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The biodistribution of novel 200-nm palmitoyl muramic acid vesicles

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Abstract

Palmitoyl muramic acid (PMA) a novel synthetic surfactant has been prepared by the reaction of palmitic acid *N*-hydroxysuccinimide ester and muramic acid. The compound was characterised by thin layer chromatography, mass spectrometry and melting point. PMA vesicles prepared from PMA, cholesterol, poly-24-oxyethylene cholesteryl ether-Solulan C24 (45:45:10) were loaded with doxorubicin (DOX) by the use of ammonium sulphate gradients. DOX PMA vesicles (mean size 250 nm) were found to exhibit no change in size when incubated over a 24-h period with rat plasma at 37°C and very little change in the level of drug associated with the niosomes-showing good plasma stability. The biodistribution of DOX PMA vesicles was studied in male BALB/c mice after intravenous administration (5 mg kg^{-1} DOX). PMA vesicles were found to avoid liver uptake but not splenic uptake when compared to DOX loaded sorbitan monostearate (Span 60) niosomes. DOX plasma levels at 5 h after the administration of DOX PMA vesicles represented 0.20% of the administered dose and 0.10% in the case of DOX Span 60 niosomes. The implications of these findings for drug delivery are discussed.

Keywords: Palmitoyl muramic acid; Thin layer chromatography; Biodistribution; Drug delivery

1. Introduction

Non-ionic surfactant vesicles (niosomes) are closed bilayer vesicles analogous to liposomes but prepared from non-phospholipid materials (Florence, 1993; Uchegbu and Florence, 1995). Niosomes have proved beneficial in experimental disease states as drug carriers of anti-cancer (Rogerson et al., 1988; Uchegbu et al., 1995) and anti-infective agents (Baillie et al., 1986). The use of synthetic surfactants to prepare vesicular drug carriers theoretically offers a rich variety of potential vesicle surfaces as vesicles may be prepared from amphiphiles bearing sugar, oxyethylene, glycerol, polyhydroxy and amino acid head groups (Uchegbu and Florence, 1995). However very little systematic study has been carried out on the biological and physicochemical effects of varying the niosome amphiphile head group. A

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comparison between niosomes prepared from polyglycerol and polyoxyethylene ether surfactants revealed that the encapsulation efficiency for a macromolecular pro-drug was affected by the nature of the head group (Uchegbu and Duncan, 1997).

While many studies have examined the fate of positively and negatively charged liposomes after intravenous administration (Gabizon and Papahadjopoulos, 1992; Kim et al., 1994; Gabizon et al., 1996), the vesicles studied inevitably contain a phospholipid component, making the assignation of surface charge difficult at physiological pH. Indeed dipalmitoyl phosphatidylcholine/cholesterol vesicles containing 10 mol% 1-*O*-palmityl-D-glucuronic acid (PGlcUA) had the same electropheretic mobility as vesicles devoid of PGlcUA (Namba et al., 1990) and once incubated with bovine serum albumin both 'positively' and 'negatively' charged vesicles had the same electrophoretic mobility at physiological pH (Law et al., 1988). Hence, it might be safer to detail vesicle behaviour as a function of the different chemical groups present on the surface of these vesicles and not the perceived charge.

A study of the biodistribution of vesicles (non-phospholipid or otherwise) carrying predominantly a carboxylic acid group and no other charged moiety appears not to have been carried out before the current work. Muramic acid, which is present as the *N*-acetyl derivative within peptidoglycan—the main skeletal component of the bacterial cell walls and is also a constituent of muramyl dipeptide (*N*-acetylmuramyl-L-alanyl-D-isoglutamine-*n*-butyl ester) a known immonological adjuvant (Lederer, 1986; Takada and Kotani, 1995). This sugar was employed as the hydrophilic moiety in the construction of a new vesicle forming surfactant; palmitoyl muramic acid (Fig. 1). The in vitro stability of these vesicles in plasma and the influence of a muramic acid surface on biodistribution was also studied.

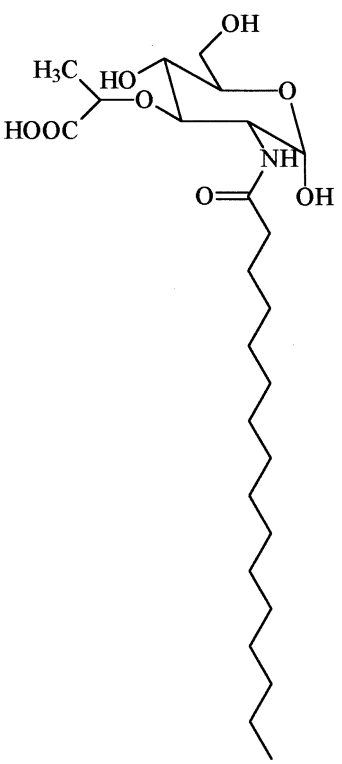

Fig. 1. Palmitoyl muramic acid (PMA).

2. Materials and methods

2.1. Materials

Muramic acid, Palmitic acid *N*-hydroxysuccinimide ester, triethylamine, dimethyl sulfoxide, sorbitan monostearate and cholesterol were all supplied by Sigma-Aldrich, Dorset, UK. Dicetyl phosphate was supplied by Fluka Chemika-Biochemika, Dorset, UK. Chloroform was supplied by Rathburn Chemicals, UK and Solulan C24 was donated by Ellis and Everard, Essex, UK. Male BALB/c mice were supplied by Harlan Olac, Blackthorn, UK.

2.2. Synthesis of N-palmitoyl muramic acid

Muramic acid (100 mg) was dissolved in dimethyl sulfoxide (17 ml) and triethylamine (108 μl). Palmitic acid *N*-hydroxy succinimide (142mg) dissolved in 2 ml of chloroform was added to this. The mixture was stirred at room temperature for 72 h and protected from light. After this chloro-

form was evaporated off at room temperature and the remaining liquid freeze-dried. The dried powder was washed with water and freeze dried yet again. Analysis of the powder was carried out by mass spectrometry (Fast atom bombardment-FAB on a ZAB-SE mass spectrometer), thin layer chromatography (aluminium foil plates, silica gel 60 F254, Merck-dichloromethane, methanol 8:2) and melting point determination (SMP 1 melting point apparatus from Stuart Scientific, UK).

2.3. Preparation of PMA vesicles and sorbitan monostearate (Span 60) niosomes

Vesicles were prepared by a modification of the method of Haran et al. (1993). A total of 300 μmol of surfactant/lipid mixture—PMA, cholesterol, Solulan C24 and dicetyl phosphate (44.5:44.5:9:2) or Span 60, cholesterol, Solulan C24, dicetyl phosphate (44.5:44.5:9:2) were dispersed in 5 ml ammonium sulphate (120 mM) solution by shaking for 1 h at 60°C. The dispersed mixture was sonicated for 4 min on an MSE PG100 probe sonicator with the instrument set at 60% maximum output. The vesicles were left to cool, separated by ultracentrifugation (150 000 $\times$ $g \times 1$ h) and incubated with 5 ml DOX (3.8 mg ml^{-1}) for 1 h at 60°C. The formulations were left to cool slowly overnight. Unentrapped DOX was separated by gel filtration over Sephadex G50. Two bands were eluted with the vesicles eluting in the void volume. PMA vesicles were then filtered first through a 0.45 μm filter and subsequently through a 0.22 μm filter. Span 60 niosomes were filtered through a 0.8 μm filter, followed by a 0.22 μm filter.

2.4. Analysis of vesicles

Vesicles were sized on a Malvern Autosizer (Malvern Instruments, UK) and analysed for DOX content by disrupting the vesicles with 10 $\times$ volume isopropanol followed by dilution with water and spectrofluorometry (Perkin Elmer LS-3 fluorometer: excitation, 480 nm; emission, 560 nm). Standard solutions of DOX were prepared in suitable isopropanol/water mixtures.

2.5. Transmission electron microscopy

The PMA vesicle dispersion was pipetted onto a precoated copper grid (400 mesh) with a support film of 1% collodion in isoamylacetate. Staining was achieved by the use of 1% phosphotungstic acid and the samples were viewed and photographed with a Philips 201 microscope at an accelerating voltage of 80 kV.

2.6. In vitro stability of PMA vesicles in plasma

Fresh rat plasma was obtained from male Wistar rats by cardiac puncture and separation of the plasma from other blood components by centrifugation (500 g $\times$ 30 min). Using 0.3 ml of DOX PMA vesicles (1.75 mg ml^{-1} DOX) and 0.8 ml of fresh rat plasma which was incubated at 37°C. At various time intervals, 100 μl of the incubation mixture was fractionated over a Sepharose 2B column (82 $\times$ 5 mm) (Uchegbu et al., 1995). There were two distinct bands which were seen, recognisable as the vesicle fraction and the plasma protein fraction. The first 0.5 ml was discarded. A subsequent 1.5 ml was collected representing the vesicle fraction and a further 3 ml collected, representing the plasma protein fraction. Blank incubation mixtures (rat plasma alone) were treated in a similar manner. Fractions were analysed by spectrofluorometry as described above using fractions obtained by the separation of the blank incubation mixtures as the reference samples. Vesicles eluting from the column were also sized by photon correlation spectroscopy.

2.7. Biodistribution of DOX vesicles

Male BALB/c mice with a mean weight $\pm$ S.D. of 25.97 $\pm$ 0.92 were allowed unlimited access to food (B and K Universal, Grimston, UK) and water. Mice were dosed intravenously via the tail vein with (a) DOX Span 60 niosomes; (b) DOX PMA vesicles or (c) DOX solution all at 5 mg kg^{-1} DOX. Mice were killed at various time intervals by carbon dioxide asphyxiation and blood collected by cardiac puncture into heparinized tubes. Plasma was separated from other blood components by centrifugation (500 $\times$ $g \times 30$

Table 1
Characterisation of PMA

Analysis method	Results
Melting point	119–124°C
TLC	Aluminium foil plates, silica gel 60 F_{254} (Merck), dichloromethane, methanol (8:2): $R_f = 0.75$
Mass spectrometry	ZAB-SE MS fast atom bmbardment (FAB): 534 (7%, $M^+ + 2Na$), 512 (59%, $M^+ + Na$), 490 (24%, $M^+ + H$), 472 (24%, M^+-OH), 239 (100%, M^+-muramic acid), 176 (M^+-palmitoyl-CH_3-CH-COOH)

min). Hearts, livers and spleens were removed, wrapped in foil and plunged into liquid nitrogen. All tissue and plasma samples were stored at −44°C until analyses could be performed.

2.8. Analysis of plasma and tissue samples for DOX

Analysis for DOX was carried out using the method of Seymour et al. (1990). To the plasma samples 50 μl daunorubicin (DNM) and 2 μg ml^{-1} (100 ng DNM) were added as the internal standard followed by 100 μl ammonium formate buffer (pH = 8.5). This solution was then extracted with 5 ml of chloroform, isopropanol (4:1) and the mixture centrifuged ($1000 \times g \times 30$min). The organic solvent was separated, dried under a stream of nitrogen and the residue reconstituted in 0.15 ml of methanol. Methanol solutions were then injected into a 150 × 3.9 mm microbondapak column using a Waters 715 autosampler (Waters Instruments, Watford, UK) and eluted with an isopropanol:water (290:710) mobile phase to which was added concentrated orthophosphoric acid to a final pH of 3.3. The mobile phase was driven by a Waters 501 solvent delivery system. Fluorescence was measured by a Waters 470 scanning fluorescence detector (excitation, 480 nm; excitation, 560 nm) and peaks quantified using a Waters 746 data module. Heart, liver and spleen samples were homogenised in 3 ml phosphate buffered saline (PBS; pH, 7.4). To 1 ml of this homogenate 100 ng of DNM was added (50 μl of 2 μg ml^{-1}) and 0.1 ml ammonium formate buffer (pH; 8.5). This mixture was extracted with 5 ml chloroform, isopropanol (4:1) and analysis for DOX was performed as described above.

3. Results and discussion

3.1. Synthesis of PMA

The synthesis of PMA was performed according to the method of Lapidot et al. (1967) in which activated esters react specifically with amino groups. The product was confirmed by mass spectrometry (Table 1). Characterisation was by a combination of thin layer chromatography and melting point (Table 1). The product contained a little unreacted muramic acid (present as a spot at the origin of the TLC plate). However the presence of this unreacted muramic acid did not prevent vesicle formation by this novel surfactant (Fig. 2).

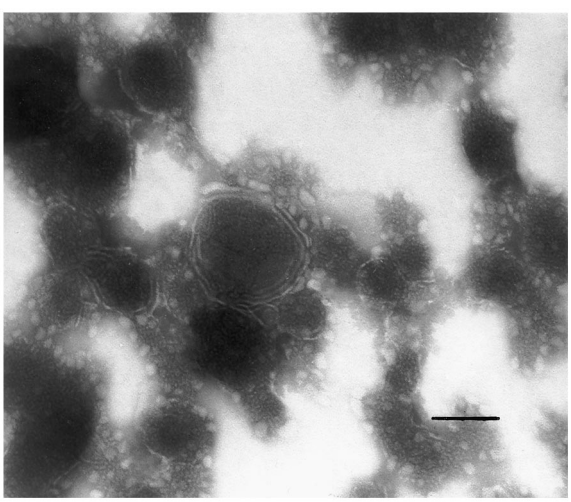

Fig. 2. Magnification × 148 000, transmission electron micrograph of PMA, cholesterol, Solulan C24 (45:45:10) vesicles, bar = 100 nm.

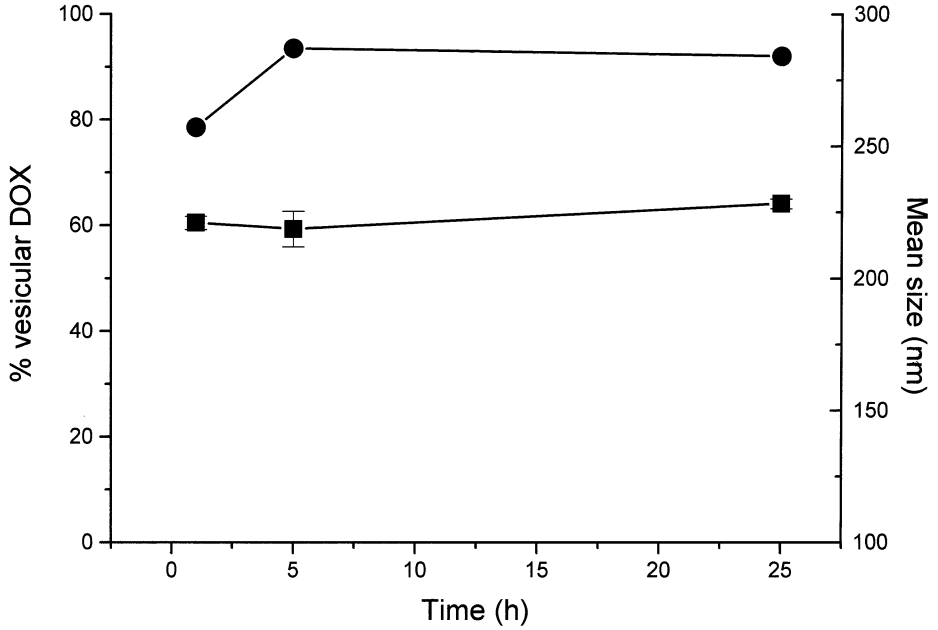

Fig. 3. The incubation of PMA vesicles with fresh rat plasma at 37°C; ●, % DOX found in the vesicle fraction (mean ± S.D.); ■, mean vesicle size.

3.2. Preparation of PMA vesicles

PMA vesicles bearing a carboxylic acid surface were prepared without the use of organic solvents and visualised by transmission electron microscopy (Fig. 2).

3.3. Stability of PMA vesicles in plasma

PMA vesicles showed excellent stability in plasma (Fig. 3). Vesicle size remained constant over a 24-h period and the amount of DOX associated with the vesicle fraction was also seen to change very little. The initial level of approximately 77% vesicle associated DOX may result in the vesicle dilution in plasma causing DOX adhering to the vesicle surface to preferentially distribute to the plasma proteins. However, over a 5-h period, equilibrium is reached with the reassociation of DOX or DOX and plasma proteins with the vesicles. After a period of 24 h over 90% of the encapsulated drug is associated with the vesicles. It is clear that various proteins/opsonins within the plasma do not cause any significant degradation of these PMA vesicles. It appears that the carboxylic acid surface confers a degree of plasma stability on these vesicles, preventing any destabilisation by plasma components.

3.4. Biodistribution of PMA

Preliminary biodistribution studies focused upon the fate of PMA vesicles at later rather than earlier time points. The vesicles administered differed only in the nature of the vesicle surface—all other aspects being largely similar (Table 2). After 5 h most of the drug had been cleared from the plasma although DOX levels on administration of PMA vesicles (0.20% of the administered dose) were significantly higher than when DOX solution (0.10% of the administered dose) or DOX Span

Table 2
Characterisation of DOX vesicles

Vesicle type	Size (nm)	DOX load (g g^{-1})
Span 60	177	0.1
PMA	239	0.07

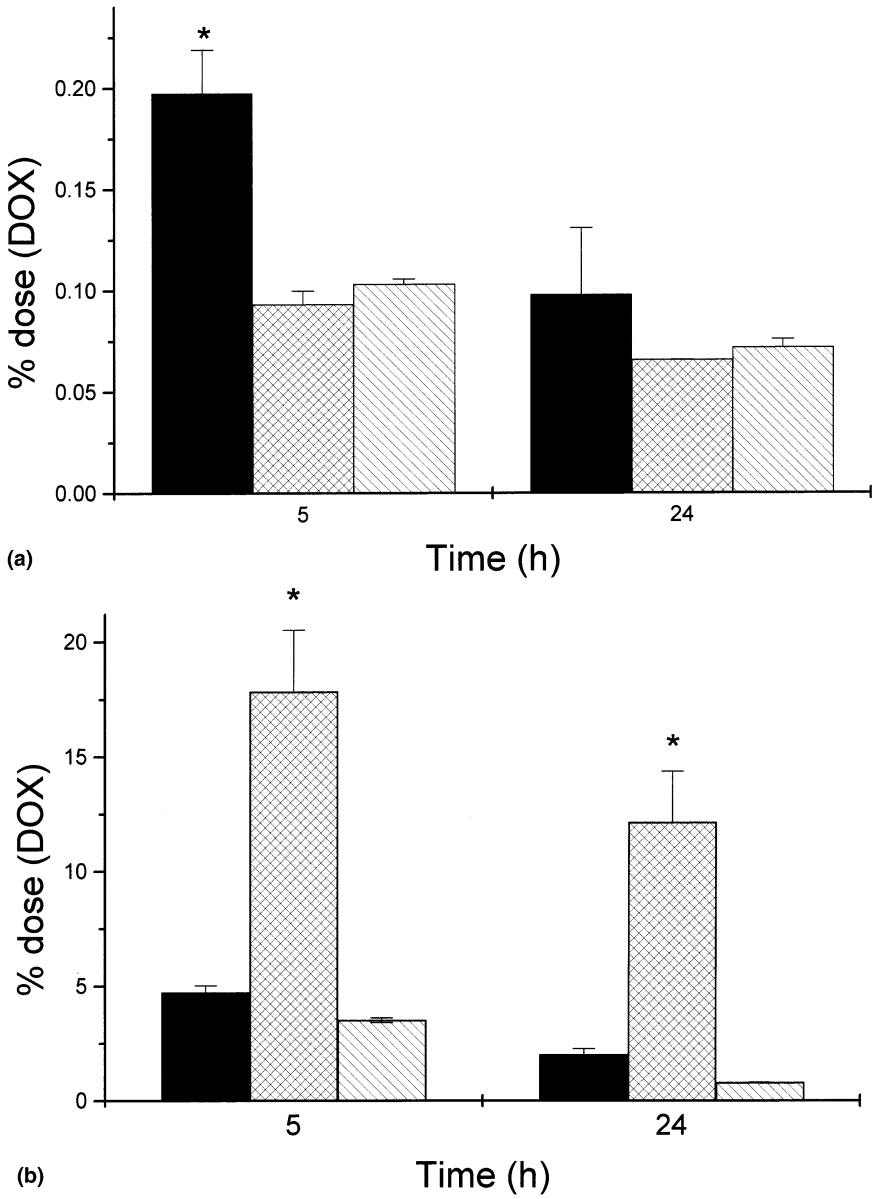

Fig. 4. DOX biodistribution (mean ± S.D.) in male BALB/c mice after the intravenous administration of DOX (5 mg ml^{-1}): (a) plasma; (b) liver; (c) spleen; solid fill, PMA vesicles; (X) fill; span 60 niosomes; (\) fill, DOX solution.

60 niosomes (0.09% of the administered dose) were administered (Fig. 4a). DOX levels in the Span 60 niosome group were lower than that found in previous studies (Uchegbu et al., 1995). This is believed to be the result of the higher DOX, surfactant ratio in the niosomes used in these studies coupled with the lower dose injected. The net result being that each particle cleared represents more of the dose. At 24 h after dosing plasma levels were similar irrespective of whether free or encapsulated drug was administered (Fig. 4a). A similar observation was reported with adri-

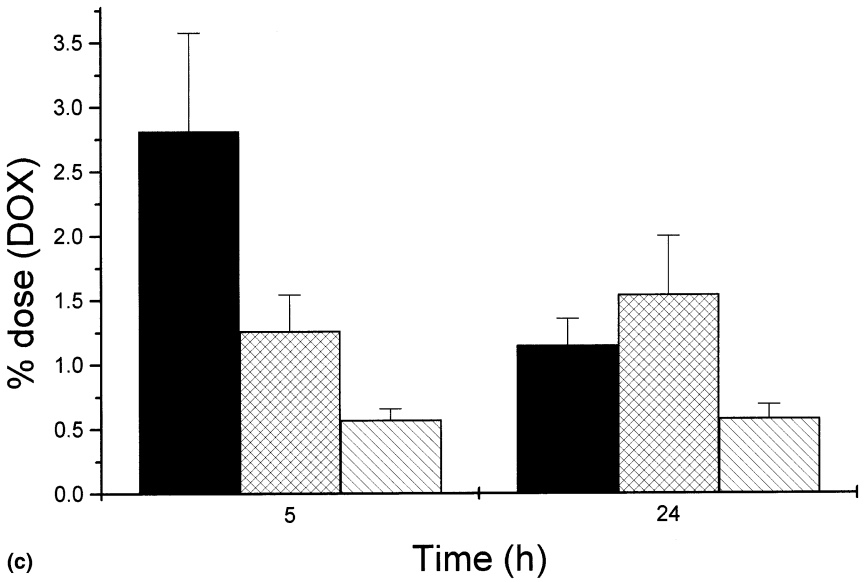

Fig. 4. (*Continued*)

amycin liposomes bearing monomethoxypolyoxyethylene glycol (2000) 2,3-dipalmitoylglycerol (Sadzuka et al., 1995).

The presence of a carbohydrate moiety and a negatively charged head group on the surface of liposomes is believed to retard liposomal clearance from the plasma (Gabizon and Papahadjopoulos, 1992) and liposomes bearing a glucuronic acid moiety (PglcUA) at the level of 10 mol% have been found to enjoy an increased plasma residence time (Namba et al., 1990). It can be concluded that the presence of a muramic acid surface did not appreciably prevent the clearance of these vesicles from the plasma; although PMA DOX was present in the plasma to a larger extent than other formulations at the time points examined (Fig. 4a). It must be emphasised that in these studies a clinically relevant entity was assayed for i.e. DOX concentration and not radioactivity, as used by other workers. The latter of which does not measure intact drug and is thus open to misinterpretation.

There was significantly less DOX present in the liver when PMA vesicles were administered compared to the administration of DOX Span 60 niosomes (Fig. 4b). DOX liver levels after the administration of Span 60 niosomes were similar to levels recorded in earlier studies (Uchegbu et al., 1995). The rapid clearance of intravenously injected liposomes by the liver is a known fact (Gregoriadis and Ryman, 1972). Niosomes also share a similar fate despite the lack of the phospholipid surface (Azmin et al., 1985; Baillie et al., 1986, Uchegbu et al., 1995, Uchegbu and Duncan, 1997). PMA vesicles on the other hand avoid liver uptake without an appreciable prolongation of plasma residence time (Fig. 4a, b). Splenic uptake of PMA vesicles was however higher than the splenic uptake of Span 60 niosomes (Fig. 4c) although differences were not statistically significant. Muramic acid has been detected in the human spleen (Hoijer et al., 1995) and it is hypothesised that peptidoglycan is present in the human spleen and stored in macrophages. It is possible that the spleen may contain receptors for muramic acid or that liver specific opsonins may avoid muramic acid vesicles while the opsonisation by spleen specific opsonins may be unavoidable. DOX heart levels were the same irrespective of the vesicles administered (Table 3).

Liposomes (Gregoriadis et al., 1996), surfactants (Hilgers et al., 1989) and muramyl dipeptide (Lederer, 1986) are all immunological adjuvants. It is logical that vesicles prepared from PMA be

Table 3
DOX heart levels 24 h after dosing

Vesicle type	% Dose (mean ± S.D.)
Span 60	0.47 ± 0.17
PMA vesicles	0.55 ± 0.24

examined for their immunological adjuvancy as is indeed planned.

4. Conclusions

Palmitoyl muramic acid—a new vesicle forming synthetic surfactant has been found to form vesicles, which are stable in plasma and on intravenous administration are rapidly cleared from the plasma. Liver uptake but not splenic uptake is suppressed by the presence of palmitoyl muramic acid in vesicles. It is hypothesised that the spleen may contain muramic acid receptors. Further studies will examine the immunological adjuvancy of these vesicles.

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