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International Journal of Pharmaceutics 298 (2005) 339–343
international journal of pharmaceutics
www.elsevier.com/locate/ijpharm

Note

Cholesterol–bile salt vesicles as potential delivery vehicles for drug and vaccine delivery

C. Martin [a], J. Thongborisute [b], H. Takeuchi [b], H. Yamamoto [b], Y. Kawashima [b], H.O. Alpar [a,*]

[a] *Vaccine Delivery Group, Centre for Drug Delivery Research, University of London, School of Pharmacy, 29–39 Brunswick Square, London WC1N 1AX, UK*
[b] *Department of Pharmaceutical Engineering, Gifu Pharmaceutical University, Mitahorahigashi, Gifu 502-8585, Japan*

Received 22 November 2004; received in revised form 21 February 2005; accepted 1 March 2005

Abstract

The aim of this study was to further investigate the interactions between cholesterol (CH) and mixed bile salts (BS) (sodium cholate and sodium deoxycholate) and their suitability for drug and vaccine delivery. Insulin was used as a model protein to assess the ability of CH:BS vesicles to entrap a therapeutically relevant macromolecule. The association of protein (FITC–insulin) with the CH:BS structure was confirmed with fluorescence microscopy, and the overall morphology of the vesicles was examined with atomic force microscopy (AFM). Results demonstrate that the nature of the vesicles formed between CH and BS is dependent not only on the concentration of BS but also on the increasing CH concentration leading to CH crystal formation.

Keywords: Cholesterol; Bile salts; Vaccine delivery

The development of oral delivery systems for protein therapeutics and vaccines has been researched extensively over the last decade (Russell-Jones, 2000; Wang, 1996). Delivery systems such as polymeric particles, gels, emulsions, liposomes and micelles have been explored for their potential to provide controlled and sustained release of macromolecules (Barenholz, 2001; Brayden, 2001; Peppas et al., 2000). Many chemical and physical barriers to successful protein and peptide delivery are present in the gastrointestinal tract (Wang, 1996); in the case of liposomes, the harsh chemical environs range from the low acidity of the stomach to the presence of bile salts (BS) in the small intestine.

The ability of BS to disrupt lipid bilayers by intercalating between the phospholipids (PL), and forming mixed micelles is well known (Venneman et al., 2002; Wacker and Schubert, 1998). During BS integration into PL:CH bilayers, the resulting vesicles can

* Corresponding author. Tel.: +44 207 753 5928; fax: +44 207 753 5942.
E-mail address: oya.alpar@ulsop.ac.uk (H.O. Alpar).

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doi:10.1016/j.ijpharm.2005.03.038

Table 1
Composition, zeta potential and entrapment of insulin for the four CH:BS vesicle formulations

CH:BS molar ratio	Mass of CH (mg)	Mass of mixed BS (mg)	Concentration of mixed BS (mM)	Zeta potential (mV) ± S.D.	Mass of entrapped insulin (μg/mg) ± S.D.
1:1	48.3	51.7	12.4	−41.2 ± 5.9	3.41 ± 0.91
1:2	31.8	68.2	16.5	−41.2 ± 2.6	3.53 ± 0.53
1:4	18.9	81.1	18.9	−25.0 ± 1.3	6.59 ± 0.19
1:10	8.5	91.5	22.0	−32.9 ± 8.1	6.52 ± 1.70

assume five potential arrangements (Venneman et al., 2002): (1) PL:CH liposomes; (2) BS micelles; (3) PL:BS micelles; (4) BS:CH micelles and (5) PL:CH:BS micelles. As the bilayer is solubilised by a progressive increase in BS intercalation and new mixed micellar and vesicular structures are formed, entrapped material will be released and recaptured accordingly.

Previously, we reported on the interaction of CH and BS (Martin et al., 2003). It was noted that when liposomes are lysed with BS, a portion of fluorescent material is not recovered; in addition, the amount of sequestered material increases with the CH content of vesicles. AFM examination of the structures formed when CH is dispersed in BS revealed crystalline morphologies, such as plate- and needle-like structures (Venneman et al., 2002). The aim of the present study therefore was to further characterise CH:BS vesicles with particular reference to their structure and ability to entrap a model protein.

To generate the CH:BS vesicles a thin film of CH (Sigma, UK) was prepared by dissolving the required mass of sterol in 1 ml chloroform (BDH Laboratories, UK) in a 50 ml volume round bottom flask. The organic solution was reduced by rotary evaporation (Büchi, Switzerland), with the round bottom flask partially submerged in a 50 °C water bath for 1 h. To ensure any residual solvent had been fully removed from the CH film, the flask was purged with nitrogen for 1 h. The film was subsequently rehydrated with the appropriate concentration of a solution of mixed BS (1:1 by mass mixture of sodium cholate and sodium deoxycholate) (Sigma, UK) and either bovine insulin (Sigma, UK) or fluorescein isothiocyanate-labelled insulin (FITC–insulin) (Sigma, UK) (at a final concentration 0.1 mg/1.0 ml), and maintained in a circular orbital shaking incubator (Gallenkamp) at 37 °C for 1 h. After rehydration, samples were decanted into sealed plastic tubes and stored at 2 °C for 24 h before analysis. Detail of composition for the four formulations is given in Table 1.

The ability of the CH:BS vesicles to sequester a model protein was assessed using insulin. Entrapment was established by bicinchoninic acid (BCA) assay (Smith et al., 1985). Compatibility of amphiphilic BS and the CH component with the BCA assay was also separately assessed, and found to cause no interference with the assay. Briefly, 20 μl of each sample was reacted with 200 μl BCA working reagent (bicinchoninic acid with 0.02% v/v copper sulphate) (Sigma, UK) and incubated at 50 °C for 30 min. Samples were allowed to cool for 5 min before results were read by spectrophotometer at 405 nm (Dynex plate reader). Results are presented in terms of the mass of insulin sequestered per mass of CH:BS vesicles (mean of three measurements ± standard deviation).

The zeta potential of the vesicles was measured by ZetaMaster (Malvern Instruments, UK). Briefly, the sample was dispersed in 20 ml of 10 mM potassium chloride (Sigma, UK) and vortexed to ensure thorough mixing. The entire sample was injected into the ZetaMaster and the mean of three measurements ± S.D. is presented.

AFM (Digital Instruments, USA) was operated in Tapping-Mode® to examine the morphology of the air-dried CH:BS vesicles. Glass cover slides were gold sputter-coated and a drop of sample placed on the slide and dried by desiccating overnight. A silicon probe (Nanoprobe SPM TIPS type RTESP7) was used and results analysed by Nanoscope® IIIa version 4.43r8 (USA) software.

The CH:BS vesicles entrapping FITC–insulin were visualised by Nikon Microphot-FXA fluorescence microscope at 343 nm excitation wavelength and images were captured digitally.

The results shown in Table 1 indicate that the two formulations containing the highest ratios of CH:BS

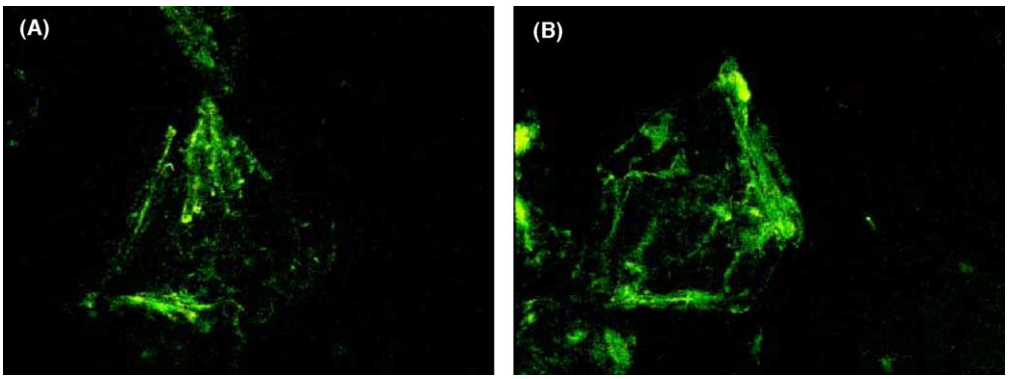

Fig. 1. Fluorescence micrographs of the crystal-like CH:BS particles. (A) A 40× magnification of the CH:BS vesicles 1:10 (molar ratio) and shows the rod-like appearance of the liquid crystal structure. (B) A 40× magnification of the CH:BS vesicles 1.0:4.0 (molar ratio). Again the rod-like appearance of the structure and the associated FITC–insulin is clearly visible within the structure.

(i.e. 1:10 and 1:4) were able to sequester almost twice as much insulin compared to the lower CH:BS ratios. CH:BS 1:10 and 1:4 molar ratio entrapped 6.52 ± 1.70 and 6.59 ± 0.19 μg/mg, respectively, compared to 3.41 ± 0.91 and 3.53 ± 0.53 μg/mg for 1:1 and 1:2 CH:BS ratio. Additionally, the zeta potential of the particles also reflected this trend, with the more negative zeta potential shown by the two formulations with lower CH:BS molar ratios: 41.2 ± 5.9 and 41.2 ± 2.6 mV for the 1:1 and 1:2, respectively. This variation in zeta potential may be due to the predominance of CH crystals at higher CH:BS molar ratio (i.e. 1:10 and 1:4), thus resulting in a less negative value. It may also result from the greater association of insulin with the high CH:BS molar ratio formulations. Conversely, at lower CH:BS molar ratios, fewer CH crystals are present, and the predominant structures are BS micelles; the higher negative zeta potential value may be attributable to the hydrophilic hydroxy groups on individual BS monomers (Narain et al., 1999).

The results for the entrapment of insulin were also confirmed when the samples were examined by fluorescence microscopy. It appears from Fig. 1 that FITC–insulin is present between the faces of adjacent CH crystals within the CH:BS structures.

Visualisation of these vesicles by AFM showed the presence of distinct structures. Fig. 2 reveals a sheet-like formation of liquid crystal structures (Cullis and Hope, 1991; Bisby, 1982). Interestingly, the dimensions of these structures correspond with the vesicle diameters as measured by dynamic laser scattering (result not shown). Fig. 2 depicts the structure of the CH:BS micelles as being needle-like crystals. When the sample was examined as a dispersed aqueous suspension with fluorescence microscopy only a few of these needle-like crystals were visible. The increased prevalence of needle-like crystals is believed to result from the increased concentration of CH on the AFM stub, as the sample was desiccated overnight and viewed in a dried state. Therefore, as the majority of the liquid had evaporated the concentration of the solution increased, leading to the promotion of CH crystallisation, possibly aided by the presence of hydrophobic BS.

The situation encountered with the association of CH and BS is analogous to that found in the small intestine during dietary fat uptake (Narain et al., 1999). BS micelles are able to transport the products of fat digestion by incorporating the less soluble lipids into mixed micellar structures. Furthermore, in liver, mixed micelles are found in bile where PL, CH and BS combine before secretion into the small intestine. If CH is not sequestered into mixed micelles and the concentration increases it begins to precipitate and form crystals (Venneman et al., 2002).

One of the BS species utilised in these experiments, i.e. deoxycholate, is a hydrophobic BS (Venneman et al., 2002), and the detergent abilities of BS increase with increasing relative hydrophobicity (Narain et al., 1999). Therefore, as more hydrophilic BS has been shown to inhibit crystallisation of CH, the deoxycholate used here at 1:10 and 1:4 molar ratios appears

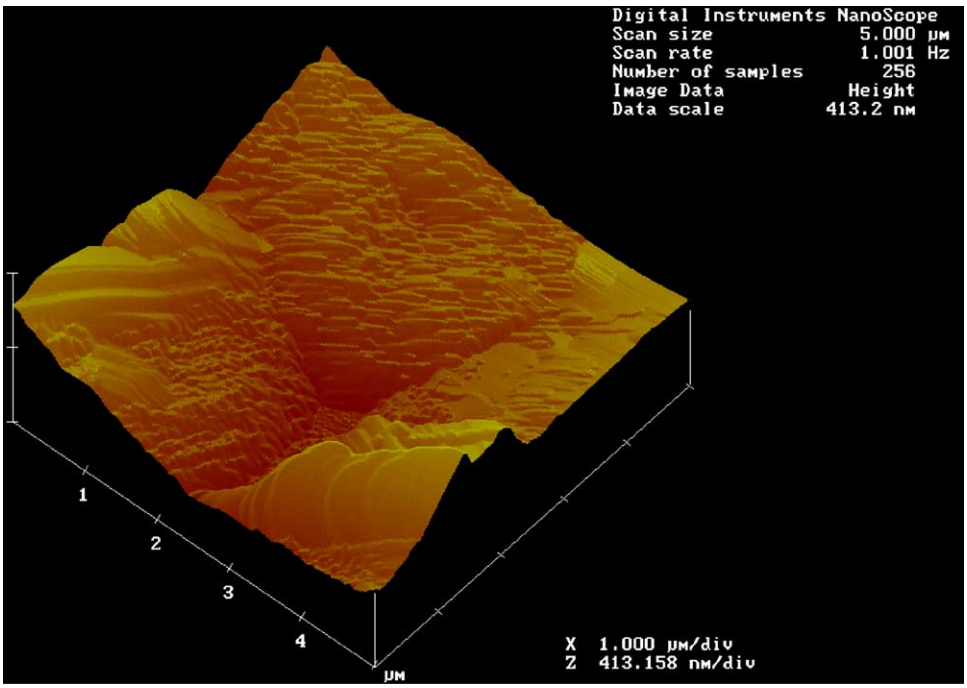

Fig. 2. ARM image of the dried CH–bile vesicles. The sheets of rod-like liquid crystals are clearly visible, and have an average length of 5 μm.

to promote the development of crystals. The results indicate that the presence of CH crystals is required to entrap more insulin, as shown by both BCA assay and fluorescence micrograph images.

These results provide a useful insight into the potential fate of liposomal carriers in vivo. It is hypothesised that upon entering the confines of the small intestine, and on exposure to BS, liposomes are effectively destroyed (Kokkona et al., 2000). This destruction is followed by a reformation, potentially between CH and BS, although not excluding the possibility of the interaction of PL. Thus, although structurally the liposomal bilayer is breached, new structures are formed which still entrap a measurable quantity of material.

Many delivery systems such as liposomes and solid lipid nanoparticles, incorporate CH into their structure (Müller et al., 2000; Gregoriadis, 1995). The results shown here give some insight into the possible mechanisms whereby extra material may be entrapped in a supersaturated layer of CH crystals. When delivered orally the CH layer may dissociate in the presence of BS, thus forming mixed micelles and enhancing the uptake of macromolecules in the small intestine.

In conclusion, CH and BS interaction resulted in structures with definable zeta potential, entrapment and morphology. Future studies aim to elucidate the 3-dimensional nature of the CH:BS structures and determine the kinetics of association between amphiphilic BS and other lipid-based molecules in vitro.

Acknowledgments

This work was conducted as part of the Research Experience for European Students (REES) program. The authors wish to gratefully acknowledge the funding provided jointly by the British Council and the Japan International Science and Technology Exchange Centre (JISTEC).

References

Barenholz, Y., 2001. Liposome application: problems and prospects. Curr. Opin. Colloid Interf. Sci. 6, 66–77.

Bisby, R.H., 1982. Lyotropic liquid crystals. In: Wyn-Jones, E., Gormally, J. (Eds.), Aggregation Processes in Solution. Elsevier Science Publishing Co. Inc.

Brayden, D.J., 2001. Oral vaccination in man using antigens in particles: current status. Eur. J. Pharm. Sci. 14, 183–189.

Cullis, P.R., Hope, M.J., 1991. Physical properties and functional roles of lipids in membranes. In: Vance, D.E., Vance, J.E. (Eds.), Biochemistry of Lipids, Lipoproteins and Membranes. Elsevier Science Publishing Co. Inc.

Gregoriadis, G., 1995. Engineering liposomes for drug delivery: progress and problems. Trends Biotechnol. 13, 527–537.

Kokkona, M., Kallinteri, P., Fatouros, D., Antimisiaris, S.G., 2000. Stability of SUV liposomes in the presence of cholate salts and pancreatic lipases: effect of lipid composition. Eur. J. Pharm. Sci. 9, 245–252.

Martin, C., Thongborisute, J., Takeuchi, H., Yamamoto, H., Kawashima, Y., Alpar, H.O., 2003. Investigation of the interactions between cholesterol and bile salts. Proc. Int. Symp. Cont. Rel. Bioact. Mater. 31, 288.

Müller, R.H., Mäder, K., Gohla, S., 2000. Solid lipid nanoparticles (SLN) for controlled drug delivery—a review of the state of the art. Eur. J. Pharm. Biopharm. 50, 161–177.

Narain, P.K., DeMaria, E.J., Heuman, D.M., 1999. Cholesterol enhances membrane-damaging properties of model bile by increasing the intervesicular-intermixed micellar concentration of hydrophobic bile salts. J. Surg. Res. 84, 112–119.

Peppas, N.A., Bures, P., Leobandung, W., Ichikawa, H., 2000. Hydrogels in pharmaceutical formulations. Eur. J. Pharm. Biopharm. 50, 27–46.

Russell-Jones, G.J., 2000. Oral vaccine delivery. J. Control. Release 65, 49–54.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., Klenk, D.C., 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. 150, 76–85.

Venneman, N.G., Huisman, S.J., Moschetta, A., vanBerge-Henegouwen, G.P., van Erpecum, K.J., 2002. Effects of hydrophobic and hydrophilic bile salt mixtures on cholesterol crystallisation in model biles. Biochim. Biophys. Acta 1583, 221–228.

Wacker, M., Schubert, R., 1998. From mixed micelles to liposomes: critical steps during detergent removal by membrane dialysis. Int. J. Pharm. 162, 171–175.

Wang, W., 1996. Oral protein drug delivery. J. Drug Target. 4, 195–232.