact that serogroup B, the predominant cause of meningococcal disease in many temperate countries, lacks polysaccharide immunogenicity has prompted the research and development of alternative vaccines based on membrane proteins. Studies have shown adjuvant-protein vaccines to have markedly improved antibody responses [38,39]. Furthermore, proteosome-based vaccines have been shown to be immunogenic [40] and outer membrane vaccines can increase immunity to group B meningococci [41–43].

Another neisserial organism for which a vaccine is being sought is *Neisseria gonorrhoeae*. It is a pathogenic organism that colonizes sites such as the urethra, cervix, pharynx, rectum and conjunctiva [44,45]. Common complications of gonococcal infections are sterility and ectopic pregnancy. Some infections can progress to salpingitis, bacteremia, septic arthritis and, in some cases, fatal meningitis. Patients with gonorrhea have been shown to produce bactericidal and opsonic antibodies to bacterial outer membrane components [46,47]. In addition, earlier studies have shown that formulations containing gonococcal protein I incorporated into liposomes elicit high titers of surface reactive, bactericidal antibodies in a rabbit model [48–50]. These studies provide encouraging results in the development of a liposomal subunit vaccine against gonococcal infections.

We have previously reconstituted gonococcal protein I into liposomes and shown that the protein is efficiently incorporated and oriented in the bilayer in a similar configuration to the native bacterial membrane [51]. The formulation was also shown to have a high degree of anti-Por antibody binding activity. In the present study, we characterize meningococcal and gonococcal membrane protein reconstitution into liposomes via detergent dialysis and we have examined the influence of different detergents, dialysis duration and protein-to-lipid ratio on the efficiency of protein reconstitution. In addition, we examine the morphology of reconstituted proteoliposomes using the technique of cryo-electron microscopy.

2. Materials and methods

2.1. Materials

1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) (M_r 760.1) was purchased from Northern Lipids, Vancouver, BC. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (POPS) (M_r 784) was purchased from Avanti Polar Lipids, Alabaster, AL. *N*,*N*-Dioleoyl-*N*,*N*-dimethylammonium chloride (DODAC) (M_r 582.5) was obtained from INEX Pharmaceuticals, Vancouver, BC. *N*-Octyl-β-D-glucopyranoside (OGP) (M_r 292.4), Hepes (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]) (M_r 238.3) and cholic acid (3α,7α,12α-trihydroxy-5β-cholan-24-oic acid) (M_r 430.6) (sodium salt) were obtained from Sigma Chemical Co., St. Louis, MO. *N*-[^{14}C]Octyl-β-D-glucopyranoside ([^{14}C]OGP) was purchased from American Radiolabeled Chemicals, St. Louis, MO. L-α-Dipalmitoyl-[2-palmitoyl-9,10-^{3}H(*N*)]-phosphatidylcholine ([^{3}H]DPPC) and [^{3}H]cholic acid were obtained from Dupont, Boston, MA. Ficoll 400 was obtained from Pharmacia, Uppsala, Sweden. The reagents for the BCA protein assay were purchased from Pierce, Rockford, IL. SM-2 Biobeads were obtained from Biorad, Hercules, CA. The gonococcal membrane protein I (Por) was supplied by Dr. Milan S. Blake, Rockefeller University, New York, NY. through DynCorp PRI. The meningococcal outer

membrane proteins containing detoxified lipooligosaccharide (2:3 w/v) (MOMP) and Empigen BB (*N*-dodecyl-*N*,*N*-dimethylglycine) (M_r 272) were supplied by Dr. Wendell Zollinger, Walter Reed Army Institute, Washington, DC.

2.2. Rate of detergent removal during dialysis

Bacterial proteins were reconstituted into liposomes by passive dialysis. The residual detergent in the sample mixture was measured at various time points. Meningococcal proteins were reconstituted from OGP and cholate/Empigen BB to determine the effects of detergent on detergent removal and efficiency of protein incorporation.

MOMP (0.5 mg/ml) in 400 mM OGP, 20 mM Hepes (pH 7.4) was prepared and added (2 ml) to 50 mg POPC. The radiolabel [^{14}C]OGP (2 μCi/ml) was included as a detergent marker. The phospholipid was dissolved by gentle vortexing at 25°C and samples then placed in SpectraPor II dialysis tubing (6.4 mm diameter) and dialyzed for 140 h at 4°C against 500 volumes of 150 mM NaCl, 20 mM Hepes (pH 7.4) with and without the presence of SM-2 Biobeads (2 g). External buffer changes were made at 20, 50 and 116 h. During dialysis, aliquots were taken from the dialysis tubes at 0, 2.5, 5, 10, 20, 30, 50, 116 and 140 h time points.

MOMP (0.5 mg/ml) in 200 mM sodium cholate, 20 mM Hepes (pH 7.4) with 5.6% Empigen BB was prepared and added to dry POPC (50 mg/ml). The phospholipid was dissolved by gentle vortexing at 25°C and the samples then placed in dialysis tubing and dialyzed for 12 days at 4°C against 500 volumes of 150 mM NaCl, 20 mM Hepes (pH 7.4) with external buffer changes at 36, 82, 142, 214 and 250 h.

The removal of OGP detergent during gonococcal Por protein reconstitution has been shown in a previous study [51]. In this study, Por protein was reconstituted into liposomes from cholate to determine the effects of detergent charge on protein incorporation efficiency.

Por protein (0.5 mg/ml) with 50 mg/ml POPC in 200 mM sodium cholate, 20 mM Hepes (pH 7.4) was prepared as described above. The radiolabel [^{3}H]cholic acid (3.3 μCi/ml) was included in the reconstitution mixture. The sample was transferred to dialysis tubing and dialyzed for 150 h at 4°C against 500 volumes of 150 mM NaCl, 20 mM Hepes (pH 7.4) with external buffer changes at 20, 50 and 130 h. During dialysis, aliquots were taken from the dialysis tubes at 0, 2.5, 5, 10, 20, 30, 50, 70, 130 and 150 h time points.

Residual detergent concentrations were determined based on scintillation counting of [^{14}C]OGP and [^{3}H]cholic acid in a Beckman LS 3801 instrument (Fullerton, CA).

2.3. Reconstitution of Por proteoliposomes from various detergents

Por protein was reconstituted at different protein-to-lipid ratios from OGP or cholate to determine the effect of protein concentration on protein incorporation efficiency.

Por protein was diluted to 0.5 mg/ml or 1.0 mg/ml protein in 400 mM OGP, 20 mM Hepes (pH 7.4). These solutions (3 ml) were then added to 150 mg of POPC and the phospholipid was dissolved by gentle vortexing at 25°C. The radiolabel [^{3}H]DPPC (0.03 μCi/mg phospholipid) was included as a lipid marker. Aliquots (1 ml) of each solution were set aside as the 0 h dialysis time point and samples were transferred to dialysis tubing and dialyzed for either 20 h or 125 h at 4°C against 500 volumes of 150 mM NaCl, 20 mM Hepes (pH 7.4). External buffer changes were made at 20 and 50 h.

Por protein (0.5 mg/ml) with 50 mg/ml POPC in 200 mM sodium cholate, 20 mM Hepes (pH 7.4) was prepared as described above. Again [^{3}H]DPPC (0.03 μCi/mg phospholipid) was included as a lipid marker. An aliquot (1 ml) of this solution was set aside as the initial time point. Two aliquots were transferred to dialysis tubing and dialyzed for either 60 h or 125 h at 4°C against 500 volumes of 150 mM NaCl, 20 mM Hepes (pH 7.4). External buffer was changed at 20, 50 and 100 h.

Following dialysis, reconstituted samples were analyzed by isopycnic density gradient centrifugation, QELS size analysis and cryo-electron microscopy as described below.

2.4. Isopycnic density gradient centrifugation

A continuous Ficoll gradient was prepared (0–10% Ficoll) (in 150 mM NaCl, 20 mM pH 7.4) using a

Gradient Maker (Hoefer Scientific Instruments). The reconstituted proteoliposomes (500 μl) were loaded on the gradient which was then centrifuged in a Beckman SW 41 Ti swinging bucket rotor on a Beckman L2-65B ultracentrifuge at $110\,000 \times g_{av}$ for 24 h at 4°C. The gradients were then fractionated into 500 μl fractions (see figure legends for details) and analyzed for protein and lipid content.

2.5. Size reduction of proteoliposomes by extrusion

Following reconstitution, proteoliposomes were size reduced using an extrusion procedure [52,53]. Briefly, reconstituted systems were placed in an Extruder (Lipex Biomembranes, Vancouver, BC) and extruded ten times through two (stacked) polycarbonate filters (Costar, Cambridge, MA) of 100 nm pore size under nitrogen pressures of 100–400 psi. Following size reduction, vesicle size distributions were determined using quasi-elastic light scattering (QELS).

2.6. Analytical procedures

Phospholipid concentrations and specific activities were determined by assaying lipid phosphorus content [54] and by liquid scintillation counting of the samples on a Beckman LS 3801 liquid scintillation counter.

Protein concentrations were determined by a modified Pierce BCA protein assay for microtiter plates (Pierce). A 50-μl aliquot of each standard, blank, or diluted unknown sample was pipetted into the appropriate microtiter plate wells. Then 50 μl of 0.5% sodium dodecyl sulfate was added followed by the addition of 100 μl of working reagent to each well. The microtiter plates were incubated at 37°C for 2 h. Absorbance was measured at 540 nm on a Biotek 96 well microtiter plate reader. Protein concentrations in the samples were determined from a bovine serum albumin (BSA) standard curve.

2.7. Quasi-elastic light scattering

Reconstituted systems were analyzed to determine vesicle size distributions by QELS analysis using a Nicomp Model 270 Submicron Particle Sizer as described previously [55].

2.8. Cryo-transmission electron microscopy

Reconstituted vesicles were analyzed using the technique of cryo-transmission electron microscopy (CTEM). Briefly, sample films were prepared in a custom-built environmental chamber under controlled temperature (25°C) and humidity conditions. The films were then vitrified by rapid freezing in liquid ethane and transferred to a Zeiss EM 902 transmission electron microscope for analysis. The specimens were kept below 108 K during the transfer and viewing procedures to prevent sample perturbation and ice formation. The microscope was operated in zero-loss, bright-field mode and at an accelerating voltage of 80 kV [56,57].

3. Results

3.1. Detergent removal during reconstitution

Reconstitution experiments were conducted using three different detergents to determine the effect of detergent properties on proteoliposome formation. The three detergents under investigation were octyl glucoside, sodium cholate and Empigen BB. Differences in ionic character and critical micellar concentrations of these detergents may influence Por protein incorporation into liposomes. The effects of these detergents on protein incorporation efficiency and rate of detergent removal during the dialysis were examined.

The non-ionic detergent octyl glucopyranoside (OGP) was used in this study because it is reported to be a relatively mild, non-denaturing detergent with a relatively high critical micellar concentration (CMC) [58,59]. In our previous reconstitution experiments, OGP removal during gonococcal protein reconstitution was quite rapid and residual detergent levels were found to be well below the CMC [51]. Here, removal of OGP (initial concentration 400 mM) during meningococcal protein reconstitution in the presence and absence of SM-2 Biobeads (Biorad) was monitored by assaying for [^{14}C]OGP during dialysis. As shown in Fig. 1A, detergent removal from the reconstitution mixture is rapid, particularly during the first 15 h. It was anticipated that Biobeads, polystyrene polymers, when added to the ex-

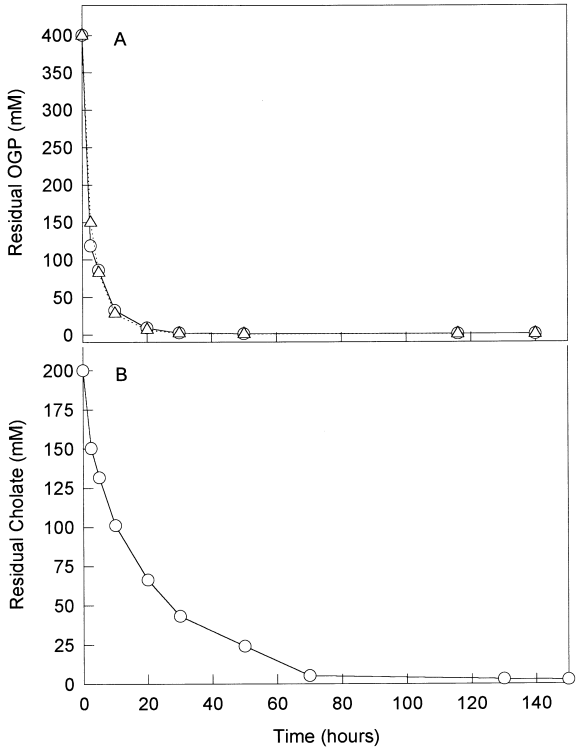

Fig. 1. Detergent levels during dialysis. The levels of *N*-octyl-β-D-glucopyranoside (OGP) and sodium cholate were measured by assaying for [^{14}C]OGP and [^{3}H]cholic acid, respectively, during the dialysis procedure. (A) OGP levels in the absence of SM-2 Biobeads (○) and in the presence of SM-2 Biobeads (△). (B) Sodium cholate levels.

ternal buffer, would bind to detergent molecules that have already been removed from the reconstitution mixture. Detergent molecules binding to the Biobeads in the external buffer would further increase the concentration gradient and lead to an increase in the rate of OGP removal from the reconstitution mixture. However, the rate of removal was similar with and without the addition of Biobeads. This suggests that either the rate limiting step in OGP removal was not the concentration gradient across the dialysis membrane or the Biobeads did not bind OGP to any great extent. Once proteoliposome formation has occurred, it is likely that further OGP removal from the vesicles will be governed by the rate of OGP 'flip-flop' or exchange from the inner monolayer to the outer monolayer. After 140 h of dialysis, OGP remaining in both samples was 0.25% of the starting concentration. This corresponds to 1 mM OGP which is well below the CMC of approximately 21 mM [58]. The kinetics of OGP removal during meningococcal protein reconstitution followed very closely the rate observed during gonococcal protein reconstitution as shown in our earlier work [51].

Removal of the ionic surfactant, cholate, during gonococcal protein reconstitution was also determined. Interestingly, cholate removal was much slower than the removal of OGP during either meningococcal (Fig. 1B) or gonococcal protein reconstitution [51]. Only after 50 h of dialysis is the residual detergent concentration at or below the CMC, which is reported to be 13–16 mM [60,61]. At 140 h of dialysis, residual cholate in the reconstitution mixture was 1.3% of initial, which corresponds to 2.6 mM, well below its CMC. In contrast, OGP levels are well below the CMC after only 20 h during

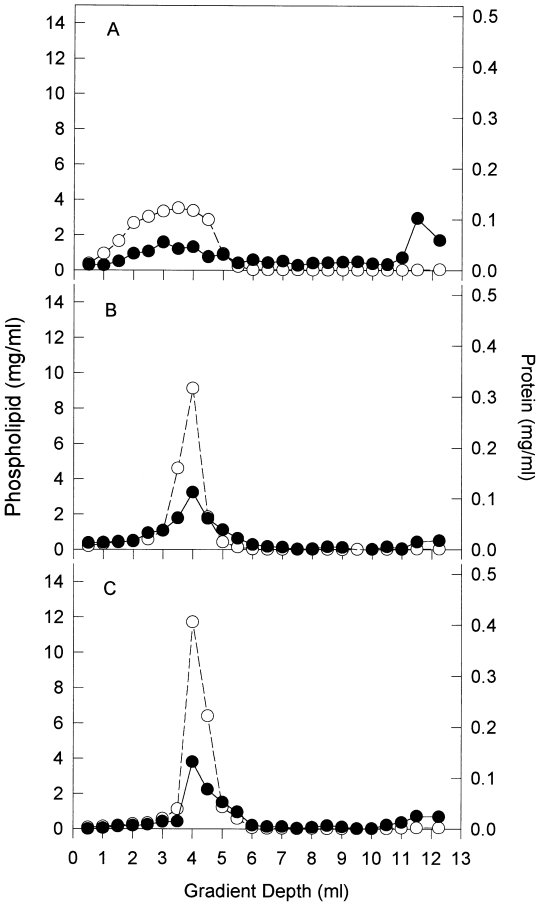

Fig. 2. Isopycnic density gradient centrifugation profiles for meningococcal OMP liposomes reconstituted from OGP: (A) 0 h, (B) 20 h and (C) 125 h. Samples were centrifuged at 110 000 × g_{av} at 4°C for 24 h on a continuous Ficoll gradient (0–10%). (●) Protein; (○) phospholipid.

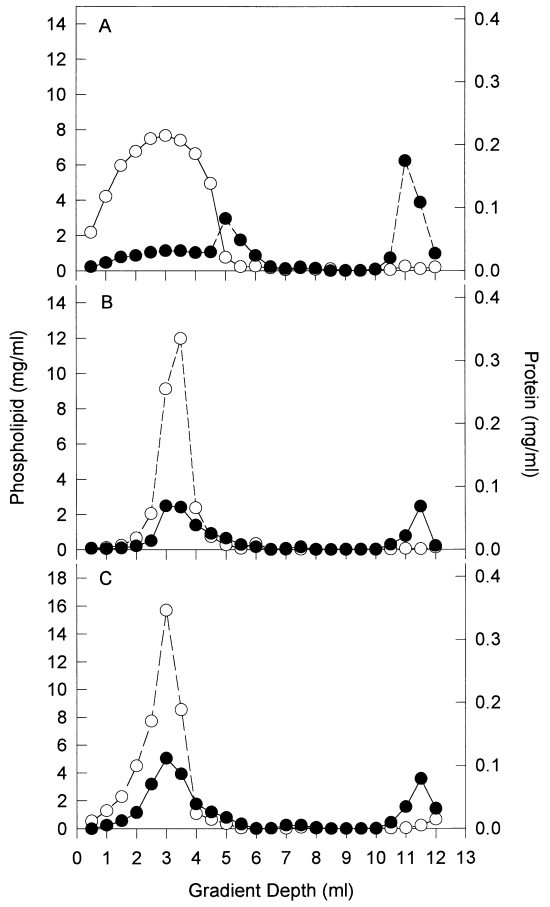

Fig. 3. Isopycnic density gradient centrifugation profiles for gonococcal Protein I liposomes (P/L = 0.01) reconstituted from cholate: (A) 0 h, (B) 60 h and (C) 125 h. Samples were centrifuged at $110\,000 \times g_{av}$ at 4°C for 24 h on a continuous Ficoll gradient (0–10%). (●) Protein; (○) phospholipid.

either meningococcal or gonococcal protein reconstitution.

3.2. Characterization of protein incorporation during dialysis

Having determined the rates of removal of OGP and cholate, we characterized the reconstitution process by following protein incorporation into liposomes as the detergent was removed. Meningococcal outer membrane proteins (MOMP) and POPC were dissolved in OGP and dialyzed for 20 and 125 h (as described in Section 2). An aliquot of the original sample was also retained. Isopycnic density gradient centrifugation was then used to follow vesicle formation and protein incorporation during the reconstitution process. The initial sample (time zero) shows a broad distribution of phospholipid in the top half of the gradient (Fig. 2). Some MOMP is also found associated with the lipid band, likely due to spontaneous vesiculation when the sample is diluted on the gradient. For this initial sample, the remaining protein is seen to migrate as a peak near the bottom of the gradient. After 20 or 125 h of dialysis, however, it can be seen that comigration of phospholipid and protein occurs to a position approximately a third of the way down the gradient. Further, the protein/lipid band extends over a relatively narrow density range. Approximately 80% of the protein is associated with the lipid, with 20% free protein at the bottom of the gradient. This profile indicates a high incorporation efficiency of MOMP incorporation during the reconstitution procedure. However, the presence of free protein at the bottom of the gradient indicates the process is incomplete which may be due to the heterogeneity of the meningococcal protein mixture.

3.3. Protein reconstitution from sodium cholate and Empigen BB

Gonococcal Por protein reconstitution was also characterized using the ionic surfactant, cholate, to determine the effects of detergent charge on protein incorporation compared to OGP-mediated protein

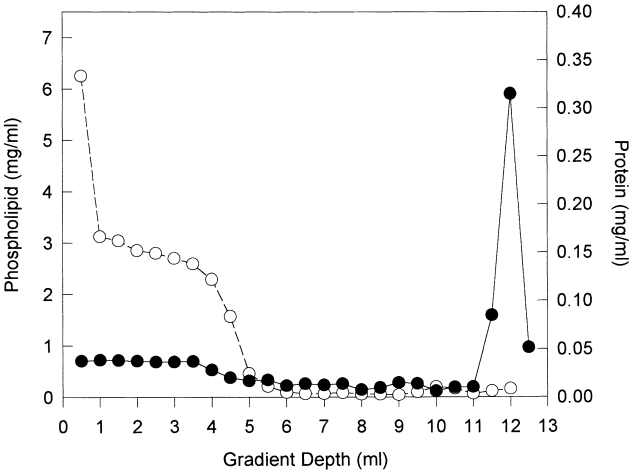

Fig. 4. Isopycnic density gradient centrifugation profile for meningococcal OMP liposomes reconstituted from Empigen BB. Sample was centrifuged at $110\,000 \times g_{av}$ at 4°C for 24 h on a continuous Ficoll gradient (0–10%). (●) Protein; (○) phospholipid.

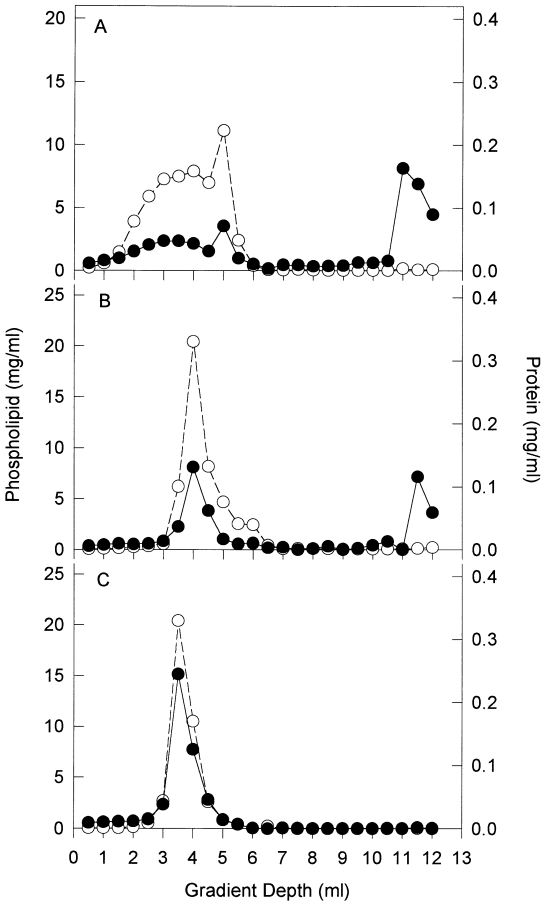

Fig. 5. Isopycnic density gradient centrifugation profiles for gonococcal Protein I liposomes (P/L = 0.01) reconstituted from OGP: (A) 0 h, (B) 20 h and (C) 125 h. Samples were centrifuged at $110\,000 \times g_{av}$ at 4°C for 24 h on a continuous Ficoll gradient (0–10%). (●) Protein; (○) phospholipid.

reconstitution. Isopycnic density gradient profiles for Por/POPC (P/L = 0.01) mixtures reconstituted for either 0, 60, 125 h from cholate are shown in Fig. 3. The initial sample (time zero) shows the lipid band dispersed over a broad density range within the top half of the gradient, whereas Por protein is observed to migrate near the bottom of the gradient. After 60 and 125 h of dialysis, there is an increase, 70% and 77%, respectively, in protein incorporation as the lipid and protein are observed to comigrate down the gradient. However, for this protein-to-lipid ratio there is a significant fraction of approximately 23% unincorporated Por, in contrast to proteoliposomes prepared from OGP at this protein-to-lipid ratio (Fig. 5). These results indicate that protein incorporation is less efficient during reconstitution from cholate.

Protein isolation studies of meningococcal membrane proteins have indicated that inclusion of zwitterionic detergent during protein solubilization and purification can restore the antibody binding capacity of membrane proteins [62]. Therefore, Empigen BB, a commonly used zwitterionic detergent, was used in meningococcal protein reconstitution experiments and the results were then compared to reconstitution with octyl glucoside (Fig. 2). Following dialysis for 12 days the protein–lipid mixtures were observed to be transparent, suggesting that either any vesicles present were very small or that no vesiculation had occurred. Density gradient centrifugation showed little or no protein associated with the phos-

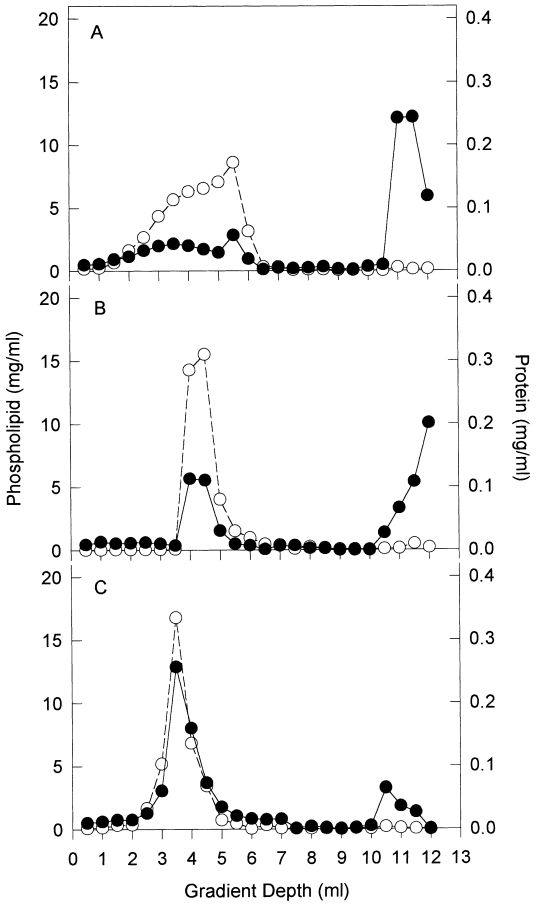

Fig. 6. Isopycnic density gradient centrifugation profiles for gonococcal Protein I liposomes (P/L = 0.02) reconstituted from OGP: (A) 0 h, (B) 20 h and (C) 125 h. Samples were centrifuged at $110\,000 \times g_{av}$ at 4°C for 24 h on a continuous Ficoll gradient (0–10%). (●) Protein; (○) phospholipid.

Table 1
QELS Size analysis of Por proteoliposomes reconstituted from OGP and cholate

Reconstitution detergent	Mean vesicle diameter (nm)	Standard deviation (nm)
OGP	317	180
Cholate	543	277
OGP/extruded vesicles	96	21

pholipid band as the phospholipid component was observed to migrate in the top half of the gradient. Most of the protein was seen in a peak fraction at the bottom of the gradient (Fig. 4). The separation of the two peaks indicates that vesicle formation did not occur during dialysis; therefore, protein incorporation could not occur.

3.4. *Influence of protein-to-lipid ratio on reconstitution efficiency*

The influence of protein-to-lipid ratio on efficiency of protein incorporation into liposomes during reconstitution was examined. Proteoliposome samples containing gonococcal protein I (Por) and POPC

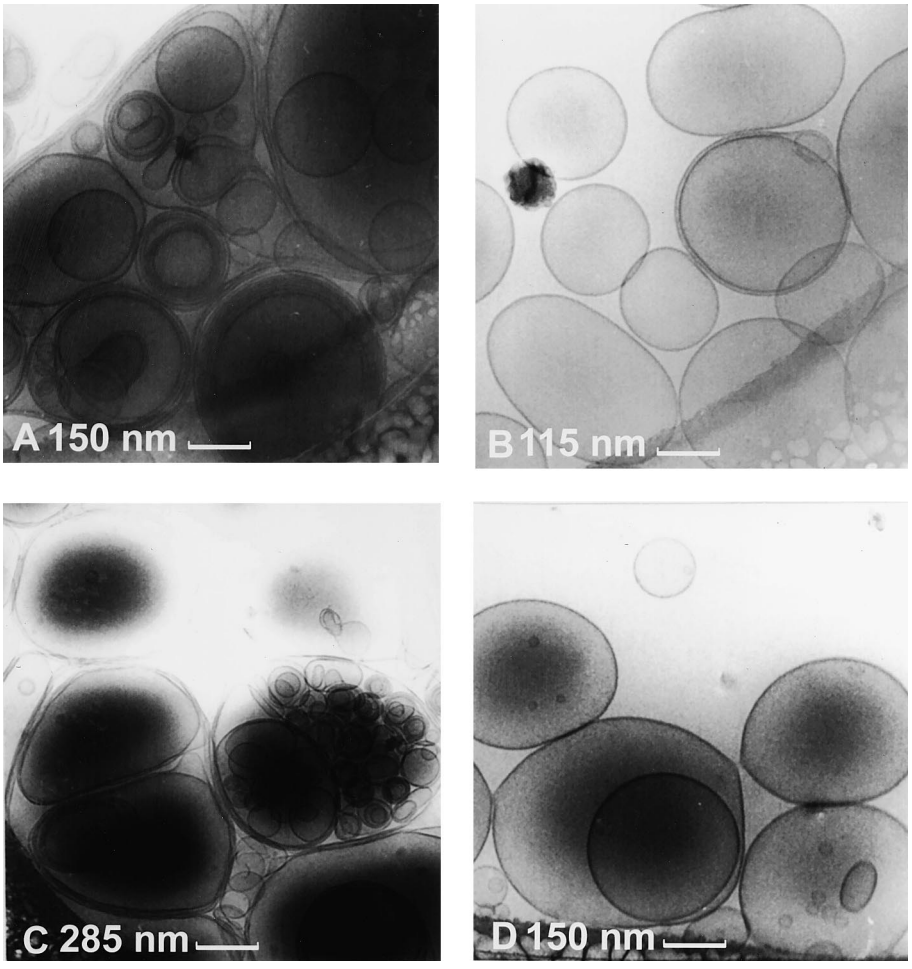

Fig. 7. Cryo-electron micrographs of reconstituted Por proteoliposome formulations. (A) POPC/Por; (B) POPC/Por; (C) POPC/DODAC/Por; (D) POPC/DODAC/Por; (E) POPC/POPS/Por; (F) POPC/POPS/Por; (G,H) Extruded POPC/Por.

were prepared from OGP (as described in Section 2) at initial protein-to-lipid ratios (w/w) of either 0.02 or 0.01. The initial samples, at both protein-to-lipid ratios, show a broad band in the top half of the gradient (Figs. 5A and 6A). Most of the protein is seen to migrate to the bottom of the gradient. Following dialysis for 20 h, a protein/lipid band extending over a relatively narrow density range is observed. However, there is still a peak fraction of free Por (30 and 50% for P/L ratios of 0.01:1 and 0.02:1, respectively) protein migrating to the bottom of the gradient, which indicates protein incorporation is incomplete after 20 h. After 125 h of dialysis, a further narrowing of the phospholipid band is seen with essentially all of the Por protein associated with this lipid band at the lower protein-to-lipid ratio (0.01:1) (Fig. 5C). It is important to note, however, that at the higher protein-to-lipid ratio (0.02:1), approximately 17% of Por protein is not associated with lipid and migrates to the bottom of the gradient (Fig. 6C). This suggests that protein incorporation is saturable and limited by the phospholipid concentration.

3.5. Proteoliposome size and morphology

Reconstituted systems were examined using QELS analysis to determine vesicle size distributions. Reconstitution from OGP resulted in proteoliposomes exhibiting a relatively broad size distribution with mean vesicle diameter about 317 nm (Table 1). It has been reported that vesicles reconstituted from cholate have mean diameters of about 50 nm, depending on lipid composition [63,64]. We observed,

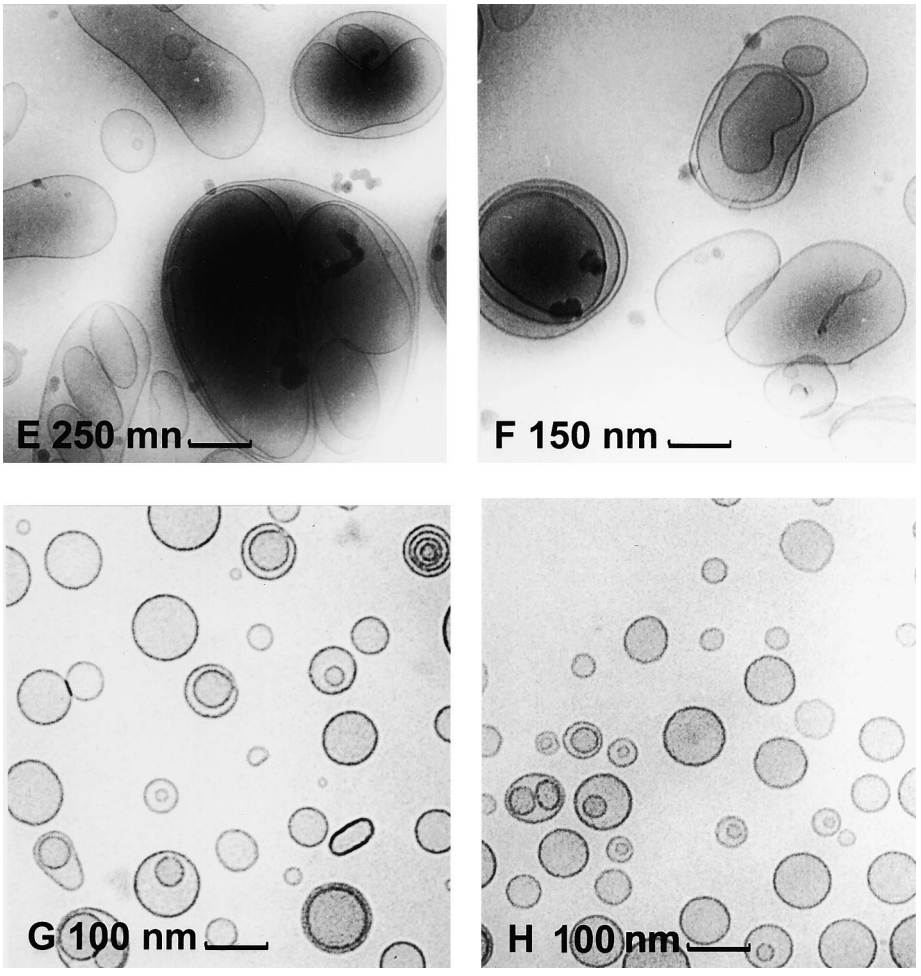

Fig. 7 (*continued*).

however, that reconstitution of Por protein into POPC vesicles generated systems with mean vesicle diameter greater than 0.5 microns.

Proteoliposome morphologies were also examined using CTEM. Por proteoliposomes reconstituted from OGP detergent are shown in Fig. 7A,B. Consistent with QELS results, CTEM analysis shows that vesicles are relatively large, multilamellar structures ranging in shape from oblong to spherical. Under low magnification (Fig. 7A), vesicles are observed as highly multilamellar and aggregated structures of varying morphology with large vesicles enclosing many smaller vesicles. These small vesicles become discernable under higher magnification; however, Por protein, due to its small size, is not visible within the lipid bilayers. Similar vesicles containing a positively charged lipid, DODAC, or a negatively charged lipid, POPS, display similar morphologies (Fig. 7C,D and E,F, respectively). Reconstituted systems were also size reduced by extrusion through two stacked polycarbonate filters of 100 nm pore size (see Section 2) in an attempt to generate unilamellar vesicles of a uniform size distribution. As shown in Fig. 7G,H, extruded proteoliposomes are uniformly unilamellar and spherical with an average diameter of about 100 nm (Table 1). Further, the extrusion procedure resulted in minimal loss of protein and lipid as reported previously [51].

4. Discussion

Due to the wide spectrum of antigenic responses to conventional whole-cell vaccine preparations, new vaccine strategies are emerging. In particular, subunit or peptide vaccine candidates are currently under investigation. However, purified peptides or antigens are often poorly immunogenic when administered alone. The antigenic properties of these immunogens can be restored when combining the antigen with an adjuvant, such as liposomes [7–9]. The incorporation of bacterial membrane proteins into liposomes has been characterized in earlier studies [50,51]. These studies have shown protein incorporation to be complete and in an orientation comparable to that seen in the native bacterial membrane. Such proteoliposomes have also been shown to exhibit a high degree of immunogenicity as determined by in vitro antibody binding assays and antibody titers in rabbits [49–51].

The subject of phospholipid and protein solubilization and subsequent proteoliposome reconstitution has been reviewed extensively [65–69]. Studies of detergents and liposomes have suggested a three-stage model of detergent–liposome interaction [70,71]. The first stage involves the partitioning of non-micellar detergent into the liposomal bilayer resulting in membrane permeability without solubilization. Stage two corresponds to the gradual disruption of the lipid and the emergence of detergent–lipid mixed micelles and detergent saturation of the liposomes. The final stage constitutes the complete solubilization and conversion of liposomes into detergent–lipid micelles. It is assumed that detergent-mediated reconstitution of liposomes or proteoliposomes follows the reverse of the solubilization process [72]. Initially, lipid/detergent and lipid/protein/detergent are present as mixed micelles. As detergent is removed, micelles become insoluble leading to the formation of structures composed of lipids and proteins that eventually form vesicles. In the final phase, residual intrabilayer detergent is removed. The incorporation of protein into the bilayer is thought to occur via two possible mechanisms. Either lipid vesicles are formed and the protein then inserts into these preformed vesicles. Alternatively, protein may become incorporated into the bilayer during the vesiculation process. Reconstitution studies have indicated that the presence of mixed micelles is required for protein incorporation to occur [72]. In addition, these studies have suggested that for initial detergent concentrations above the critical micellar concentration, complete protein incorporation could be obtained. Researchers have suggested that the composition of resulting proteoliposomes will be determined by the relative initial concentrations of lipid, protein and detergent [59]. Initial detergent concentrations have to be sufficiently above the CMC for complete vesiculation of the phospholipid. When the amount of detergent was at or near the minimal required for obtaining a clear solution, only about 50% of the lipid was found to be in vesicular form, the remainder consisting of other kinds of aggregates [59]. Initial detergent-to-lipid and initial protein-to-lipid ratio may affect the protein incorporation efficiency.

The present study compares protein reconstitution

into liposomes generated from different detergents. The detergents used octyl glucopyranoside (OGP), sodium cholate and Empigen BB, have been employed in either protein isolation and/or reconstitution studies [70–73]. OGP is a non-ionic detergent that has previously been used in the reconstitution of various membrane proteins [74]. One advantage to using this detergent is that it has a relatively high critical micellar concentration and can be rapidly removed during the dialysis procedure [51,58]. As the present study illustrates, complete Por protein incorporation into liposomes can be achieved by OGP-mediated reconstitution as has been reported for other proteins [51,71,72]. Analyses of reconstituted proteoliposomes have shown residual detergent levels to be well below the CMC, at levels that are non-membrane lytic [71].

Sodium cholate, an ionic detergent, has also been used in the reconstitution of membrane proteins into liposomes [63,72]. Studies report proteoliposomes generated by cholate-mediated reconstitution to be smaller in diameter than vesicles generated from OGP [74]. We observed, however, that Por containing vesicles generated from cholate were as large or larger than vesicles prepared from OGP. In addition, the rate of removal of cholate was much slower than that of OGP. Furthermore, cholate-mediated reconstitution resulted in lower protein incorporation efficiencies than for OGP-mediated reconstitution. The differences between these two detergents may be attributed to two factors. First, the anionic charge on the cholate may be involved in an electrostatic interaction with the protein residues. This interaction could have resulted in some protein denaturation, thus preventing complete incorporation of the gonococcal membrane protein. Second, the steroid-like structure, lower CMC and negative charge on cholate may retard or substantially reduce the rate of dissociation of detergent molecules from the lipid–protein complexes resulting in slower detergent removal. Studies suggest that the presence of polar hydroxyl groups of cholate in the hydrophobic core of liposomes and its lower CMC may play a role in the time required to generate proteoliposomes [72,75]. Slowing the reconstitution process may favor protein denaturation and/or aggregation and hence reduce the efficiency of protein incorporation.

A third detergent that was employed in proteoliposome reconstitution was Empigen BB. Empigen BB is a relatively mild, zwitterionic detergent that has been commonly used in protein solubilization and purification studies [73,76–79]. Many detergents have been used in protein isolation experiments; however, protein purification often results in loss of the antigenic determinants thus the antigenicity of the protein [79]. Empigen BB-extracted antigen preparations exhibit an ability to elicit greater antibody responses than preparations extracted with various other detergents [80,81]. These studies indicate Empigen BB to be milder and less denaturing on isolated proteins allowing for the retention of antigenic activity. Based on these properties, meningococcal proteoliposomes were reconstituted from a mixture of sodium cholate and Empigen BB to compare these vesicles to proteoliposomes formed from OGP. The present study demonstrates, however, that no vesiculation or protein incorporation occurred during reconstitution from a cholate/Empigen BB mixture. Despite prolonged dialysis, effective removal was not achieved and hence no protein reconstitution was possible. The CMC for Empigen BB (1.2 mM) [78] is lower than those of OGP and sodium cholate but this factor alone does not appear to be sufficient to explain why detergent removal was not achieved. One possibility is that within mixed micelles containing phospholipid, Por, cholate and Empigen BB, the effective CMC of each detergent compound is significantly lower than for the individual pure surfactant. In this regard it should be noted that Empigen BB has a charge distribution similar to that of membrane phospholipids [82].

Analysis of the detergent-mediated reconstitution experiments presented in this paper indicate octyl glucoside to be the detergent of choice for proteoliposome reconstitution, in particular, for the incorporation of bacterial membrane proteins into a liposomal subunit vaccine.

As mentioned earlier, it has been suggested that the composition and characteristics of the reconstituted system will be determined by the relative initial concentrations of lipid, protein and detergent [59]. With an initial detergent concentration below the CMC, only partial protein incorporation was attainable [72]. Complete protein incorporation into liposomes was achieved when initial detergent levels were above the CMC. Studies involving the incorporation

of the cytochrome P450 enzyme system into liposomes using OGP have reported the efficiency of protein incorporation to be dependent on the initial protein-to-lipid ratio [74]. This is consistent with the present results which indicate that protein incorporation is saturable for a given lipid concentration. As observed, initial protein-to-lipid ratios above this saturation limit result in incomplete protein incorporation with consequent denaturation and aggregation of non-incorporated protein. For a subunit vaccine preparation to be most effective, it would be advantageous to have maximum presentation of the antigenic determinants on the surface of the liposome. In addition, complete antigen incorporation into liposomes may reduce toxicity and increase immunogenicity of the antigen, subsequently yielding a vaccine with increased potency and efficacy.

Earlier studies have shown that proteoliposomes generated by OGP mediated dialysis are largely unilamellar with a mean diameter of about 200 nm [59]. In contrast, the cryo-electron micrographs presented in this paper indicate that reconstituted systems are heterogeneous in their size, lamellarity and morphology. Reconstituted proteoliposomes composed of neutral or charged lipids were observed to be multilamellar with very large diameter (>500 nm). These systems could be reduced in size to a homogeneous system of unilamellar vesicles of 100 nm using an extrusion procedure [52,53]. There are several advantages to vesicles of smaller diameter in a liposomal vaccine formulation. Studies have shown smaller vesicles to have prolonged circulation lifetimes compared to larger vesicles [83]. Increased circulation lifetimes could possibly increase vaccine efficacy by increasing the degree of antigen presentation and uptake by macrophages resulting in an improved immune response. Also, smaller vesicles would be favorable with regards to sterilization and safety for human use. Conventional heat sterilization or irradiation of the vaccine formulation would not be suitable sterilization methods as they would likely cause denaturation of the protein and loss of the immunogenic properties. In order to retain the immunogenic characteristics of the vaccine, the preparation would have to be sterilized under non-denaturing conditions. One method that would be suitable is terminal filtration. This procedure involves passing the reconstituted sample through a 0.2-μm filter that effectively removes any microbial organisms present in the sample. Clearly, this requires that the proteoliposomes be less than 200 nm in diameter.

The antigenic and immunogenic properties of these proteoliposome systems are currently being evaluated in animal models.

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