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Surface-associated serum proteins inhibit the uptake of phosphatidylserine and poly(ethylene glycol) liposomes by mouse macrophages

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Abstract

Serum proteins, acting as opsonins, are believed to contribute significantly to liposome–macrophage cell association and thus regulate liposome uptake by cells of the mononuclear phagocytic system (MPS). We studied the effect of serum protein on binding and uptake of phosphatidylglycerol-, phosphatidylserine-, cardiolipin-, and *N*,*N*-dioleyl-*N*,*N*-dimethylammonium chloride- (DODAC) containing as well as poly(ethylene glycol)- (PEG) containing liposomes by mouse bone marrow macrophages in vitro. Consistent with the postulated surface-shielding properties of PEG, protein-free uptake of liposomes containing 5 mol% PEG and either 20 mol% anionic phosphatidylserine or 20 mol% cationic DODAC was equivalent to uptake of neutral liposomes. In contrast to previous reports indicating that protein adsorption to liposomes increases uptake by macrophages, the presence of bound serum protein did not increase the uptake of these liposomes by cultured macrophages. Rather, we found that pre-incubating liposomes with serum reduced the uptake of liposomes containing phosphatidylserine. Surprisingly, serum treatment of PEG-containing liposomes also significantly reduced liposome uptake by macrophages. It is postulated that, in the case of phosphatidylserine liposomes, the bound serum protein can provide a non-specific surface-shielding property that reduces the charge-mediated interactions between liposomes and bone marrow macrophage cells. In addition, incubation of PEG-bearing liposomes with serum can result in a change in the properties of the PEG, resulting in a surface that is better protected against interactions with cells.

Keywords: Liposome; Macrophage; Anionic lipid; Cationic lipid; Serum; Dysopsonin

Abbreviations: DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine; Chol, cholesterol; DSPE-PEG, (*N*-monoethoxy poly(ethylene glycol) succinyl)1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine M_r 2000; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; PG, 1,2-dioleoyl-*sn*-glycerol-3-phosphoglycerol; DODAC, *N*,*N*-dioleyl-*N*,*N*-dimethylammonium chloride; PS, 1,2-dioleoyl-*sn*-glycerol-3-phosphoserine; FBS, fetal bovine serum; PFHMII, protein-free hybridoma medium; CHE, [^{3}H]cholesteryl-hexadecyl ether; G_{M1}, monosialoganglioside; HBSS, Hank's buffered salt solution; PBS, phosphate-buffered saline; P_B, protein bound; MPS, mononuclear phagocytic system; BMM, bone marrow macrophage; BCA, bicinchoninic acid

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1. Introduction

Macrophage particle uptake occurs by absorptive endocytosis and strong binding of liposomes to cells is a critical stage in the process [1–3]. Limiting the degree of liposome–cell association should, therefore, reduce mononuclear phagocytic system (MPS) uptake. Adsorption may result from direct interaction between the liposome and cell surface [2], or through intermediary serum proteins, called opsonins [4]. Opsonin proteins adsorb to the surface of blood-born particles such as bacteria, directing the particles to the MPS for uptake. While a liposome-opsonizing role has not been firmly established for any specific protein in vivo [5], opsonization by purified serum proteins is known to modify the interactions between liposomes and cells in vitro [6] and is generally considered fundamental to liposome recognition by phagocytic cells in vivo [5,7]. Thus liposome modifications that reduce serum opsonization are predicted to extend circulation longevity of liposomes. Conversely, it has also been suggested that long-circulating liposomes may interact with 'dysopsonins' [8], serum proteins that can reduce liposome uptake.

To date, the most significant advance towards prolonging liposome circulation times has been achieved through the inclusion of lipid polymers such as monosialoganglioside (G_{M1}) or poly(ethylene glycol) (PEG)-grafted lipid [9–12]. PEG-derivatized phosphatidylethanolamine contains a long flexible hydrophobic polymer moiety attached to the amino group of PE, a modification that converts the zwitterionic PE to an anionic lipid with a grafted linear polymer. It has been proposed that PEG can assume a 'mushroom' or a 'brush' conformation [13] depending on the PEG surface density on the liposome [11]. Extension of hydrophilic chains of PEG over the surface of liposomes creates a field of hydration; the shape and density of the hydrated field are dependent on the orientation of PEG in space. For liposomes with 5 mol% PEG_{2000} the size of this barrier has been estimated to be 5–6 nm [11,14,15]. This fixed aqueous layer on the surface of PEG-containing liposomes may either sterically interfere with interactions between cells and lipid headgroups or it may act as a barrier to the binding of serum protein to the surface of the liposomes [7,10,11,16].

There are many observations yet to be reconciled with theoretical concepts of liposomal behavior in the circulation. For instance, liposomes containing PEG have extended circulation times [9,10], however while PEG-lipids can reduce the rate of loss of liposomes from the circulation, this occurs without altering the cumulative liposome uptake by the cells of the MPS [5,10,11]. Another anomaly is that, unlike conventional liposomes, MPS uptake of liposomes containing PEG is less dependent on physicochemical factors such as charge, acyl chain saturation and dose [12,17]. Interestingly, it has also been observed that MPS blockade extends the circulation time of PEG liposomes [18]. A related observation is that PEG does not prevent MPS blockade induced by liposome-encapsulated doxorubicin [19]. It is important to note that PEG does not completely conceal liposomes as proteins attached to PEG liposomes are highly immunogenic [20,21]. Similar issues exist when reconciling extended circulation times with the protective effects of G_{M1} [18].

Clearly more information about interactions between liposomes, serum protein and macrophages is required to develop an encompassing hypothesis for behavior of liposomes in vivo. We have re-examined the roles of charge, protein coating and a steric polymer barrier on binding and uptake by macrophages in culture. The macrophage target population used in this study was mouse bone marrow cells. Bone marrow provides a readily accessible source of macrophage cells, without requiring proteolytic enzyme isolation or chemical induction. Uptake of liposomes into isolated bone marrow macrophage (BMM) cells is inversely proportional to circulation longevity [22], thus isolated macrophages can provide information regarding uptake mechanisms in vivo. We have tried to reconcile novel observations of macrophage uptake of liposomes in vitro with circulation characteristics of liposomes in vivo.

2. Materials and methods

2.1. Materials

Mouse serum was purchased from Cedarlane (Mississauga, Canada). Fetal bovine serum (FBS) was purchased from Hyclone Laboratories (Logan, UT, USA) and horse serum was obtained from (ICN

Biomedicals Inc., Aurora, OH, USA), PFHMII (protein-free hybridoma medium) was purchased from Gibco (Life Technologies, Burlington, Canada). RPMI and Hank's buffered salt solution (HBSS) were obtained from Stem cell technologies (Vancouver, Canada). CHE ([^{3}H]cholesteryl-hexadecyl ether) was purchased from New England Nuclear Research Products (Mississauga, Canada) (1 mCi/ml). Lipids and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-m PEG_{2000} were purchased from Avanti Polar Lipids (Birmingham, AL, USA), or Northern Lipids (Vancouver, Canada). *N*,*N*-Dioleyl-*N*,*N*-dimethylammonium chloride (DODAC) was a generous gift from Inex Pharmaceuticals (Vancouver, Canada). Latex beads were obtained from Molecular Probes (Eugene, OR, USA). All other chemicals were from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Isolation of BMMs

Mouse BMMs were obtained by flushing the femur bones of adult CD-1 mice with HBSS. Bone marrow cells were collected by density gradient centrifugation of this cell suspension. Cells were seeded on non-tissue culture treated polystyrene plates in HBSS. After 4 h, the HBSS and the non-attached cells were removed and discarded. The macrophage cells which remained were cultured for 5–7 days in a 2:1 mixture of macrophage media (RPMI containing 5% FBS, 5% horse serum) and L929 cell-conditioned media. The cultured macrophages were $>95\%$ viable by trypan blue exclusion. For experiments, BMMs were plated at 10^6 cells in 6 well tissue culture plates (Falcon) 24 h before use. All uptake studies were performed in PFHMII.

Uptake of latex beads was monitored as a means of ensuring macrophage integrity under serum-free conditions. Beads (100 nm fluorescent microspheres, Molecular Probes) (10^7/well) were added to BMMs in 6 well plates and incubated for up to 4 h and cells at either 4°C or 37°C. BMMs were rinsed three times with ice cold phosphate-buffered saline (PBS), then collected in PBS containing 0.2% sodium dodecyl sulfate (SDS). Uptake was calculated by subtracting the amount of cell-associated beads at 4°C from the amount of cell-associated beads at 37°C. Fluorescence per bead was calibrated in order to quantify bead uptake. PFHMII was identified as a protein-free growth medium capable of maintaining BMMs as a viable phagocytic population. All protein assays were preformed using a micro bicinchoninic acid (BCA) protein assay kit with BSA as standard (Pierce, Rockford, IL, USA). Under our assay conditions, 1 μg of protein corresponds to approximately 1.3×10^4 macrophages.

2.3. Preparation of liposomes

Liposomes were prepared by aqueous reconstitution of dried lipid films in HEPES-buffered saline (10 mM HEPES (pH 7.4), 150 mM NaCl) [23]. [^{3}H]CHE (0.08 Ci/mol) was added as radioactive tracer. The sample was subjected to five freeze thaw cycles prior to repeated high-pressure extrusion through stacked 100 nm pore size polycarbonate membrane filters (Nuclepore, Pleasanton, CA, USA) using an extruder purchased from Lipex Biomembranes (Vancouver, Canada). Liposome size was determined by quasi-elastic light scattering using a Nicomp submicron particle size analyzer. All liposomes exhibited averaged diameters of $90–120\pm25$ nm.

2.4. Serum treatment of liposomes

Mouse serum was frozen and thawed no more than two times to prevent inactivation of sensitive proteins. 10 μmol liposomes were incubated with mouse serum (1:4 liposomes:serum, v/v) for 60 min at 37°C with gentle agitation. Separation of liposomes from bulk serum protein was achieved by passing liposomes over 12 ml Sepharose CL-4B (Pharmacia, Uppsala, Sweden) gel filtration column. Fractions, 0.25 ml, were collected and aliquots were assayed directly for the presence of protein and for liposomes by scintillation counting. Liposome fractions were pooled and used immediately for liposome uptake studies or for analysis of associated protein. The lipid-associated protein of each liposome fraction was quantified following extraction of the lipid with chloroform and methanol [24].

2.5. Proteins associated with liposomes

Protein extracted from column-recovered liposomes was also analyzed by protein gel electropho-

resis. Extracted proteins were analyzed by SDS–electrophoresis on 7.5% polyacrylamide gels [25], developed with silver stain (Bio-Rad Silver Stain Plus Bio-Rad Laboratories, Hercules, CA, USA).

2.6. Liposome uptake by tissue culture BMMs

Liposomes recovered from gel filtration columns were diluted to a final concentration of 1 mM total lipid in PFHMII. For liposome uptake studies BMMs were plated in 6 well tissue culture plates. Tissue culture medium was removed from the wells and cells were rinsed with HBSS then 2 ml of media containing liposomes was added to each well. Unless otherwise indicated, cells were incubated for 4 h at 37°C in a humidified atmosphere. As a control for liposome binding to cell surfaces, BMMs were incubated on ice at 4°C. BMMs were viable by trypan blue exclusion after 4 h incubation at this temperature. Medium was removed and wells were rinsed twice with PBS then lysed in PBS containing 0.2% SDS. Protein content was determined with the micro BCA assay and liposome content was determined by liquid scintillation counting in PicoFluor 40 (Canberra Packard, Mississauga, Canada).

2.6.1. Statistics

Statistical analysis applied a two-tailed Student's *t*-test assuming unequal variances. Differences with $P < 0.05$ were considered significant. Data are presented as mean ± S.E.M.

3. Results

3.1. Macrophage bead uptake

We have re-examined PEG and the effects of bound serum proteins on liposome–phagocyte interaction in vitro with BMMs, a population of readily accessible macrophage cells. Rather than simply eliminate the serum from tissue culture media, BMMs were cultured in protein-free media. To assess the integrity of our cell preparation for monitoring uptake of liposomes in the absence of serum, we first ensured that the phagocytic capacity of macrophages was intact and maintained during protein-free incubation. To do this, the ability of the BMM preparations to phagocytose latex beads was assessed. Phagocytosis of latex beads as a function of bead to cell ratio in either RPMI media containing 10% serum or in protein-free media is shown in Fig. 1. We demonstrated that during protein-free culture the total uptake of beads at a ratio of 200 beads/cell was reduced by 30% relative to cells cultured in 10% serum, however cells did maintain vigorous phagocytic activity. The reduction in bead uptake may be due to the absence of serum factors that mark beads for uptake (opsonins) or of serum factors that affect the macrophage cell directly. It is important to note that serum factors are not obligatory for phagocytic activity by the BMMs. Phagocytosis, measured by bead uptake, was linear with respect to time and was temperature-dependent during protein-free culture (inset Fig. 1). The protein-free medium in these studies supports the growth and maintains the phagocytic activity of BMMs. Thus the

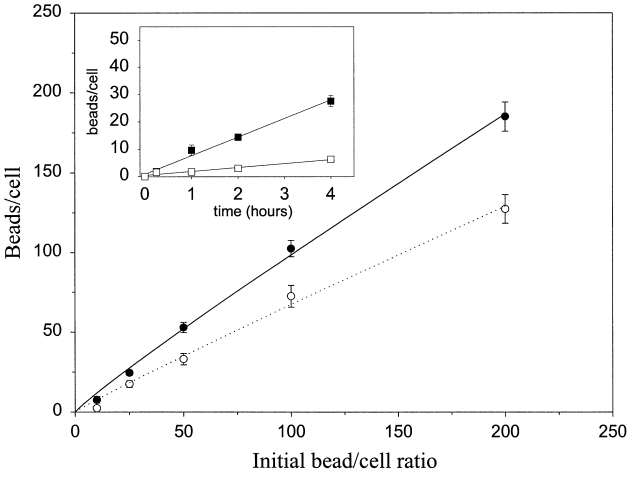

Fig. 1. Phagocytosis of latex beads by cultured BMMs. One assessment of the integrity of the macrophage preparation was retention of macrophage phagocytic capacity determined by uptake of fluorescent latex beads. Macrophage cells (2×10^6) were incubated in 6 well Falcon plates with 1.1 μM latex beads for 2 h in the presence of serum (●) or protein-free medium (PFHMII) (○). Cells were lysed and the uptake of beads was determined by particle counting. The data presented are the average mean of 2–10 replicates of triplicate experiments; bars, S.E.M. Inset: temperature dependence of binding and uptake of latex beads. Macrophages were seeded at 2×10^5 cells/well in 24 well plates and were incubated in PFHMII with 1×10^7 latex beads/well at 37°C (■) or at 4°C (□). At indicated times the cells were collected and the bead uptake per cell was determined. Data are averaged means from two triplicate experiments; bars, S.E.M.

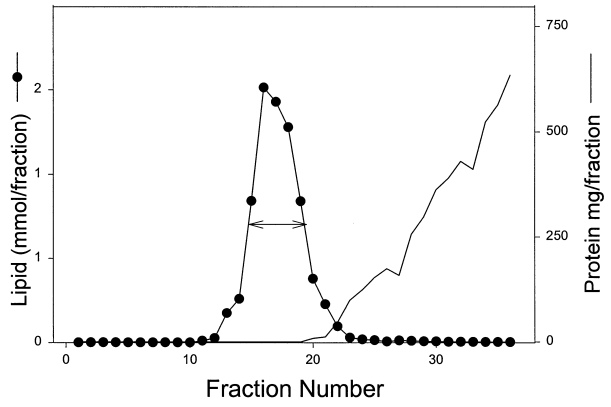

Fig. 2. Fractionation of serum-coated liposomes on CL-4B columns. Liposomes were incubated in serum for 1 h before being chromatographed on CL-4B Sepharose columns. Fractions of 0.25 ml were collected and assayed for relative protein content by the micro BCA (see Section 2) assay or for liposome content by scintillation counting. The lipid elution profile (●) and the protein elution profile (no symbols) of DSPC:Chol (55:45) liposomes is shown as an example of a typical chromatograph. The lipid recovered was 88% of the total loaded onto the column. The fractions between the arrows were pooled for macrophage uptake studies.

effect of protein bound to the liposomes could be isolated from any direct effects of serum factors on these cells.

3.2. Separation of liposomes from bulk serum protein and quantification of bound protein

To study the effect of serum protein binding on liposome uptake, liposomes were pre-exposed to serum then recovered by gel filtration. Certain serum proteins strongly associate with liposomes, and thus can be separated along with the liposomes from the rest of the serum components by column chromatography. The results of a typical chromatographic separation using a Sepharose CL-4B column are shown in Fig. 2, where liposomes could be readily separated from unbound protein. We could not achieve this separation using the mini-spin column procedure described by [6]. To limit the presence of unbound serum protein, column fractions were pooled conservatively, as indicated by the arrows.

Following recovery of liposomes from the gel filtration column, lipid was extracted and the protein residue was measured by the BCA assay to quantify the amount of serum protein associated with liposomes. The relative protein to lipid ratios for the different liposome formulations studied are shown in Table 1. For purpose of comparison, protein binding to 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC)/cholesterol (Chol) liposomes (8.4 μg protein/μmol lipid) was compared to other liposomes of differing phospholipid acyl chain length, phospholipid headgroup charge and presence of PEG. Protein binding to 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC)/Chol liposomes (4.4 μg protein/μmol lipid) or to DMPC/Chol/1,2-dioleoyl-*sn*-glycerol-3-phosphoglycerol (PG) (5.4 μg protein/μmol lipid) was not significantly different from protein binding to DSPC/Chol liposomes or DSPC/Chol/PG, suggesting that the acyl chain length was not a major factor dictating protein binding. As expected, the degree of serum protein binding was dependent on the presence of charged lipids. Liposomes containing certain negatively charged phospholipids bound considerably higher levels of protein than did the neutral liposomes. Specifically, DSPC/Chol/1,2-dioleoyl-*sn*-glycerol-3-phosphoserine (PS) liposomes and DSPC/Chol/cardiolipin liposomes bound two ($P < 0.02$) and three ($P < 0.005$) times more protein, respectively, than did DSPC/Chol liposomes. This increased serum protein association, however, was not observed with negatively charged PG-containing liposomes (3.9 μg protein/μmol lipid). Importantly, the results in Table 1 suggest that protein binding to liposomes

Table 1
Protein binding as a function of liposome composition

Lipid composition	Lipid mol ratio	P_B (μg protein/μmol lipid)
DSPC:Chol	55:45	8.4 ± 1.8
Acyl chain length		
DMPC:Chol	55:45	4.4 ± 1.3
Acyl chain length and charge		
DMPC:Chol:PG	35:45:20	5.4 ± 3.1
Charge		
DSPC:Chol:PS	35:45:20	20.2 ± 4.7
DSPC:Chol:cardiolipin	45:45:10	38.1 ± 7.1
DSPC:Chol:DODAC	35:45:20	11.3 ± 2.4
DSPC:Chol:PG	35:45:20	3.9 ± 1.7
5% PEG		
DSPC:Chol:PEG	50:45:5	13.4 ± 3.4
DSPC:Chol:PS:PEG	30:45:20:5	15.2 ± 3.8
DSPC:Chol:DODAC:PEG	30:45:20:5	35 ± 5.5

Average of the means of 3–5 experiments ± S.E.M.

was not decreased by incorporation of 5 mol% PEG. For instance, the decrease in protein binding observed with liposome containing both PS and PEG (15.2 μg protein/μmol lipid) relative to PS liposomes (20.0 μg protein/μmol lipid) was not statistically significant. Interestingly liposomes containing the cationic lipid DODAC formulated with 5 mol% PEG bound significantly higher levels of associated protein than corresponding DODAC liposomes without PEG-modified lipids ($P < 0.005$). The serum binding results show that incorporation of 5 mol% PEG-lipids does not impede the ability of liposomes to interact with serum proteins under the assay conditions employed here. We were unable to demonstrate that 5 mol% PEG blocked binding of non-specific serum proteins to the surface of the liposomes as suggested previously [14,26,27].

3.3. SDS–PAGE liposome-associated serum protein

While PEG does not alter the amount of protein bound to the liposomes, it is possible that protein binding profiles are altered for PEG liposomes prepared with zwitterionic or charged phospholipids. We analyzed the proteins extracted from serum-treated liposomes by SDS–PAGE. The protein-banding pattern obtained following analysis of six liposome compositions is shown in Fig. 3 (lanes 2–7). Consistent with the data in Table 1, the amount of protein bound increased as the liposome composition changed from neutral to anionic and cationic liposome (lanes 2, 4, and 6, respectively). All major bands can be attributed to protein in the serum protein samples seen in lane 1. We observed only minor qualitative or quantitative differences in liposome-associated protein as a function of PEG incorporation for either the DSPC/Chol (lane 2 versus lane 3) or DSPC/Chol/PS liposomes (lane 4 versus lane 5), consistent with the results in Table 1. PEG did increase the liposome-associated protein of DSPC/Chol/DODAC liposomes (lane 7 versus lane 6). Some distinct protein bands are observed on various liposome compositions and are indicated with dots. One band with a M_r in the range of 100 kDa appears uniquely associated with PS liposomes, and bands of M_r 104, 116, and 23.5 kDa appear only in the PS liposomes with PEG. A protein of M_r 45 kDa is apparent only on DODAC-containing liposomes

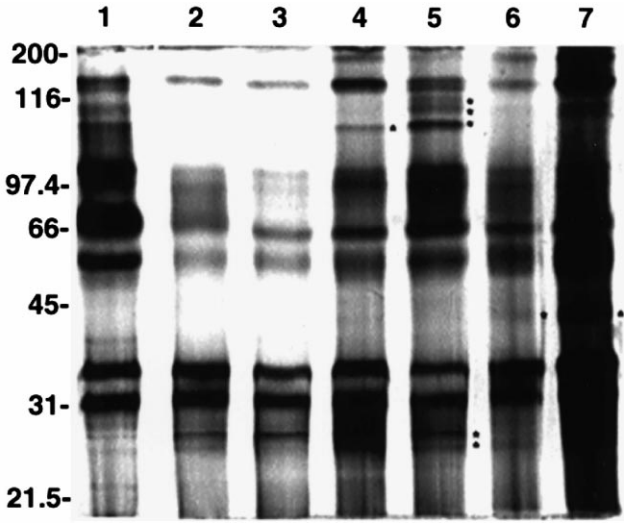

Fig. 3. Protein was extracted from column-purified liposomes (1 μmol lipid) then separated on 7.5% acrylamide gels. Proteins were visualized with silver stain. Lane 1 total mouse serum, lane 2 DSPC:Chol, lane 3 DSPC:Chol:PEG, lane 4 DSPC:Chol:PS, lane 5 DSPC:Chol:PS:PEG, lane 6 DSPC:Chol:DODAC, lane 7 DSPC:Chol:DODAC:PEG. Molecular weight standards are indicated on the left.

with PEG. Even in the presence of PEG, the liposome composition, and most importantly the presence of charged lipid, determined the nature of interactions with serum proteins.

3.4. Effect of bound serum factors on liposome uptake by BMMs

Section 3.3 details recovery of liposomes from serum with associated protein. To explore the interrelationship between PEG incorporation and serum protein on liposome uptake by BMMs, we first looked at the effect of bound protein on uptake of liposomes prepared without PEG. Liposome uptake by macrophages was measured for selected liposome formulations listed in Table 1. Protein-free liposomes or liposomes with selectively bound proteins were added to BMMs in protein-free media for 4 h and cell uptake values for liposomes pre-incubated in serum (shaded bars) or saline (filled bars) are shown in Fig. 4A,B. In the absence of protein, liposomes bound to, and were taken up by, BMMs. The amount of liposome uptake by BMMs was dependent on liposome composition. The lowest uptake was observed for neutral liposomes, DSPC/Chol

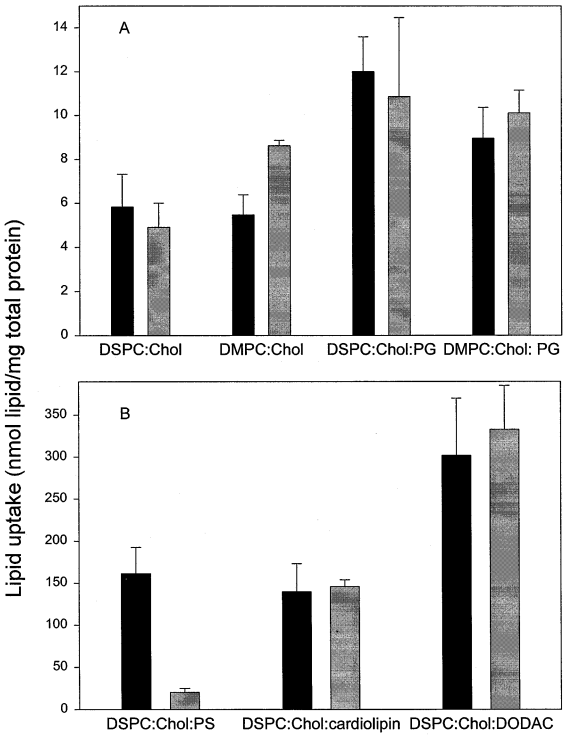

Fig. 4. Effect of serum coating on liposome uptake by macrophages. Liposomes were pre-incubated under serum-free conditions (■) or pre-coated with mouse serum protein (▨). The net uptake of liposomes by macrophages was determined after 4 h of incubation under serum-free conditions. Lipid compositions of liposomes: DSPC:Chol (55:45), DMPC:Chol (55:45), DSPC:Chol:PG (35:45:20), DMPC:Chol:PG (35:45:20), DSPC:Chol:PS (35:45:20), DSPC:Chol:cardiolipin (35:45:20), DSPC:Chol:DODAC (35:45:20). Average of the means of 3–5 triplicate experiments; bars, S.E.M.

(55:45 mol/mol) and DMPC/Chol (55:45 mol/mol) liposomes, with uptake values of 5.8 and 5.4 nmol/mg cellular protein, respectively. Uptake was not influenced by acyl chain length, as uptake of DMPC/Chol and corresponding DSPC-containing liposomes was the same. High levels of uptake were observed for liposomes containing the anionic lipids cardiolipin (139 nmol/mg cellular protein) and PS (161 nmol/mg cellular protein). However, when liposomes were prepared with 20 mol% PG, there was not a statistically significant increase in uptake over DSPC/Chol or DMPC/Chol liposomes. The highest uptake was observed with liposomes containing the cationic lipid, DODAC (301 nmol/mg cellular protein). The avid uptake of cationic liposomes probably is not solely a result of an elevated level of association with BMMs, since the binding of PS liposomes and the binding of DODAC liposomes to cells at 4°C are not significantly different (data not shown).

Our results with serum-treated liposomes were unexpected. In particular PS-containing liposomes were taken up by macrophages less avidly following protein adsorption. The uptake of 20 nmol lipid/mg cellular protein in the presence of bound serum protein represents an 8-fold decrease relative to the uptake measured in the absence of protein ($P < 0.005$). It is worth noting that the uptake of protein bound PS liposomes was still 2–3-fold higher than either the DSPC/Chol liposomes or PG liposomes, with or without bound serum protein. Uptake of either cardiolipin or DODAC liposomes was not different after serum treatment, thus associated protein had no effect on uptake of these liposomes. From these results we conclude that serum protein is not required for liposome uptake, and the presence of protein does not determine the relative uptake of charged or neutral liposomes. Nevertheless, our results are consistent with previous studies that have shown that the liposomes that are taken up more avidly by macrophages in vitro are the liposomes that are eliminated more rapidly following intravenous (i.v.) administration and bind greater levels of serum protein in vitro.

3.5. *Effect of bound serum protein on pegylated liposome uptake by BMMs*

BMM uptake was then measured for liposomes containing PEG-grafted lipids (Fig. 5), in the presence (shaded bars) and absence (filled bars) of serum protein, for neutral, anionic (PS) and cationic (DODAC) liposomes. In the absence of protein, inclusion of 5 mol% PEG reduced uptake of anionic PS-containing or cationic DODAC-containing liposomes by BMMs by 3-fold ($P < 0.02$) and 7-fold ($P < 0.01$), respectively (compare Figs. 4 and 5). Thus our protein-free BMM model reproduces previous work demonstrating inhibition of macrophage uptake of liposomes with surface-associated PEG [22]. We observed an increase in uptake of DSPC/Chol prepared with 5 mol% PEG liposomes (50 nmol/mg cellular protein) relative to the corresponding liposomes prepared without PEG (5.8 nmol/mg cellular protein). We showed in Table 1 that PEG did not inhibit

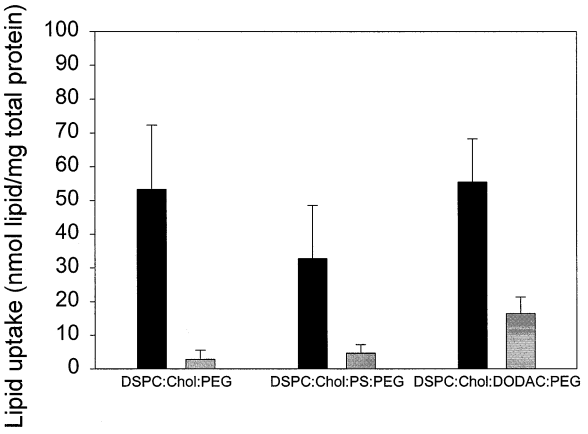

Fig. 5. Effect of serum coating on uptake of liposomes containing PEG by macrophages. Liposomes generated with 5% DSPE-PEG were pre-incubated under serum-free conditions (■) or pre-coated with mouse serum protein (▨). The net uptake of liposomes by macrophages was determined after 4 h of incubation under serum-free conditions. Lipid compositions: DSPC:Chol:PEG (50:45:5), DSPC:Chol:PS:PEG (30:45:20:5), DSPC:Chol:DODAC:PEG (30:45:20:5). Average of the means of 2–3 triplicate experiments; bars, S.E.M.

serum binding; yet serum treatment of PEG liposomes significantly reduced BMM uptake of neutral, anionic and cationic liposomes, by 10-fold ($P < 0.05$), 8-fold ($P < 0.01$) and 3-fold ($P < 0.05$), respectively (Fig. 5). The combined effect of PEG inclusion and serum treatment provided nearly complete inhibition of liposome uptake for the neutral and cationic liposomes. Similar to results obtained for liposomes without bound protein, the levels of cell association and liposome uptake were similar for all PEG-containing formulations with bound serum protein. This is interesting considering that the amount of protein bound differs significantly between these preparations (see Table 1).

Alterations in net liposome uptake by cultured macrophages could be a consequence of changes either in the binding step or in the internalization of bound liposomes. In order to assess this, the uptake data in Figs. 4 and 5 were replotted to show the internalization of liposomes as a function of liposome cell surface binding at 4°C (Fig. 6). For each composition we calculated the surface bound lipid as a percent of the total cell-associated lipid. As shown in Fig. 6A,B, after 4 h of incubation, the fraction of liposomes without protein associated with the outside of the ranges from 25.2 to 68.7%. Liposome composition fundamentally regulated the nature of the effect of serum. Reduced uptake of serum-treated PS liposomes (Fig. 4B) corresponds to reduced macrophage binding and thus the ratio of cell-associated to surface bound PS is the same in the presence and absence of associated serum proteins (Fig. 6A). The ability of serum treatment to hinder the cell binding step of PS liposome uptake argues for a dysopsonin effect of serum proteins. This effect was not observed

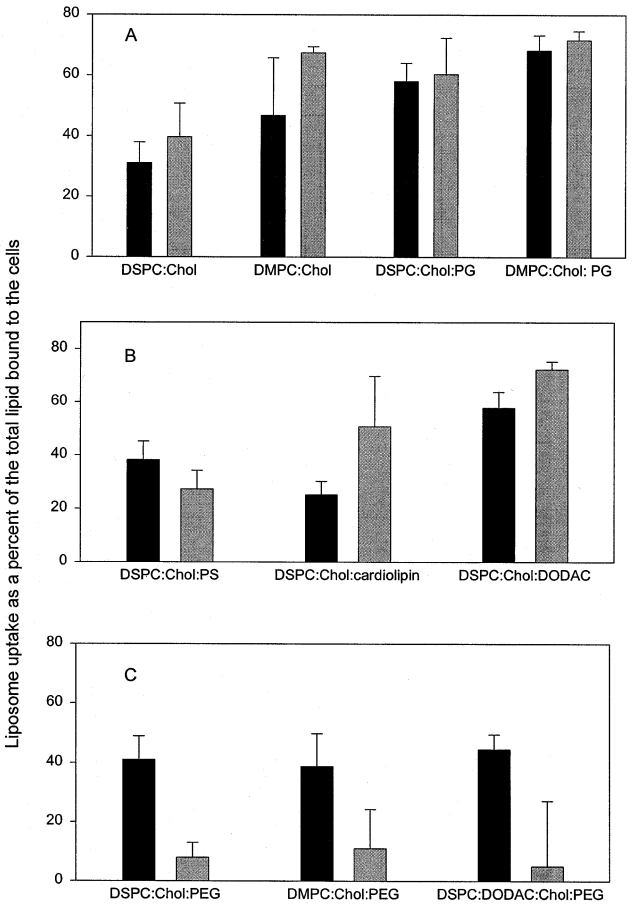

Fig. 6. Effect of serum coating on the percent of associated lipid that represents liposomes incorporated into the cells. Liposomes were pre-incubated under serum-free conditions (■) or pre-coated with mouse serum protein (▨). The fraction of incorporated liposomes by macrophages was determined by calculating the lipid uptake at 37°C as a fraction of the total lipid bound at 37°C. Lipid compositions: A: DSPC:Chol (55:45), DMPC:Chol (55:45), DSPC:Chol:PS (35:45:20), DSPC:Chol: cardiolipin (35:45:20); B: DSPC:Chol:PG (35:45:20), DMPC:Chol:PG (35:45:20), DSPC:Chol:DODAC (35:45:20); C: DSPC:Chol:PEG (50:45:5), DSPC:Chol:PS:PEG (30:45: 20:5), DSPC:Chol:DODAC:PEG (30:45:20:5). Mean of two or five triplicate experiments; bars, S.E.M.

with cardiolipin liposomes. Protein-free cardiolipin liposomes surface association at 4°C was higher in comparison to the surface association of liposomes with bound serum protein, resulting in a higher ratio of internalized to cell bound lipid for the former case (Fig. 6B).

The effect of serum treatment of PEG liposomes on the ratio of uptake was quite distinct (Fig. 6C). Interestingly, serum treatment of PEG liposomes affected both binding and internalization of cell-associated lipid. The presence of bound serum protein on PEG liposomes not only reduced the amount of surface bound liposomes by 50–68% (Fig. 5) but also reduced the uptake of cell-associated lipid to as little as 10% of total cell-associated lipid (Fig. 6C). Similar to data shown in Fig. 5, the percent of cell-associated liposomes for formulations containing PEG was comparable for neutral, anionic and cationic liposomes suggesting the different compositions appear similar to macrophages in culture.

4. Discussion

Our results contradict the assertion that high uptake of anionic liposomes is a function of recognition of bound serum proteins by macrophages and also contradict the claim that PEG-grafted lipids reduce macrophage uptake by reducing serum protein binding. First, we demonstrated that serum treatment reduced uptake of only PS liposomes. Second, we showed that serum reduces cell association of cardiolipin liposomes without altering uptake of these liposomes. Third, we showed that in the presence or absence of serum protein, neutral, anionic and cationic liposomes with 5 mol% surface-grafted PEG are taken up identically by cultured macrophages. PEG incorporation results in liposome surfaces that appear identical to macrophages, regardless of the charge of the lipid components. Thus, the presence of PEG takes precedence over the presence or absence of charged lipids for BMM uptake of liposomes. Finally, the results strongly suggest that serum protein binding to liposomes containing surface-grafted PEG polymers provides a change in the liposomal surface properties that reduces liposome binding and uptake by BMMs. To reconcile these findings with the prevalent view that serum proteins act as opsonins and govern liposome phagocytosis, we have considered previous reports on the role of protein binding in governing liposome uptake. Further, we present a model that suggests that reduced cell uptake of PEG liposomes is due, in part, to a structural change in the grafted PEG polymer induced by protein binding.

PEG-lipids tend to increase the circulation longevity of i.v. injected liposomes [10,11,17,28]. PEG-lipid-mediated reduction of uptake of liposomes by isolated macrophages has been shown in most [29] but not all studies [30]. The mechanism for these PEG effects has never been thoroughly explained, however three possibilities have been suggested. PEG is postulated to stabilize liposomes by preventing serum protein-mediated lipid degradation [11] or to obstruct interactions between liposomes and cell surfaces [9,10]. The most widely accepted theory, however, is that the presence of PEG sterically hinders opsonin protein binding [5]. Opsonins are best documented in studies assessing the phagocytosis of bacteria. By analogy to this process, immune and non-immune opsonin proteins are hypothesized to adsorb non-specifically to liposome surfaces and influence uptake by phagocytic cells [4] and accordingly, PEG interferes with this process. Results presented here agree with reports that macrophages and liposomes can associate directly [2] and that liposome uptake does not require opsonins [31]. PEG can interfere with this interaction in the absence of protein, however proteins bound to liposomes can enhance the ability of PEG to interfere with cell association.

There is a significant body of circumstantial evidence supporting the existence of opsonins capable of influencing liposome uptake by macrophages and perfused tissue. Liposomes have been shown to interact with blood proteins [32,33] and rapid clearance from the circulation was found to correlate with liposome compositions having high serum protein binding [33]. Further, serum treatment of liposomes enhanced uptake of PS liposomes by perfused liver [34–38]. There have also been in vitro studies suggesting serum [2,39] or known bacterial opsonins, including immunoglobulins, fibronectin, vitronectin and complement increase the rates of uptake of liposomes into cultured cells [5,6,40–47]. In addition, the amount of complement bound in vitro [6] or β2 macroglobulin bound in vivo [48] to various liposome

preparations was found to be inversely proportional to its circulation longevity. Support for an in vivo role for complement in liposome clearance was shown in a study demonstrating that K-76COOH, a compound which interferes with complement activation, reduced liposome clearance in rats [49]. However results from other studies do not support the opsonin hypothesis, suggesting either that there is no serum requirement for liposome uptake, or more interestingly, that dysopsonins can reduce liposome uptake by macrophages. For instance, several studies have observed that serum either decreased liposome uptake [38,50,51] or did not alter liposome uptake [52] by perfused liver. Ahl et al. [46] reported that, for two differing liposome preparations, the level of complement binding did not correlate with circulation times.

Despite the mixed evidence presented above for opsonization of liposomes, the theory that PEG-lipids extend liposome circulation lifetime by preventing opsonins from binding to liposomes is a reasonable and attractive proposal. This theory is supported by the demonstration that PEG-lipids reduced liposome partitioning in an aqueous/PEG two-phase system [53]. In addition, protein binding to a PEG monolayer decreased with increasing PEG concentration [54]. Protein–liposome interactions as measured by complement activation [4,47,51] or biotin binding to liposome-associated avidin [15] were shown to be decreased on liposomes containing PEG-lipids suggesting PEG can block protein interactions with substances on liposome surfaces. A recent study showed that a minimum of 10–15 mol% (*N*-monoethoxy poly(ethylene glycol) succinyl)1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine M_r 2000 (DSPE-PEG 2000) was required to prevent PS liposome activation of the prothrombinase complex [55]. Importantly, 5 mol% DSPE-PEG, a quantity sufficient to prolong circulation [56], was not sufficient to prevent this binding. This implies that higher concentrations of PEG may be required to fully block interactions between liposomes and proteins than to block uptake. In fact, Harvie et al. [57] noted that liposomes, prepared without PEG or with approximately 10 mol% PEG, had identical bound protein levels when isolated from mice 30 min post-injection. In addition, cell uptake of PC liposomes pre-exposed to whole plasma was the same when prepared with or without 10 mol% PEG [58].

A recent report identifies a receptor on phagocytic cells which recognizes PS exposed on the plasma membrane of apoptotic cells. Importantly, target cell recognition by this receptor can be blocked by PS liposomes [59]. Further, in accordance with the present study, Kamps [60] showed that uptake of PS-containing liposomes by rat liver macrophage and endothelial cells in vitro was inhibited by serum in a manner that was dependent on the cell type and the source of sera. Liposome uptake by various cell populations in vitro allied more closely with liposome distribution in vivo when the incubations were made in the presence of serum. This provided the basis for a suggestion of specific and non-specific uptake mechanisms for PS. This suggests that inhibition of liposomes uptake by serum protein, observed here with BMMs, may be relevant for regulating tissue uptake in vivo, for instance limiting uptake in the RES tissues observed by Woodle [61]. We suggest protein associated with the liposome may limit recognition of PS liposomes by specific or non-specific cell receptors (see Fig. 7A). The hindrance of liposome uptake by protein may be through an increase in the hydrophilicity of liposomes, thereby limiting interactions with cell membranes or cell proteins. Alternatively, the serum protein may sterically hinder interaction between PS and the cell membrane. While we observed that mouse serum protein reduced uptake of PS liposomes, BMM uptake of all liposomes with or without serum was still proportional to expected plasma elimination rates of liposomes without surface-grafted PEGs.

Serum not only has specific effects on PS liposome uptake, but also lipid composition-dependent effects on liposome cell association. Serum treatment reduced cell association of cardiolipin liposomes without altering cell uptake. While liposome charge is known to be important, but not determinant, this result suggests that cell surface association is also insufficient to promote uptake by macrophages. This observation could be accounted for by the existence of productive surface association, characterized as liposome binding to the cells which leads to uptake, and non-productive surface association which does not lead to uptake. Thus, in contrast to results

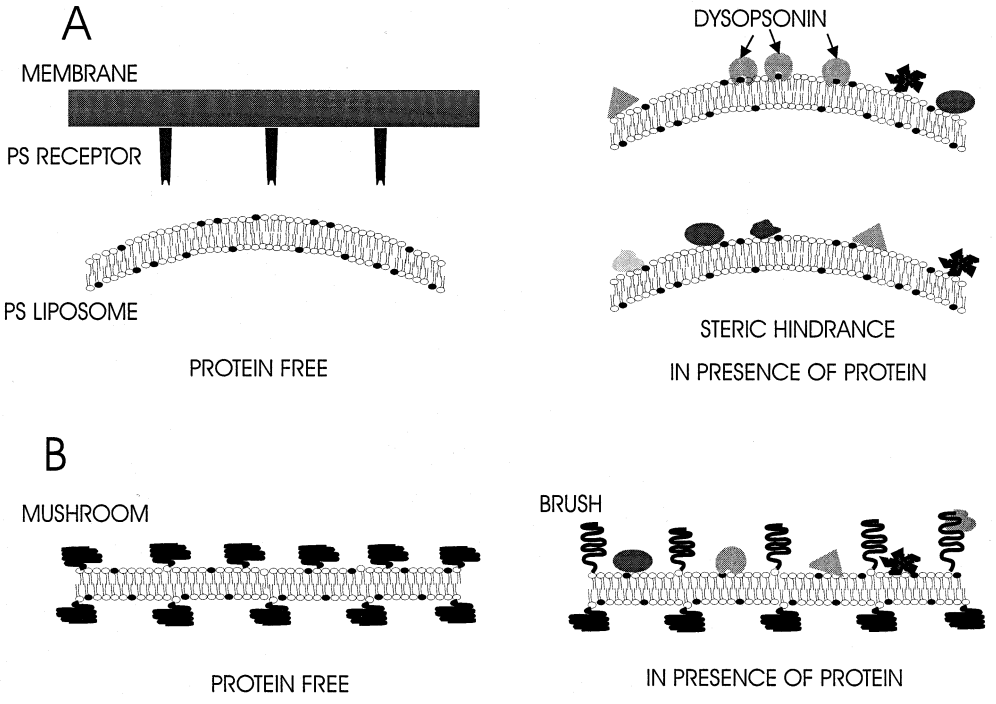

Fig. 7. Models for serum protein regulation of liposome–macrophage interactions. A: Serum inhibition of PS liposome uptake. Under serum-free conditions, liposomal PS interacts with macrophage membranes, possibly via a PS-specific receptor. Serum protein interaction with the liposomal surface reduces interaction with cell membranes. Both a specific recognition of PS by dysopsonins (arrows) or a steric hindrance by bulk protein interactions are consistent with this inhibition. B: Serum inhibition of uptake of liposomes containing PEG. In the absence of serum, PEG reduces the uptake of liposomes containing charged lipids, however it does not completely block uptake, suggesting the surface of the liposome is accessible. This may be due to PEG being in the mushroom configuration, however allowing for protein and cell surface interactions. Hydrostatic interactions between PEG and serum protein induce the PEG to adopt a more extended configuration, preventing uptake by phagocytic cells.

observed with PS, in the case of cardiolipin protein association only inhibits non-productive binding.

An important conclusion of this paper is that PEG does not prevent interactions with serum proteins. PEG is thought to form a shroud on the surface of the liposomes partially shielding hydrostatic charges [27], however this may still allow for protein interactions with the surface of the liposome. Perhaps even more controversial, PEG itself may provide a scaffolding onto which protein can bind. We propose that serum proteins and PEG act together to influence liposome–macrophage cell association. This additive effect may be a consequence of a change in the conformation of the surface-grafted PEG. For instance PEG incorporated to 5 mol% may be in the mushroom conformation. Following protein binding, steric constraints imposed on the PEG may result in a change in the conformation to the brush conformation (see Fig. 7B). The increased extension of the PEG brought about by this conformational change is thus more effective at preventing association and uptake of liposomes by macrophages.

It is worth noting that the combined effect of PEG and serum protein is reminiscent to observations made with polymer-coated nanoparticles and serum proteins. Hydrophobic latex particles share some properties with liposomes, including being avidly taken up by macrophages. Like liposomes, macrophage uptake of nanoparticles increases with size and surface charge [62]. Nanoparticles coated with hydrophilic PEG-like materials such as poloxamine-908 [63] have prolonged circulation in vivo and macrophage uptake of particles in vitro decreases with increased thickness of the polymer coating [64]. Serum enhances the effect of polymer coating, further reducing particle uptake by isolated macrophages, suggesting that a combined effect of hydrophobicity, opsonization and dysopsonization determines uptake

of coated particles by macrophages [63]. In an analogous fashion, we suggest that it is the combination of PEG and serum, altering the overall hydrophobicity of liposomes, which determines macrophage uptake.

Understanding macrophage–liposome interactions is critical to developing long-circulating liposome preparations. Modification of the liposome physiochemistry alters circulation longevity and engenders corresponding changes in macrophage uptake in vitro. We have concluded that PEG liposomes can bind to macrophages and be internalized. Further, PEG does not a priori need to reduce interaction between serum proteins and liposomes in order to provide its steric stabilization effects. We suggest that the primary effect of surface-grafted PEG polymers is to reduce cell uptake of liposomes and that the liposome bound proteins play a role by affecting the PEG conformation.

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References

[1] J. Dijkstra, M. van Galen, G. Scherphof, Biochim. Biophys. Acta 813 (1985) 287–297.
[2] K.D. Lee, K. Hong, D. Papahadjopoulos, Biochim. Biophys. Acta 1103 (1992) 185–197.
[3] K.D. Lee, S. Nir, D. Papahadjopoulos, Biochemistry 32 (1993) 889–899.
[4] N.M. Wassef, G.R. Matyas, C.R. Alving, Biochem. Biophys. Res. Commun. 176 (1991) 866–874.
[5] H.M. Patel, Crit. Rev. Ther. Drug Carrier Syst. 9 (1992) 39–90.
[6] A. Chonn, S.C. Semple, P.R. Cullis, Biochim. Biophys. Acta 1070 (1991) 215–222.
[7] A. Chonn, S.C. Semple, P.R. Cullis, J. Biol. Chem. 267 (1992) 18759–18765.
[8] S. Moghimi, H. Patel, Biochim. Biophys. Acta 984 (1989) 384–387.
[9] A.L. Klibanov, K. Maruyama, A.M. Beckerleg, V.P. Torchilin, L. Huang, Biochim. Biophys. Acta 1062 (1991) 142–148.
[10] A. Mori, A.L. Klibanov, V.P. Torchilin, L. Huang, FEBS Lett. 284 (1991) 263–266.
[11] D.D. Lasic, F.J. Martin, A. Gabizon, S.K. Huang, D. Papahadjopoulos, Biochim. Biophys. Acta 1070 (1991) 187–192.
[12] M.C. Woodle, K.K. Matthay, M.S. Newman, J.E. Hidayat, L.R. Collins, C. Redemann, F.J. Martin, D. Papahadjopoulos, Biochim. Biophys. Acta 1105 (1992) 193–200.
[13] P.G. de Gennes, in: P. Bondgrand (Ed.), Physical Basis of Cell–Cell Adhesion, CRC Press, Boca Raton, FL, 1988, pp. 39–60.
[14] V.P. Torchilin, V.G. Omelyanenko, M.I. Papisov, A.A. Bogdanov Jr., V.S. Trubetskoy, J.N. Herron, C.A. Gentry, Biochim. Biophys. Acta 1195 (1994) 11–20.
[15] D. Needham, D.V. Zhelev, T.J. McIntosh, in: A.S. Janoff (Ed.), Liposomes – Rational Design, Marcel Dekker, 1999, pp. 13–62.
[16] J. Senior, G. Gregoriadis, Life Sci. 30 (1982) 2123–2136.
[17] T.M. Allen, C. Hansen, Biochim. Biophys. Acta 1068 (1991) 133–141.
[18] M.J. Parr, M.B. Bally, P.R. Cullis, Biochim. Biophys. Acta 1168 (1993) 249–252.
[19] H.J. Lim, D. Masin, T.D. Madden, G. Zhang, S.A. Johnstone, M.B. Bally, Clin. Cancer Res. 6 (2000) 4449–4460.
[20] N.C. Phillips, J. Dahman, Immunol. Lett. 45 (1995) 149–152.
[21] P.G. Tardi, E.N. Swartz, T.O. Harasym, P.R. Cullis, M.B. Bally, J. Immunol. Methods 210 (1997) 137–148.
[22] T.M. Allen, G.A. Austin, A. Chonn, L. Lin, K.C. Lee, Biochim. Biophys. Acta 1061 (1991) 56–64.
[23] M.J. Hope, M.B. Bally, L.D. Mayer, A.S. Janoff, P.R. Cullis, Chem. Phys. Lipids 40 (1986) 89–107.
[24] D. Wessel, U.I. Flugge, Anal. Biochem. 138 (1984) 141–143.
[25] U.K. Laemmli, Nature 227 (1970) 680–685.
[26] G. Blume, G. Cevc, Biochim. Biophys. Acta 1146 (1993) 157–168.
[27] M.C. Woodle, M.S. Newman, J.A. Cohen, J. Drug Target. 2 (1994) 397–403.
[28] G. Blume, G. Cevc, M.D. Crommelin, I.A. Bakker-Woudenberg, C. Kluft, G. Storm, Biochim. Biophys. Acta 1149 (1993) 180–184.
[29] R. Zeisig, K. Shimada, S. Hirota, D. Arndt, Biochim. Biophys. Acta 1285 (1996) 237–245.
[30] A. Vertut-Doi, H. Ishiwata, K. Miyajima, Biochim. Biophys. Acta 1278 (1996) 19–28.
[31] D. Liu, Q. Hu, Y.K. Song, Biochim. Biophys. Acta 1240 (1995) 277–284.
[32] F. Bonté, M.J. Hsu, A. Papp, K. Wu, S.L. Regen, R.L. Juliano, Biochim. Biophys. Acta 900 (1987) 1–9.
[33] A. Chonn, P.R. Cullis, D.V. Devine, J. Immunol. 146 (1991) 4234–4241.
[34] D. Hoekstra, G. Scherphof, Biochim. Biophys. Acta 551 (1979) 109–121.

[35] E. Mayhew, C. Gotfredsen, Y.J. Schneider, A. Trouet, Biochem. Pharmacol. 29 (1980) 877–886.
[36] V.P. Torchilin, V.G. Omel'yanenko, A.L. Klibanov, A.I. Mikhailov, V.I. Gol'danskii, V.N. Smirnov, Biochim. Biophys. Acta 602 (1980) 511–521.
[37] S.M. Moghimi, H.M. Patel, Biochim. Biophys. Acta 1135 (1992) 269–274.
[38] H. Aoki, K. Fuji, K. Miyajima, Int. J. Pharm. 149 (1997) 15–23.
[39] S.M. Moghimi, H.M. Patel, Biochem. Soc. Trans. 21 (1993) 128S.
[40] H.R. Petty, J.W. Francis, Biophys. J. 47 (1985) 731–734.
[41] M.J. Hsu, R.L. Juliano, Biochim. Biophys. Acta 720 (1982) 411–419.
[42] J.T. Derksen, H.W. Morselt, D. Kalicharan, C.E. Hulstaert, G.L. Scherphof, Exp. Cell Res. 168 (1987) 105–115.
[43] N.M. Wassef, C.R. Alving, Methods Enzymol. 149 (1987) 124–134.
[44] D.G. Perry, P. Wisniowski, G.L. Daugherty, J. Downing, W.J. Martin II, Am. J. Respir. Cell Mol. Biol. 17 (1997) 462–470.
[45] R. Bumrah, H.M. Patel, Biochem. Soc. Trans. 22 (1994) 86S.
[46] P.L. Ahl, S.K. Bhatia, P. Meers, P. Roberts, R. Stevens, R. Dause, W.R. Perkins, A.S. Janoff, Biochim. Biophys. Acta 1329 (1997) 370–382.
[47] A.J. Bradley, D.V. Devine, S.M. Ansell, J. Janzen, D.E. Brooks, Arch. Biochem. Biophys. 357 (1998) 185–194.
[48] A. Chonn, S.C. Semple, P.R. Cullis, J. Biol. Chem. 270 (1995) 25845–25849.
[49] T.M. Huong, H. Harashima, H. Kiwada, Biol. Pharm. Bull. 22 (1999) 515–520.
[50] H. Harashima, S. Komatsu, S. Kojima, C. Yanagi, Y. Morioka, M. Naito, H. Kiwada, Pharm. Res. 13 (1996) 1049–1054.
[51] Q. Hu, D. Liu, Biochim. Biophys. Acta 1284 (1996) 153–161.
[52] F. Liu, D. Liu, Biochim. Biophys. Acta 1278 (1996) 5–11.
[53] J. Senior, C. Delgado, D. Fisher, C. Tilcock, G. Gregoriadis, Biochim. Biophys. Acta 1062 (1991) 77–82.
[54] H. Du, P. Chandaroy, S.W. Hui, Biochim. Biophys. Acta 1326 (1997) 236–248.
[55] G.N.C. Chiu, M.B. Bally, L.D. Mayer, Biochim. Biophys. Acta (in press).
[56] T.M. Allen, C. Hansen, F. Martin, C. Redemann, A. Yau-Young, Biochim. Biophys. Acta 1066 (1991) 29–36.
[57] P. Harvie, A. Desormeaux, M.C. Bergeron, M. Tremblay, D. Beauchamp, L. Poulin, M.G. Bergeron, Antimicrob. Agents Chemother. 40 (1996) 225–229.
[58] T. Allen, Adv. Drug Deliv. Rev. 13 (1994) 285–309.
[59] V.A. Fadok, D.L. Bratton, D.M. Rose, A. Pearson, R.A. Ezekewitz, P.M. Henson, Nature 405 (2000) 85–90.
[60] J.A. Kamps, H.W. Morselt, G.L. Scherphof, Biochem. Biophys. Res. Commun. 256 (1999) 57–62.
[61] M.C. Woodle, Chem. Phys. Lipids 64 (1993) 249–262.
[62] H. Ayhan, A. Tuncel, N. Bor, E. Piskin, J. Biomater. Sci. Polym. Ed. 7 (1995) 329–342.
[63] S.M. Moghimi, I.S. Muir, L. Illum, S.S. Davis, V. Kolb-Bachofen, Biochim. Biophys. Acta 1179 (1993) 157–165.
[64] L. Illum, L.O. Jacobsen, R.H. Muller, E. Mak, S.S. Davis, Biomaterials 8 (1987) 113–117.