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# In vitro interaction of zinc(II)-phthalocyanine-containing liposomes and plasma lipoproteins

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**Abstract**

We have studied the interaction of small unilamellar liposomes containing zinc(II)-phthalocyanine (Zn-Pc) with human plasma lipoproteins. High-, low- and very low-density lipoproteins (HDL, LDL and VLDL) were purified from plasma and combined in amounts reflecting their natural abundance in plasma. After short periods of incubation at 37 °C, the bulk of Zn-Pc was incorporated into HDL and LDL; very little $^{14}$C-labelled palmitoyl oleoyl phosphocholine, the most abundant phospholipid in the formulation, was associated with lipoproteins. When liposomes were incubated in pooled plasma, 73%–85% of Zn-Pc and 27%–34% of radiolabelled phospholipid were recovered with HDL and LDL, indicating a possible role for plasma lipid transfer proteins in the incorporation of phospholipid into lipoproteins. Some Zn-Pc was also found in association with VLDL. The buoyant density of Zn-Pc liposomes increased in a dose-dependent fashion when the particles were incubated with plasma, and it is suggested that this was due, at least in part, to opsonization of liposomes by plasma proteins.

*Keywords:* Photodynamic therapy; Zinc(II)-phthalocyanine; Lipoproteins; Low-density lipoprotein; Liposomes; Phospholipid

## 1. Introduction

A number of photosensitizing agents are known to localize in neoplastic tissues and this forms the basis of their ability to cause selective tumour damage on light activation [1]. When introduced into the bloodstream, hydrophobic photosensitizers bind to, and are transported by, lipoproteins [2, 3]. Epidemiological data, analysis of solid tumours and studies performed with various cell lines grown in culture indicate that tumours have a high demand for cholesterol which they may satisfy by upregulation of low-density lipoprotein (LDL) receptor activity [4]. Thus LDL may function in vivo as a vehicle for the selective delivery into tumour cells of hydrophobic photosensitizers that have partitioned into the lipoprotein particles, although the relative importance of such a pathway is, at present, unresolved [5, 6].

A number of workers have incorporated hydrophobic photosensitizers into liposomes in order to facilitate their administration and to improve their pharmacokinetic profile [3, 7]. There is evidence that certain liposomal formulations can interact with plasma lipoproteins, although greater emphasis has been placed on studies of their interaction with high-density lipoprotein (HDL) than with LDL [8, 9]. The lipophilic photosensitizing agent zinc(II)-phthalocyanine (Zn-Pc) has been incorporated into a number of liposomal formulations by Jori and coworkers [3, 10], who found that, following their incubation with human serum or intravenous administration to rabbits, the liposome-derived photosensitizer was bound exclusively to the three major lipoprotein components of plasma.

In order to gain insight into the mechanism of Zn-Pc translocation from liposomes to LDL, we incubated small unilamellar vesicles incorporating Zn-Pc with purified human LDL [11]; there was a rapid, vigorous interaction between the two particles that resulted in the incorporation of liposomally derived Zn-Pc and phospholipid into the lipoprotein by a non-fusogenic process. In this paper, we report the interaction of Zn-Pc liposomes with pooled human lipoprotein and with human plasma.

## 2. Materials and methods

### 2.1. Human plasma and lipoproteins

Plasma was obtained from fasting (12 h) volunteers; heparinized blood from four individuals was pooled in

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order to minimize sample-to-sample variation. LDL apoprotein concentrations in pooled plasma were calculated from values obtained for total cholesterol, triglycerides and HDL cholesterol, as determined using commercially available colorimetric assay kits (Boehringer Mannheim, Germany). The three major lipoprotein classes were isolated by density gradient centrifugation [12]; pooled plasma (3.8 ml) was adjusted to $d = 1.21$ g ml$^{-1}$ using KBr and overlain with KBr–NaCl solutions of $d = 1.063$, d = 1.019 and $d = 1.0063$ g ml$^{-1}$ (2.8 ml each). After centrifugation to equilibrium, bands representing very low-density lipoprotein (VLDL; $d < 1.006$ g ml$^{-1}$), LDL ($1.024 < d < 1.055$ g ml$^{-1}$) and HDL ($1.10 < d < 1.21$ g ml$^{-1}$) were removed. Lipoprotein-deficient serum (LPDS) containing plasma proteins was recovered at $d > 1.21$ g ml$^{-1}$. The identity of each lipoprotein fraction was confirmed by sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS–PAGE). LDL was radioiodinated at pH 10 with carrier-free $^{125}$I as described by Van Tol et al. [13]. Free $^{125}$I was removed by Sephadex G-50 gel filtration followed by dialysis against repeated changes of phosphate-buffered saline (PBS) containing 0.25 mM ethylenediaminetetraacetic acid (EDTA) (pH 7.4) at 4 °C.

### 2.2. Zn-Pc liposomes

Zn-Pc (obtained from CIBA-Geigy Ltd., Basel, Switzerland) was formulated into small (90–100 nm), predominantly unilamellar vesicles using 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 1,2-dioleoyl-sn-glycero-3-phospho-1-serine (OOPS) as described previously [11] to give a final Zn-Pc to POPC to OOPS ratio of 1 : 70 : 30 (w/w/w). $^{14}$C-POPC (New England Nuclear, Stevenage, UK) was usually incorporated into the preparation [11]. Incorporated Zn-Pc was predominantly in the monomeric state as determined by a comparison of the absorbance at 671 and 605 nm [14].

### 2.3. Agarose gel electrophoresis

Samples were rapidly mixed with an equal volume of agarose (60 °C), applied to the gel and electrophoresed (70 mA for 3 h) in 0.75% w/v agarose at pH 8.3 using a buffer containing 0.089 M tris(hydroxymethyl)aminomethane (Tris), 0.089 M boric acid and 3 mM EDTA. Gels were stained with Coomassie brilliant blue R-250 or sliced into 0.5 cm fractions for quantification of Zn-Pc or $^{14}$C-POPC. Zn-PC was measured fluorometrically ($\lambda_{ex} = 610$ nm; $\lambda_{em} = 676$ nm) after extraction of the gel slices with 1-methyl-2-pyrrolidinone. Radioactivity was measured in a Beckman LS 1801 liquid scintillation analyser using Lumagel as scintillant.

### 2.4. Density gradient ultracentrifugation

Zn-Pc liposomes were incubated in plasma or reconstituted lipoproteins; 1.24 g of KBr was added to 1 ml aliquots and the volume was adjusted to 3.8 ml with PBS–EDTA (pH 7.4) ($d = 1.21$ g ml$^{-1}$). Samples were overlain with KBr–NaCl solutions of $d = 1.063$ g ml$^{-1}$ (2.8 ml), 1.019 g ml$^{-1}$ (2.8 ml) and 1.0063 g ml$^{-1}$ (2.6 ml) and centrifuged to equilibrium using a Beckman XL-80 ultracentrifuge and SW 40 Ti swing-out rotor (22 h, 4 °C, 285 000*g*). Gradients were fractionated by pumping out the liquid from the bottom of the tube at a flow rate of 1.2 ml min$^{-1}$ and fractions were analysed for Zn-Pc and $^{14}$C-POPC as detailed above.

### 2.5. Other analytical methods

SDS–PAGE was performed using 10% polyacrylamide slab gels. Proteins were transferred electrophoretically from polyacrylamide gels to nitrocellulose filters and identified using polyclonal antisera obtained from the usual commercial sources.

## 3. Results

### 3.1. Interaction of liposomes and pooled lipoproteins

Patsch et al. [15] determined that the HDL to LDL to VLDL protein ratios in pooled human plasma were 1.8228 : 0.769 : 0.084. Therefore, to study the interaction of Zn-Pc liposomes with the lipoprotein fraction of serum, HDL, LDL and VLDL were purified, checked for homogeneity and purity by agarose gel electrophoresis and combined in these protein ratios at concentrations reflecting their abundance in plasma using 8 mM PBS (pH 7.4). Pooled lipoproteins were incubated at 37 °C with different Zn-Pc liposome concentrations and the incubation mixtures were electrophoresed. Fig. 1 shows the result obtained when lipoproteins and liposomes were incubated for 5 min. Liposomes and lipoproteins could be readily discriminated using this technique; the electrophoretic mobility (bromophenol blue = 1.00) of Zn-Pc liposomes was 0.88 (lane 9), whereas the $R_f$ values for the three lipoproteins resolved from the mixture (lane 1) were 0.21 for LDL, 0.39 for VLDL and 0.54 for HDL. VLDL was difficult to visualize by protein staining due to its low concentration in the lipoprotein pool. The presence of Zn-Pc liposomes in the incubation mixture resulted in an increase in the electrophoretic mobility of LDL and HDL, and the mobility of these particles increased in direct proportion to the concentration of the liposomes. For example, with an LDL apoprotein B to Zn-Pc molar ratio of 1 : 1, the $R_f$ values for LDL and HDL were 0.22 and

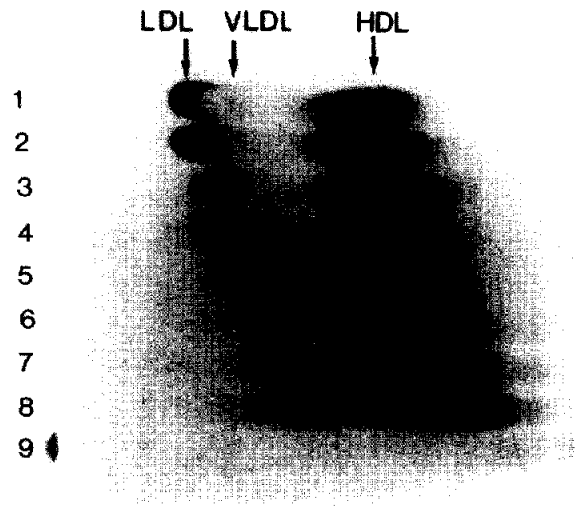


Fig. 1. Agarose gel electrophoresis of pooled human HDL, LDL and VLDL incubated at 37 °C for 5 min with different concentrations of Zn-Pc liposomes. Lipoproteins were combined to give 1.009, 0.426 and 0.0467 mg $ml^{-1}$ for HDL, LDL and VLDL protein respectively. The gel was stained with Coomassie brilliant blue R-250. Lane 1 contained only pooled lipoproteins (25 $\mu$g apoprotein); lanes 2–8 contained pooled lipoproteins (25 $\mu$g apoprotein) incubated with different amounts of Zn-Pc liposomes, expressed as the following molar ratios of LDL apoprotein B to Zn-Pc: lane 2, 1 : 1; lane 3, 1 : 4; lane 4, 1 : 8; lane 5, 1 : 15; lane 6, 1 : 30; lane 7, 1 : 50; lane 8, 1 : 80. Lane 9 contained Zn-Pc liposomes (12.5 $\mu$g Zn-Pc); Zn-Pc liposome bands could be readily visualized prior to staining due to the intense blue colour of the dye (absorption maximum, 676 nm).

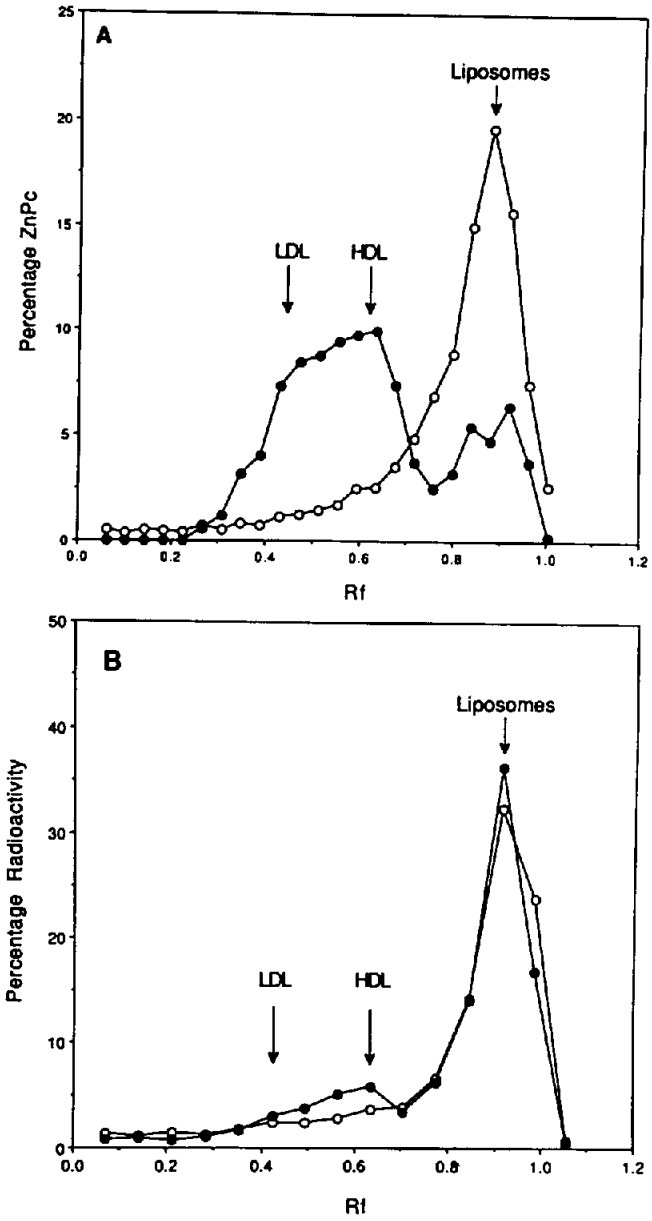


Fig. 2. Distribution of Zn-Pc (A) and $^{14}$C-POPC (B) in 0.75% agarose gels. Lanes contained Zn-Pc–POPC–OOPS liposomes (○) or liposomes incubated at 37 °C for 5 min with pooled lipoproteins at an LDL apoprotein B to Zn-Pc molar ratio of 1 : 80 (●).

0.55; at a molar ratio of 1 : 15, the corresponding values were 0.29 and 0.60, and at a 1 : 80 ratio, the values were 0.4 and 0.67 respectively (lanes 2, 5 and 8). At all molar ratios, modified LDL and HDL particles behaved electrophoretically as homogeneous populations (lanes 2–8), although there was some broadening of the bands at high Zn-Pc liposome concentrations. No protein was detected in the application slots of the gel at any protein to Zn-Pc ratio examined, indicating that modified particles had a diameter of less than 200 nm [11]. Essentially identical gels were obtained when mixtures were incubated for shorter (2 min) or longer (30 min) periods of time.

The distribution of Zn-Pc and $^{14}$C-POPC in agarose gels following incubation of liposomes for 5 min at 37 °C with pooled lipoproteins at an LDL apoprotein B to Zn-Pc molar ratio of 1 : 80 is shown in Fig. 2. When the liposomes alone were electrophoresed, Zn-Pc (Fig. 2(A)) and $^{14}$C-POPC (Fig. 2(B)) gave sharp bands coincident at $R_f = 0.88$. Following incubation with lipoproteins, about 75% of Zn-Pc was associated with LDL- and HDL-containing portions of the gel (Fig. 2(A)), whereas there was only a very small shift in the distribution of radiolabel (Fig. 2(B)). Very little Zn-Pc or radiolabel was recovered from the application slot after electrophoresis. There was no modification of the $R_f$ value of residual liposomes. Similarly, when the incubation products were examined by density gradient ultracentrifugation, there was no change in density of the residual liposomal band.

### 3.2. Interaction of liposomes and pooled plasma

The pooled plasma used for these experiments contained 0.473 mg $ml^{-1}$ HDL cholesterol, 1.273 mg $ml^{-1}$ LDL cholesterol and a calculated LDL protein con-

centration of 0.445 mg $ml^{-1}$. A trace amount of $^{125}I$-LDL was added to the plasma in order to locate LDL on agarose gels following electrophoresis; the electrophoretic mobility of LDL in plasma was thus determined to be 0.22. On incubation with Zn-Pc liposomes at an LDL apoprotein B to Zn-Pc molar ratio of 1 : 80, the $R_f$ value increased to 0.29 after 5 min at 37 °C and to a maximum of 0.44 after 30 min. Similar, smaller increases in $R_f$ values were noted with a molar ratio of 1 : 8.

After incubation of $^{14}C$-labelled Zn-Pc liposomes (molar ratio of 1 : 80) with plasma and subsequent electrophoresis, 75%–85% of Zn-Pc was recovered from the lipoprotein-containing region ($R_f$=0.1–0.6) of the gel (Fig. 3(A)). A similar pattern was obtained when the components were mixed and immediately electrophoresed (0 min) and when incubated at 37 °C for 5 or 30 min. In contrast, only 27% of $^{14}C$-POPC was recovered in the lipoprotein region after 0 min (Fig. 3(B)); this increased slightly after incubation for 5 min (32%) and 30 min (34%). The peak at $R_f$=0.82 (Figs. 3(A) and 3(B)) represents residual liposomes; the reduction in the electrophoretic mobility from 0.91 may be due to the selective removal of liposomal components or to opsonization, or to both phenomena.

When Zn-Pc liposomes were subjected to density gradient ultracentrifugation, they were found to have a buoyant density of 1.0602 g $ml^{-1}$, a value similar to that obtained for native LDL. After incubation with pooled plasma for 5 min, there was an increase in the density of the liposome band; thus the bulk of $^{14}C$-POPC was recovered at 1.0910 g $ml^{-1}$ at an LDL apoprotein B to Zn-Pc molar ratio of 1 : 80. As the ratio of plasma was increased, there was a corresponding increase in density to 1.1314 g $ml^{-1}$ at a ratio of 1 : 8 and to 1.1500 g $ml^{-1}$ at 1 : 1. The distribution of Zn-Pc and $^{14}C$-POPC following centrifugation to equilibrium of incubation mixtures at ratios of 1 : 8 and 1 : 80 is shown in Fig. 4. Whereas the bulk of Zn-Pc redistributed to HDL-, LDL- and VLDL-containing fractions, a high proportion of $^{14}C$-POPC remained associated with the modified liposomes.

The deposition of opsonins was examined using SDS-PAGE and electroblotting techniques. After 5 min incubation at 37 °C with pooled plasma, liposomes were recovered using density gradient ultracentrifugation. Proteins with molecular weights of greater than 200, and 102, 75, 68, 60, 45, 33 and 28 kDa were associated with the liposome fraction. The 102 kDa and 33 kDa bands reacted with an anti-C3 $\alpha$-chain serum and thus represent products of complement component C3 activation. No bands reacted with antiserum against complement factors C4 and C9, S-protein or fibronectin. Bands at 75, 68 and 28 kDa correspond to C3 $\beta$-chain, albumin and apo A-1 respectively.

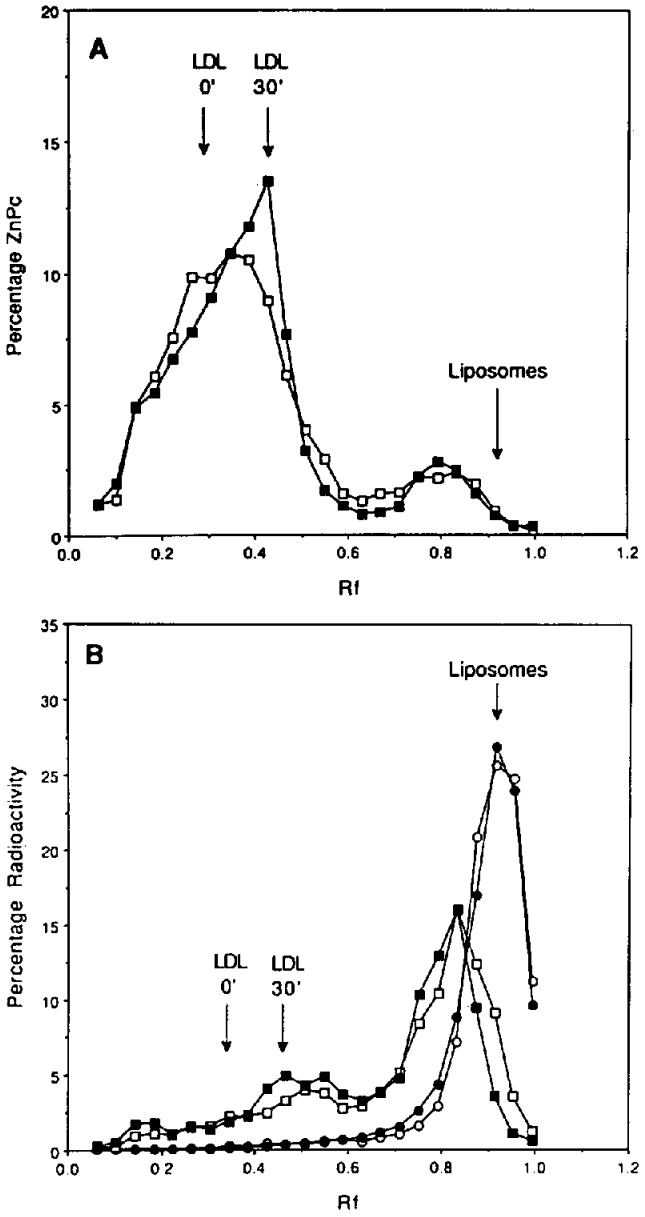


Fig. 3. Distribution of Zn-Pc (A) and $^{14}C$-POPC (B) in 0.75% agarose gels. Zn-Pc–POPC–OOPS liposomes were mixed with pooled plasma (LDL apoprotein B to Zn-Pc molar ratio of 1 : 80) and either electrophoresed immediately (□) or incubated at 37 °C for 30 min (■). The location of Zn-Pc liposomes electrophoresed in the absence of plasma is indicated by the arrow in each panel; as indicated in (B), incubation of the liposomes for 30 min (○) had no effect on the electrophoretic mobility of this preparation compared with 0 min incubation (●). The location of $^{125}I$-LDL at the appropriate time intervals is also indicated.

## 4. Discussion

In a recent study [11], we examined the in vitro interaction of Zn-Pc liposomes and isolated LDL in an attempt to gain an understanding of the mechanism of transfer of lipophilic photosensitizers into plasma

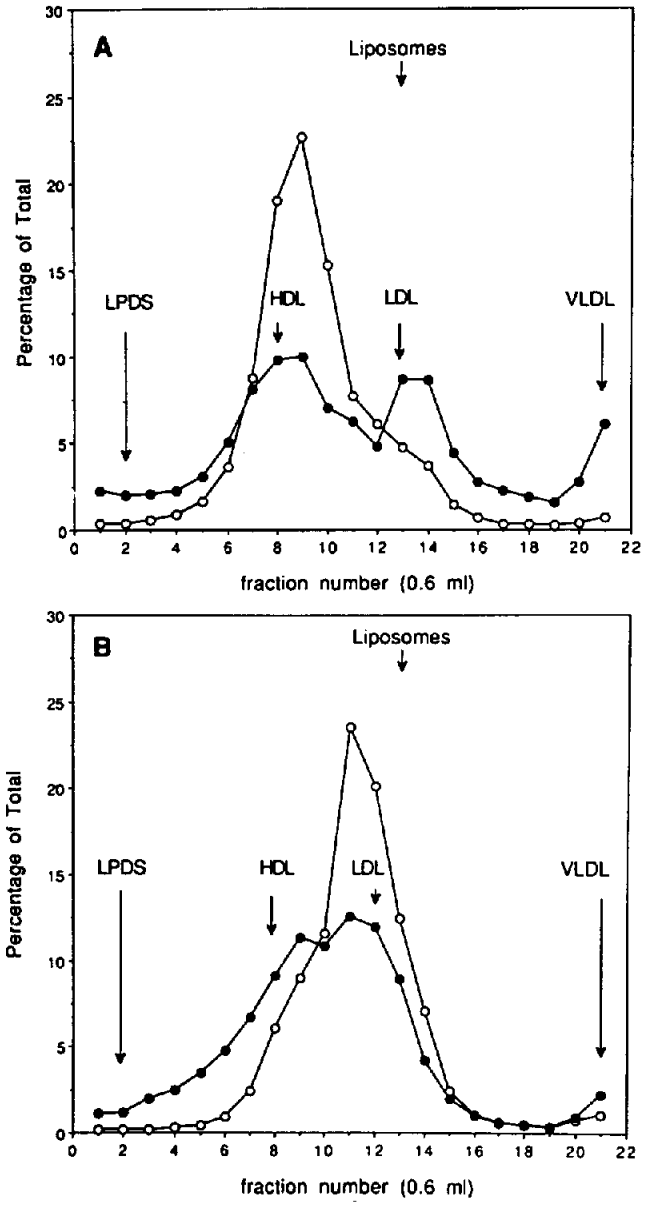


Fig. 4. KBr–NaCl density gradient ultracentrifugation of Zn-Pc liposomes and pooled human plasma incubated at 37 °C for 5 min. Liposomes and plasma were combined in LDL apoprotein B to Zn-Pc molar ratios of either 1 : 8 (A) or 1 : 80 (B). The Zn-Pc (●) and $^{14}$C-POPC (○) contents of each fraction were determined. Zn-Pc values were corrected for protein fluorescence in the denser region of the gradient.

lipoproteins. We concluded that a rapid incorporation of Zn-Pc and phospholipid into LDL occurred by a process that could not be explained by fusion or simple aggregation of particles; under certain conditions, over 80% of Zn-Pc and 70% of POPC were found in association with LDL. Although analogous mechanisms for the transfer of phospholipids and lipophilic molecules between particles may occur in vivo, the situation in biological systems is undoubtedly more complex; for example, both plasma cholesteryl ester transfer protein [16] and phospholipid transfer protein [17] can facilitate the transfer of phospholipids between liposomes and lipoproteins and the transfer of cholesterol esters and/or phospholipids between HDL and LDL [18]. Similarly, the presence of other classes of lipoprotein particles may influence transfer, since apoproteins present on HDL and VLDL are known to be adsorbed by otherwise intact liposomes [8] and, in the present study, we have presented evidence that there is some transfer of apolipoprotein A-1, the major apoprotein associated with HDL, to the liposomes following incubation in plasma.

We felt it appropriate, therefore, to investigate the interaction of Zn-Pc liposomes with both pooled lipoproteins and pooled human plasma in order to understand better the mechanism of photosensitizer and phospholipid transfer. Thus, incubation of liposomes with purified HDL, LDL and VLDL, reconstituted to give those proportions and concentrations found in normal human plasma, resulted in the transfer of the bulk of the Zn-Pc (75%) to the HDL and LDL classes. However, in contrast with our earlier experiments with LDL alone [11], only very small amounts (less than 5%) of $^{14}$C-POPC were found to be associated with the lipoproteins following agarose gel electrophoresis. This high degree of selective transfer suggests that a component of the lipoprotein mixture may be able to inhibit the transfer of phospholipid into potential recipient particles, or that the liposomes are stabilized in the presence of the lipoprotein mixture. Indeed, it has been established that the apolipoprotein A-1 component of HDL can reduce particle-to-particle contact in systems containing LDL and phospholipid microemulsions [19], probably by interacting with exposed hydrophobic domains on LDL [20]. Thus the selective transfer of Zn-Pc into HDL and LDL, observed in our study, may result from apolipoprotein A-1-mediated inhibition of sufficient liposome–lipoprotein contact to facilitate phospholipid transfer; Zn-Pc transfer would occur by a different mechanism, presumably as a result of the greater capacity of Zn-Pc to partition into lipoprotein in comparison with liposomal phospholipid or, possibly, due to the transfer of the dye through the aqueous phase. The observed asymmetric transfer of liposomal components supports our previous suggestion [11] that Zn-Pc transfer does not involve a fusion process.

Of note was the fact that the incubation of lipoproteins with liposomes led to an increase in the electrophoretic mobility of all particles in both the HDL and LDL fractions (Fig. 1), even when the concentration of liposomes in the mixture was low. The only negatively charged component of the liposome is OOPS and it is tempting to assume that the increase in mobility is due to transfer of this phospholipid into the lipoprotein particles; however, this implies selective transfer of OOPS over POPC. Unfortunately, we are unable to

test this possibility directly as labelled OOPS is not available.

Agarose gel electrophoresis proved to be a useful technique for the detection of labelled liposomes after incubation in human plasma, although it was not possible to visualize directly the liposomes or the various lipoproteins in the gel due to the presence of large amounts of plasma proteins. Similar care must be taken in the interpretation of the ultracentrifugation data, as the buoyant density of liposomes may be practically identical with that of one or other of the lipoproteins, and particularly LDL. In addition, liposomes will interact with opsonizing proteins in plasma, a process likely to result in changes in their biophysical characteristics. However, using these two techniques, it was possible to show that extensive Zn-Pc transfer from liposomes to HDL, LDL and VLDL occurs in plasma–liposome incubation mixtures. It was also clear that more liposomal phospholipid became incorporated into lipoproteins in plasma (27%–34%) compared with that in the reconstituted lipoprotein fraction (less than 5%). This suggests a role for phospholipid transfer factors present in human plasma; indeed, Tall et al. [8] have shown that plasma phospholipid transfer protein(s) can account for the more rapid transfer of liposomal phospholipid into HDL when vesicles are incubated in plasma rather than with centrifugally isolated HDL. We observed no transfer of $^{14}$C-POPC into HDL when liposomes were incubated with purified HDL [21]. A further indication of a role for transfer proteins was the observation (Fig. 3) that the electrophoretic mobility of LDL in plasma changed over the 30 min incubation period, whereas it did not when liposomes were mixed with either purified LDL [11] or reconstituted lipoproteins. As phospholipid transfer proteins are enzymes, a time-dependent process is to be expected.

The agarose gel electrophoresis and ultracentrifugation data suggest that Zn-Pc–POPC–OOPS liposomes are opsonized by plasma proteins. Analysis of liposomes recovered from plasma incubation mixtures demonstrated the presence of C3 activation products; the absence of C4 products suggests that deposition occurred via the alternative pathway of complement activation. Recently, Chonn et al. [22] have shown that a number of liposomal formulations can be opsonized by complement C3 products. Other opsonins were also detected and almost certainly account for the increase in buoyant density and modified electrophoretic behaviour of liposomes when incubated in plasma, but not in the reconstituted lipoprotein mixture. The binding of plasma proteins and interaction with plasma lipoproteins will exert a profound influence on the in vivo fate of liposomes; for this reason, we have initiated studies in suitable animal models that are designed to determine the role of these factors in the localization of Zn-Pc–POPC–OOPS vesicles.

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