Available online at www.sciencedirect.com
ScienceDirect
International Journal of Pharmaceutics 332 (2007) 192–195
INTERNATIONAL JOURNAL OF PHARMACEUTICS
www.elsevier.com/locate/ijpharm

Note

Cage-like complexes formed by DOTAP, Quil-A and cholesterol

D.G. Lendemans, A.M. Egert, S. Hook, T. Rades *

School of Pharmacy, University of Otago, Dunedin, New Zealand

Received 8 February 2006; received in revised form 10 September 2006; accepted 19 September 2006
Available online 26 September 2006

Abstract

In this note we describe the substitution of the phospholipid component in classical ISCOMs (immune-stimulating complexes) with the cationic lipid dioleoyl-trimethyl-ammonium-propane (DOTAP). The self-assembled colloidal structures formed by DOTAP, Quil-A and cholesterol were characterised using transmission electron microscopy and laser Doppler velocimetry. Samples were prepared using the lipid-film hydration and dialysis techniques. Cage-like structures containing DOTAP were obtained in high numbers using dialysis, but not lipid-film hydration on day 4 post-preparation. The formation of cage-like structures was however only observed at compositions with low weight proportions of DOTAP (less than 25%) resulting in neutral to negative ζ-potentials of the colloidal particles. Compositions with high weight proportions of DOTAP displayed phase separation phenomena. Thus, whilst DOTAP can be incorporated with Quil-A and cholesterol to form cage-like particles, it appears to be unsuitable to prepare cationic equivalents of ISCOMs.

Keywords: ISCOMs; Cationic; Vaccine delivery; *Quillaja* saponins; Quil-A; Cholesterol; DOTAP

Immune-stimulating complexes (ISCOMs) constitute a potent particulate carrier platform for subunit vaccines and contain *Quillaja* saponins as built-in immuno-potentiators (Sanders et al., 2005). Phospholipids are a necessary constituent of ISCOMs and allow the formation of well-defined cage-like structures with a typical size of 40–100 nm (Kersten et al., 1991). Due to the anionic saponins, ISCOMs have a strongly negative ζ-potential (Kersten et al., 1991). We have previously described the successful substitution of cholesterol in ISCOMs with DC-cholesterol to form cationic cage-like structures (Lendemans et al., 2005). In this note we describe attempts to formulate cage-like structures containing the cationic lipid dioleoyl-trimethyl-ammonium-propane (DOTAP, as substitute for phospholipids), cholesterol and the semi-purified *Quillaja* saponin fraction Quil-A (Dalsgaard, 1974). Such particles may be useful as cationic alternatives to ISCOMs, potentially allowing efficient adsorption of DNA and protein subunit antigens.

1.5 ml TRIS buffered saline pH 7.4 (TBS; 65 mM TRIS, 80 mM NaCl) was used for the preparation of all samples. The total concentration of the components DOTAP (Northern Lipids, Canada), Quil-A (Brenntag Biosector, Denmark) and cholesterol (Sigma, USA) in all formulations was 6.7 mg/ml. Colloidal structures were prepared using the dialysis and lipid-film hydration techniques described previously (Kersten et al., 1991; Demana et al., 2004b; Lendemans et al., 2005). A membrane with a molecular weight cut-off of 1000 Da and 40 mg/ml octylglucoside (Sigma) were utilised for dialysis, which was performed at 4 °C over a period of 48 h against 4× 1 l TBS. On day 4 post-preparation, samples were characterised by transmission electron microscopy (TEM) and laser Doppler velocimetry on as previously described (Lendemans et al., 2005).

A series of samples was prepared with compositions representing several cuts through a pseudo-ternary phase diagram (cuts A–D, rows 1–5, Fig. 1). Corresponding ζ-potentials were determined in TBS (Fig. 1), and measured values were in good agreement with a hypothetical line of charge inversion around 1:1 molar ratios of Quil-A:DOTAP, assuming an average $M_R = 1650$ of the saponin molecules in Quil-A (Mitra and Dungan, 1997), and $M_R = 698.5$ for DOTAP (as chloride).

The system containing DOTAP as single lipid component (sample A1) produced DOTAP vesicles with a diameter in the range between 20 and 500 nm (not shown). In the samples containing binary mixtures of DOTAP and cholesterol (row 1) vesicles were observed (Fig. 2A) and colloidal structures became bigger towards higher weight proportions of cholesterol. In the

* Corresponding author at: School of Pharmacy, University of Otago, Dunedin, PO Box 913, New Zealand. Tel.: +64 3 479 5410; fax: +64 3 479 7034.
E-mail address: thomas.rades@stonebow.otago.ac.nz (T. Rades).

0378-5173/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2006.09.029

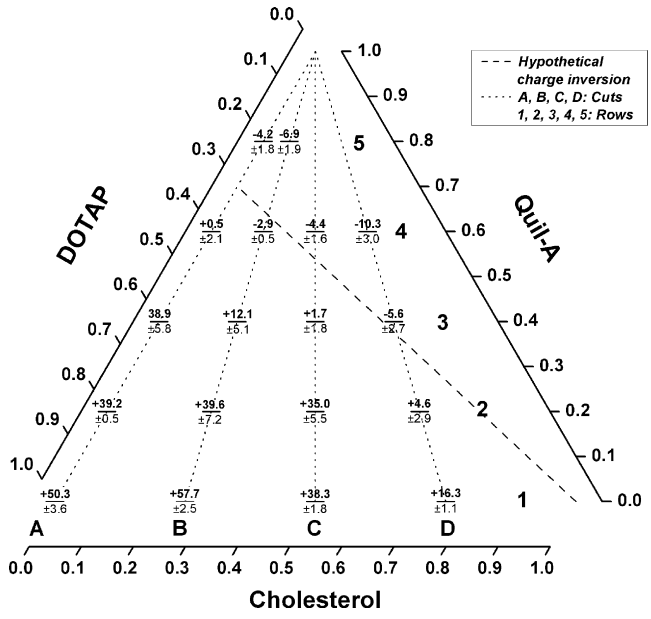

Fig. 1. Designations and ζ-potentials (mean ± S.D.) of samples prepared by lipid-film hydration. ζ-potentials were determined 10 times in TBS. The dashed line indicates 1:1 molar ratios of DOTAP and Quil-A (hypothetical charge inversion).

binary mixtures of DOTAP and Quil-A, the presence of 20% Quil-A (sample A2) decreased the overall particle size compared to structures formed by pure DOTAP (Fig. 2B). A similar observation was made previously in phosphatidylcholine:Quil-A systems (Demana et al., 2004b). Some of the smaller structures showed a stacked morphology (Fig. 2B, arrow). These stacked structures may constitute bilayered fragments of vesicles (Demana et al., 2004b). An increase in Quil-A concentration (40% and 60%, samples A3 and A4) did however not lead to a further reduction in particle size and instead resulted in re-formation of bigger structures with a distinct lamellar fine structure (Fig. 2C). The re-formation of larger aggregates is very likely related to the decrease in ζ-potential (Fig. 1). Aggregation of structures at an equimolar ratio of cationic and anionic amphiphile is characteristic for catanionic systems in the presence of excess salt and water (Horbaschek et al., 1998, 2000). Addition of more Quil-A (80%, sample A5) led to formation of smaller particles in the range between 7.5 and 90 nm with a negative ζ-potential (not shown). In conclusion, the binary systems DOTAP:Quil-A (cut A) showed an initial decrease in size upon addition of anionic saponin molecules to the cationic DOTAP, which was followed by aggregation towards charge neutrality. Further addition of Quil-A then resulted in re-segregation into structures with a negative ζ-potential.

In agreement with the pseudo-binary DOTAP:cholesterol systems (row 1, Fig. 1), colloidal structures in ternary systems became larger towards higher weight proportions of cholesterol. At lower and intermediate concentrations of Quil-A (rows 2 and 3 in cuts B–D, Fig. 1), these larger structures often showed a high number of pores in a hexagonal arrangement as previously described in the context of classical ISCOMs (Bangham and Horne, 1962; Kersten et al., 1991; Demana et al., 2004b). The number of these structures increased towards higher cholesterol concentrations. Importantly, these were found in combination with a second colloidal structure in which pores were absent. These structures had a distinct lamellar fine-structure and were found especially at higher DOTAP concentrations. The presence of two distinct colloidal structures may indicate non-ideal mixing or even phase separation. Fig. 2D illustrates this (sample C3, pore-containing [P] and lamellar [L] sections are indicated). The size pattern described above for the pseudo-binary mixtures (cut A) was also found within the pseudo-ternary mixtures (cuts B–D). Fig. 2E shows a system with aggregation tendency (sample B4, with a ζ-potential of -2.9 ± 0.5 mV). A small number of cage-like-structures with typical size and narrow size distribution (32.9 ± 3.3 nm, $n = 50$, solid arrow) was also present in those systems. Sample B5 with a negative ζ-potential of -6.9 ± 1.9 mV produced a high proportion of ring-like micelles which were similar in size and morphology to the well-known mixed micelles composed of Quil-A and cholesterol (Kersten et al., 1991; Demana et al., 2004a). These ring-like micelles were sometimes found as single units and sometimes as aggregates in ISCOM-like structures (Fig. 2F). It has been proposed that ring-like micelles as precursor structures may aggregate to well-defined ISCOMs over time (Demana et al., 2004a). Summarising the results obtained using lipid-film hydration as the preparation method, aggregation phenomena were observed in samples with equimolar amounts of DOTAP and Quil-A. The use of DOTAP did not result in the formation of cage-like particles in the cationic area of the phase diagram. Instead, the observations suggested a phase separation (Fig. 2D), with domains predominantly containing Quil-A:DOTAP or Quil-A:cholesterol, respectively, co-existing with each other. The three components DOTAP, cholesterol and Quil-A may have only combined to a saturation level, thus causing the absence of cage-like particles at compositions with high weight proportions of DOTAP. Using other phospholipids (such as PC) in conjunction with lipid-film hydration, ISCOMs were reported to form at high weight proportions of phospholipids (Demana et al., 2004a,b). The incompatibility of higher DOTAP proportions with the Quil-A:cholesterol pore structures may be due to its permanently charged headgroup, as the ISCOM model proposed by Kersten et al. states that the lipid headgroups show towards the inside of the pores (Kersten et al., 1991). However, due to charge repulsion and space restrictions the pore structures formed by Quil-A:cholesterol may only have a limited capacity to take up DOTAP. With smaller DOTAP proportions, formation of some cage-like structures was observed, albeit only close to charge neutrality or at compositions with a negative ζ-potential. This however is a clear indicator that DOTAP was able to insert into Quil-A:cholesterol structures to some extent as spherical cage-like structures would have otherwise not formed at all (Kersten et al., 1991; Demana et al., 2004a). Alternatively, the poor mixing or assumed phase separation may also be related to the lipid-film hydration method as this method is known to produce heterogeneous particle populations and samples which require equilibration (Copland et al., 2000; Demana et al., 2004b). Thus, dialysis was utilised as an alternative preparation technique in a second set of experiments. Sample compositions were restricted to a small area of the phase diagram. Fig. 3 gives an overview

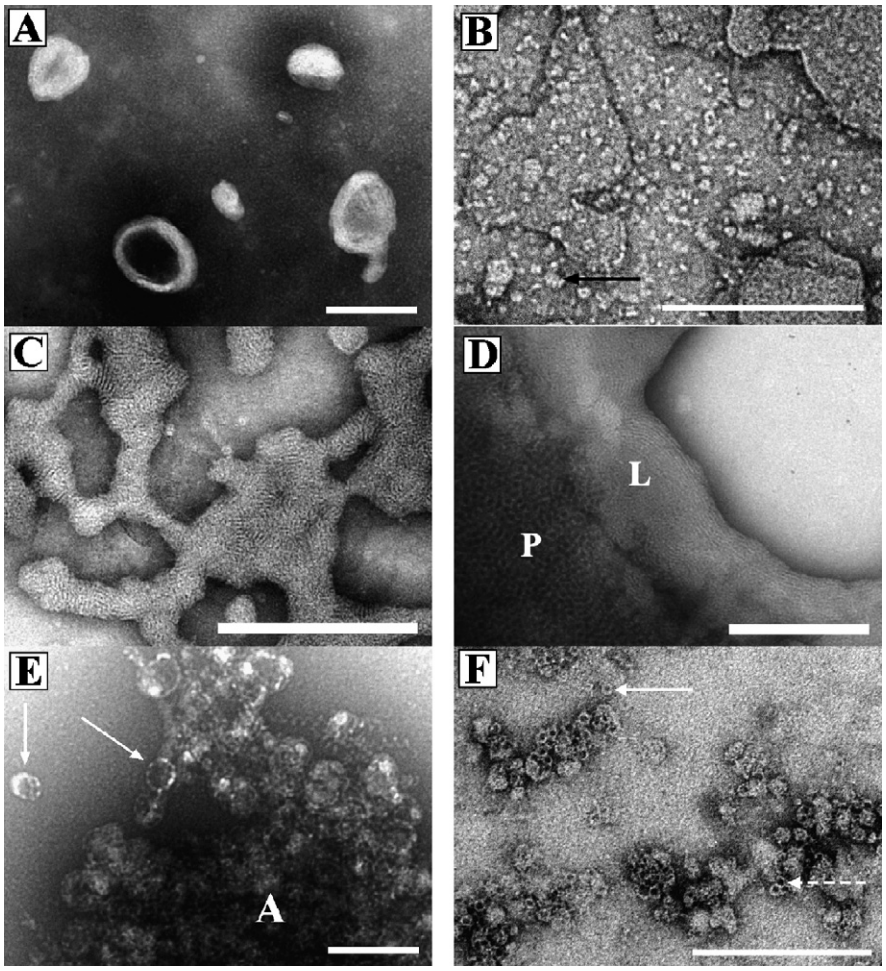

Fig. 2. TEM micrographs of selected samples prepared by lipid-film hydration. Samples are designated as in Fig. 1. (A) Sample B1; (B) sample A2—arrow indicates stacked structures, bar = 200 nm; (C) sample A4, bar = 200 nm; (D) sample C3—pore domains "P" and lamellar domains "L" are indicated, bar = 200 nm; (E) sample B4—cage-like structures (solid arrow) and aggregates ("A") are shown, bar = 100 nm; (F) sample B5—structures with a negative ζ-potential re-separated and distinct ring-like micelles (solid arrow) or aggregates of these (dashed arrow) were found, bar = 200 nm.

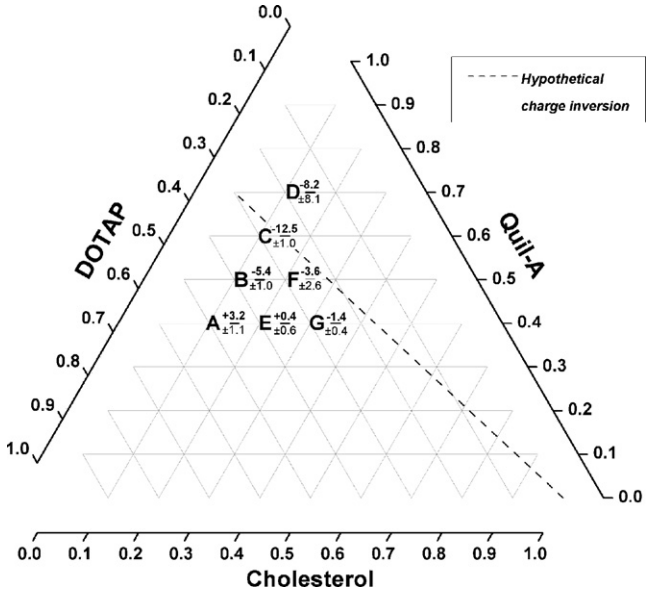

Fig. 3. Designations and ζ-potentials (mean ± S.D.) of samples prepared by dialysis. ζ-potentials were determined 10 times in TBS. The dashed line indicates 1:1 molar ratios of DOTAP and Quil-A (hypothetical charge inversion).

of compositions and ζ-potentials. With the exception of sample C, decreasing trends in ζ-potentials were observed towards higher concentrations of Quil-A. In agreement with the samples prepared by lipid-film hydration, colloidal structures with ζ-potentials close to charge neutrality were found as aggregates with domains which either displayed or lacked pores ("P" and "L" domains, Fig. 4A). Sample C produced ISCOM-like particles with a negative ζ-potential of -12.5 ± 1.0 mV (Fig. 4B). The sample of similar composition prepared by the hydration method (Fig. 1, B4) had formed predominantly bigger aggregates with a ζ-potential of -2.9 ± 0.5 mV. This may suggest that following dialysis the negatively charged saponin molecules in Quil-A had interacted more strongly with the lipid components than following lipid-film hydration. Alternatively, there may be a partial loss of DOTAP during dialysis. The diameter of the ISCOM-like structures was 54.4 ± 4.8 nm ($n = 75$). Closer inspection of the micrograph reveals that small lipidic particles (21.5 ± 2.4 nm, $n = 25$) were attached to the particles (Fig. 4B, arrow), and this may again indicate that cage-like particles incorporated DOTAP to saturation and that excess amounts of DOTAP separated from these. The extensive formation of cage-like structures *per se*, however, suggests incorporation of

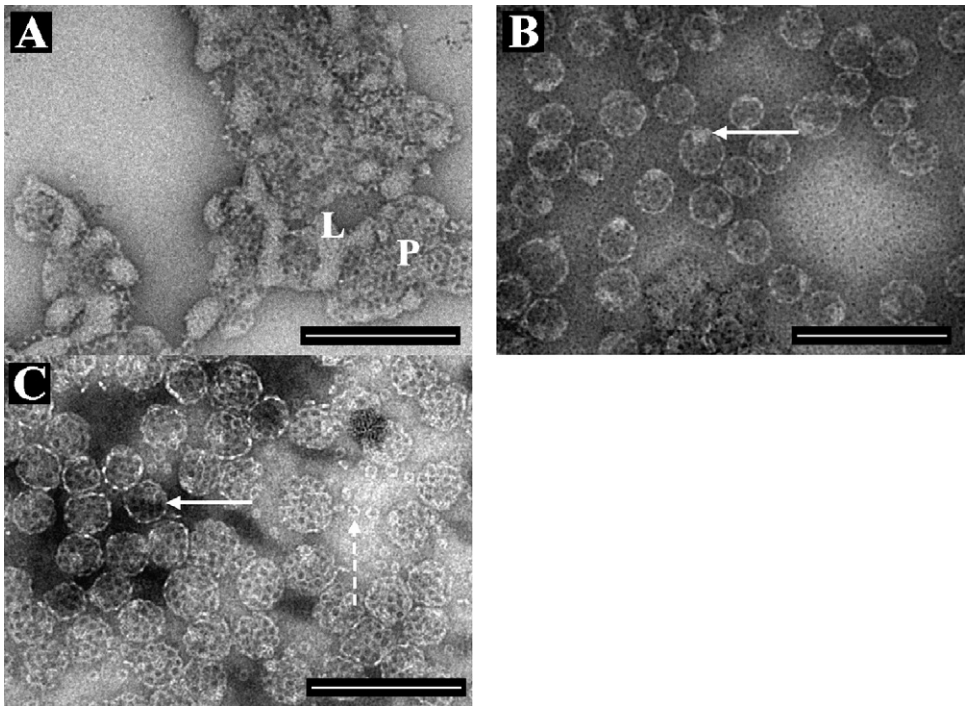

Fig. 4. TEM micrographs of selected samples prepared by lipid-film hydration. Samples are designated as in Fig. 3. (A) Sample F—pore domains "P" and lamellar domains "L" are indicated; (B) sample C—small lipidic particles are attached to cage-like particles (solid arrow); (C) sample D—cage-like particles (solid arrow) were found with ring-like micelles (dashed arrow), bar = 200 nm.

DOTAP (Kersten et al., 1991). Cage-like structures were also observed in sample D and the size of these ISCOM-like particles increased to 61.3 ± 5.8 nm ($n = 100$) in this sample (Fig. 4C). Small lipidic particles however, were not attached to the ISCOM-like particles in this sample. Instead, the sample D did contain some ring-like micelles (Fig. 4C, dashed arrow). This suggested that with the experimental protocol used, the saturation limit of DOTAP within Quil-A:cholesterol structures was between 15–25% by weight. The fact that the particles (and their pores) in sample D were larger than those in sample C may be due to a different lipid packing and arrangement of cationic charges within the pores. This may result in head group repulsion and size expansion.

In conclusion, cage-like structures containing DOTAP were obtained in high numbers using dialysis, but not lipid-film hydration, on day 4 post-preparation. Samples prepared by dialysis thus appeared to equilibrate faster than those prepared by lipid-film hydration. However, the formation of cage-like structures was only observed at compositions leading to neutral or negative ζ-potentials. Compositions with higher proportions of DOTAP displayed phase separation phenomena, regardless of the preparation method used. Thus, unlike the substitution of cholesterol with DC-cholesterol (Lendemans et al., 2005), the replacement of phospholipids by DOTAP does not appear to be suitable to prepare cationic Quil-A containing cage-like structures.

Acknowledgment

This work would not have been possible without the support of the Cancer Society of New Zealand.

References

Bangham, A.D., Horne, R.W., 1962. Action of saponin on biological cell membranes. Nature 196, 952–953.

Copland, M.J., Rades, T., Davies, N.M., 2000. Hydration of lipid films with an aqueous solution of Quil A: a simple method for the preparation of immune-stimulating complexes. Int. J. Pharmaceut. 196, 135–139.

Dalsgaard, K., 1974. Saponin adjuvants. III. Isolation of a substance from *Quillaja* saponaria Molina with adjuvant activity in foot-and-mouth disease vaccines. Archiv fuer die gesamte Virusforschung 44, 243–254.

Demana, P., Davies, N., Berger, B., Vosgerau, U., Rades, T., 2004a. A comparison of pseudo-ternary diagrams of aqueous mixtures of Quil A, cholesterol and phospholipid prepared by lipid-film hydration and dialysis. J. Pharm. Pharmacol. 56, 573–580.

Demana, P.H., Davies, N.M., Vosgerau, U., Rades, T., 2004b. Pseudo-ternary phase diagrams of aqueous mixtures of Quil A, cholesterol and phospholipid prepared by the lipid-film hydration method. Int. J. Pharmaceut. 270, 229–239.

Horbaschek, K., Hoffmann, H., Hao, J., 2000. Classic La phases as opposed to vesicle phases in cationic-anionic surfactant mixtures. J. Phys. Chem. B 104, 2781–2784.

Horbaschek, K., Hoffmann, H., Thunig, C., 1998. Formation and properties of lamellar phases in systems of cationic surfactants and hydroxy-naphtoate. J. Colloid Interf. Sci. 206, 439–456.

Kersten, G.F., Spiekstra, A., Beuvery, E.C., Crommelin, D.J., 1991. On the structure of immune-stimulating saponin-lipid complexes (ISCOMs). Biochim. Biophys. Acta 1062, 165–171.

Lendemans, D.G., Myschik, J., Hook, S., Rades, T., 2005. Cationic cage-like complexes formed by DC-cholesterol, Quil-A and phospholipid. J. Pharmaceut. Sci. 94, 1794–1807.

Mitra, S., Dungan, S.R., 1997. Micellar properties of *Quillaja* saponin. 1. Effects of temperature, salt, and pH on solution properties. J. Agric. Food Chem. 45, 1587–1595.

Sanders, T.S., Brown, L.E., Deliyannis, G., Pearse, M.J., 2005. ISCOM-based vaccines: the second decade. Immunol. Cell Biol. 83, 119–128.