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Protein adsorption to polyethylene glycol modified liposomes from fibrinogen solution and from plasma

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Abstract

Unmodified and polyethylene glycol (PEG) modified neutral and negatively charged liposomes were prepared by freeze–thaw and extrusion followed by chromatographic purification. The effects of PEG molecular weight (PEG 550, 2000, 5000), PEG loading (0–15 mol%), and liposome surface charge on fibrinogen adsorption were quantified using radiolabeling techniques. All adsorption isotherms increased monotonically over the concentration range 0–3 mg/ml and adsorption levels were low. Negatively charged liposomes adsorbed significantly more fibrinogen than neutral liposomes. PEG modification had no effect on fibrinogen adsorption to neutral liposomes. An inverse relationship was found between PEG loading of negatively charged liposomes and fibrinogen adsorption. PEGs of all three molecular weights at a loading of 5 mol% reduced fibrinogen adsorption to negatively charged liposomes. Protein adsorption from diluted plasma (10% normal strength) to four different liposome types (neutral, PEG-neutral, negatively charged, and PEG-negatively charged) was investigated using gel electrophoresis and immunoblotting. The profiles of adsorbed proteins were similar on all four liposome types, but distinctly different from the profile of plasma itself, indicating a partitioning effect of the lipid surfaces. α2-macroglobulin and fibronectin were significantly enriched on the liposomes whereas albumin, transferrin, and fibrinogen were depleted compared to plasma. Apolipoprotein AI was a major component of the adsorbed protein layers. The blot of complement protein C3 adsorbed on the liposomes suggested that the complement system was activated.

Keywords: Polyethylene glycol; Plasma protein; Protein adsorption; Liposome; Immunoblot

1. Introduction

The use of liposomes for drug delivery has been limited due to their rapid removal from the circulation by macrophages of the reticuloendothelial system (RES). It is believed that adsorption of proteins (opsonization) makes liposomes more susceptible to phagocytosis. A number of studies have investigated protein interactions with conventional liposomes and lipid bilayers [1–5]. These studies have identified common opsonins that bind to liposomes in vivo such as the immunoglobulins and complement proteins, non-immune opsonins such as fibronectin and vitronectin, cell-binding proteins such as fibrinogen, and lipoproteins. In general, these studies have not investigated the compositional profile of the proteins adsorbed from blood. Liposome properties such as surface charge, rigidity, and thermal phase transitions have also been identified as significant factors for protein–liposome interactions [1,2,6,7].

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Over the past several years progress has been made in extending liposome lifetimes in the circulation by incorporating polyethylene glycol (PEG) into the membranes. PEG modification has been shown to decrease liposome uptake by macrophages of the RES [8]. From various studies it has been shown that PEG molecular weight and loading have significant influence in prolonging the circulation lifetimes [9–14]. Small, stable liposomes with 4–10 mol% of PEG molecular weight of 2000 or greater are typically used in commercial applications [14].

It has been widely assumed, mainly by analogy with PEG modified surfaces generally, that PEG modification inhibits the adsorption of opsonins to liposomes, probably by steric exclusion. However, there have been few studies of the adsorption of proteins to PEG modified liposomes, and the evidence is not sufficient to allow the unequivocal conclusion that PEG modification inhibits adsorption. Senior et al. concluded, using an indirect method involving aqueous two-phase partitioning of liposomes [15], that PEG modification slows the adsorption of plasma components. Xu et al. [16] have shown a 2–3-fold reduction in total protein adsorbed to PEG modified liposomes from plasma. Others have reported on the effects of PEG incorporation in lipid bilayers. For example, Ephremova et al. [17] showed that protein adsorption was influenced by the presence of PEG mainly by masking the effects of the charge on the lipid head groups. Du et al. have reported that PEG reduces the adsorption of proteins to spread lipid bilayers [18]. Xu and Marchant [19], on the other hand, found no effect of PEG on total protein adsorption from plasma to lipid bilayers as measured by total internal reflection fluorescence.

In this communication we report on the effects of PEG modification on protein adsorption to liposomes. We investigated adsorption to unmodified and PEG modified liposomes with emphasis on the effects of liposome charge and PEG molecular weight and loading. In vitro adsorption studies on unmodified and PEG modified liposomes were carried out from solutions of fibrinogen in buffer and from human plasma. In the fibrinogen experiments, adsorbed protein amounts were measured quantitatively by radiolabeling techniques. In the plasma experiments, total adsorbed protein was measured and qualitative indications regarding individual proteins were obtained using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting.

2. Materials and methods

2.1. Materials

All phospholipids were purchased from Avanti Polar Lipids. Cholesterol ($>99\%$ purity) was obtained from Sigma. Pooled human plasma was prepared from citrated blood (25 donors) and stored at $-70°C$. $Na^{125}I$ was from ICN Radiochemicals and Iodo-gen Reagent from Pierce Chemicals. 2-Mercaptoethanol was from BDH. Goat antisera to human factor XI, factor XII, prekallikrein, high molecular weight kininogen (HMWK), fibrinogen, albumin, and protein C, and sheep antisera to human antithrombin III (ATIII), vitronectin, and thrombin were obtained from Cedarlane Laboratories (Hornby, ON, Canada). Goat antisera to human plasminogen, transferrin, IgG, and β-lipoprotein, and rabbit antisera to α2-macroglobulin, β2-microglobulin, and hemoglobin were from Sigma. Goat antisera to human complement factor C3, factor B, factor H, and factor I were from Calbiochem. Goat antiserum to human α1-antitrypsin and rabbit antiserum to fibronectin were from Enzyme Research Laboratories (South Bend, IN, USA). Goat antiserum to human protein S was from American Diagnostica (Greenwich, CT, USA) and to apolipoprotein AI was from ESBE (Markham, ON, Canada). Rabbit antiserum to human factor V was a generous gift of Dr. F. Ofosu (McMaster University). Affinity purified goat-antirabbit IgG–alkaline phosphatase conjugate was from Bio-Rad; affinity-purified rabbit-antigoat IgG–alkaline phosphatase conjugate was from Sigma, and rabbit-antisheep IgG–alkaline phosphatase conjugate was from Bethyl Laboratories (Montgomery, TX, USA).

2.2. Liposome preparation

Liposomes were prepared by rotoevaporating a mixture of phospholipids, cholesterol, and varying amounts of mPEG-conjugated phosphatidylethanolamine (PE-mPEG) in chloroform. In a typical prep-

aration, 40 mg total phospholipid plus cholesterol at 30 mol% was used. The resulting thin film was hydrated with sucrose solution (180 mM sucrose, 10 mM Tris, 24 mM NaCl, pH 7.4) and freeze–thawed five times. The suspension was extruded ten times through two stacked polycarbonate membranes of 100 nm pore size and 47 mm diameter (Avestin) at 60°C. To remove external sucrose the sized liposomes were chromatographed on a Sepharose CL-4B column (Sigma) equilibrated with Tris-buffered saline (TBS, pH 7.4). The eluted liposome fractions were pooled for further analysis. PE-mPEG inclusion in the liposomes was assumed to be 100%. The size distribution of the liposomes was determined by dynamic light scattering (Variable Angle QELS, Brookhaven Instruments) and the concentration was determined by phosphate assay [20].

2.3. Protein adsorption

2.3.1. Adsorption from fibrinogen solution

Aliquots of liposome suspension (1.5 ml, concentration approximately 1 mg/ml) were centrifuged at 55 000 rpm for 30 min at 25°C. Tubes with TBS only were used as controls. The supernatant was partially removed leaving 150 μl total volume. The centrifuge tubes were vortexed briefly to resuspend the liposome pellet in the remaining supernatant. Stock solutions of fibrinogen in TBS were prepared of which 1% was radiolabeled with ^{125}I using the Iodogen technique (Pierce). Radioiodinated fibrinogen (850 μl) was added to each centrifuge tube giving a total volume of 1 ml and final protein concentrations of 0.5, 1.5, and 3.0 mg/ml. A control was run consisting of fibrinogen solution at 3.0 mg/ml without liposomes. Adsorption was carried out for 3 h at 22°C.

The liposomes were separated from the fibrinogen solution by centrifuging four times at 55 000 rpm and 25°C for 30 min. After each spin the supernatant was removed, replaced with 1 ml TBS, and vortexed to resuspend the pellet. The radioactivity of each TBS wash was determined. Following the final wash, the liposome pellets were removed from the centrifuge tubes by adding 200 μl TBS, vortexing to break up the pellet, and removing the suspension. This operation was repeated five times. The radioactivity of each 200-μl aliquot was recorded and the total radioactivity determined. The quantity of liposomes removed was determined by phosphate assay. The radioactivity of the empty tubes was also determined.

2.3.2. Adsorption from plasma

Aliquots of liposome suspension (1.5 ml, varying composition, in triplicate) were placed in centrifuge tubes, and the volumes were made equal by adding TBS. Tubes with TBS only were used as controls. The tubes were centrifuged at 70 000 rpm for 30 min at 25°C to isolate the liposomes. The supernatant was partially removed leaving 150 μl total volume. The centrifuge tubes were vortexed briefly to resuspend the liposome pellet in the remaining supernatant.

Plasma was diluted with TBS to 12% of normal strength. Diluted plasma (850 μl) was added to the 150 μl of liposome suspension in each centrifuge tube to give a final plasma strength of 10% normal. Adsorptions were carried out for 2 h at 22°C. In some experiments a small amount of radiolabeled albumin (ICl method [21]) was added to the plasma to monitor the supernatant washes (not for quantification of bound protein).

The liposomes were separated from the plasma by multiple centrifugation (100 000 rpm, 30 min, 25°C) and washing steps. In experiments to establish protocol, washing was continued until the radioactivity of the wash fluid reached background levels. The liposomes were removed from the centrifuge tubes by adding 100 μl TBS and vortexing to break up the pellet. This was done three times to generate a total volume of 300 μl. A portion of the liposome suspension (20 μl) was diluted 1:2 for phosphate assay. The remaining 280 μl was solubilized in 70 μl of 10% SDS. Portions of this fluid were taken for SDS–PAGE and for total protein assay.

2.4. Total protein assay

The quantity of protein eluted from the liposome surfaces was determined using the Bio-Rad DC Total Protein Assay. This is a colorimetric assay designed to determine protein concentration following detergent solubilization. Solutions of purified albumin (Behring Diagnostics) ranging in concentration from 0.1 to 1.0 mg/ml in 2% SDS, were used to generate a standard curve. Standards, as well as the protein samples eluted from the liposomes, were as-

sayed in a microtiter plate format in triplicate. Absorbance was measured at 690 nm using a microtiter plate reader.

2.5. SDS–PAGE and Western blotting

SDS–PAGE (4% stacking gel, 12% separating gel, reducing conditions) and Western blotting were used to identify proteins bound to the liposomes after plasma exposure as described previously [22].

3. Results and discussion

3.1. Liposome preparation

Neutral and negatively charged liposomes were prepared with saturated C_{14} lipids and with varying loadings and molecular weights of PEG. The compositions are summarized in Table 1. The neutral liposomes were formulated with 4 mol% DMPG to prevent agglomeration. All preparations included cholesterol at a 70:30 phospholipid/cholesterol mol ratio. PEGs of molecular weight 550, 2000, and 5000 were incorporated into the liposomes at loadings in the range of 0–15 mol% of the total phospholipid. Liposomes were prepared in a sucrose solution to increase density and thus facilitate pelleting. It was assumed that no leakage of encapsulated sucrose occurred since phospholipid bilayer vesicles have been shown to be impermeable to sucrose [23]. Liposomes of consistent size were prepared by freeze–thaw and extrusion followed by chromatography. Over all preparations, the average size of liposomes prepared using 0.1 μm extrusion membranes was 134 ± 15 nm at an average concentration of 1.77 ± 0.63 mg/ml. The size distribution was relatively constant from preparation to preparation, whereas there was some variability in concentration.

3.2. Adsorption from fibrinogen solution

Fibrinogen was studied in this work because it is an abundant cell-adhesive plasma protein and an important coagulation factor [24]. It circulates in plasma at a concentration of approximately 3 mg/ml. It is a dimeric structure each half of which consists of three polypeptide chains of mass 66, 52, and 46.5 kDa. It has the form of a tri-domainal 'dumbbell' 45 nm in length and about 9 nm in diameter [25]. Fibrinogen has a dual role in thrombosis and hemostasis: it is required for platelet aggregation, and polymerizes to from fibrin under the influence of thrombin. It has been shown that on many surfaces in contact with blood, fibrinogen is adsorbed rapidly and then displaced by less abundant proteins of higher affinity [26]. Fibrinogen adsorption to phospholipid surfaces has been studied in detail [27], but adsorption to PEG liposomes has not been investigated.

The experimental procedure consisted of five stages: liposome isolation, incubation in fibrinogen solution, separation of liposomes from solution by centrifugation, pellet removal from centrifuge tubes, and measurement of radioactivity associated with liposome pellets and centrifuge tubes. During the liposome isolation step, the effect of the initial centrifugation and resuspension on the liposomes was of concern. For example, it seemed possible that the pelleting process could cause the liposomes to aggregate in a random manner, thus causing variability in the surface area available for adsorption. This possi-

Table 1
Lipid compositions

Liposome type	Lipid components	Molar composition (%)	PE-mPEG loading (mol% of total phospholipid)
Neutral	PC:PG:PE:Ch	69:4:4:23	0
Neutral PEG (2000 mol. wt.)	PC:PG:PE-mPEG:Ch	69:4:4:23	5
Negative	PA:PE:Ch	73:4:23	0
Negative PEG (550, 2000, 5000 mol. wt.)	PA:PE-mPEG:Ch	75:2:23	2
		73:4:23	5
		69:8:23	10
		65:12:23	15

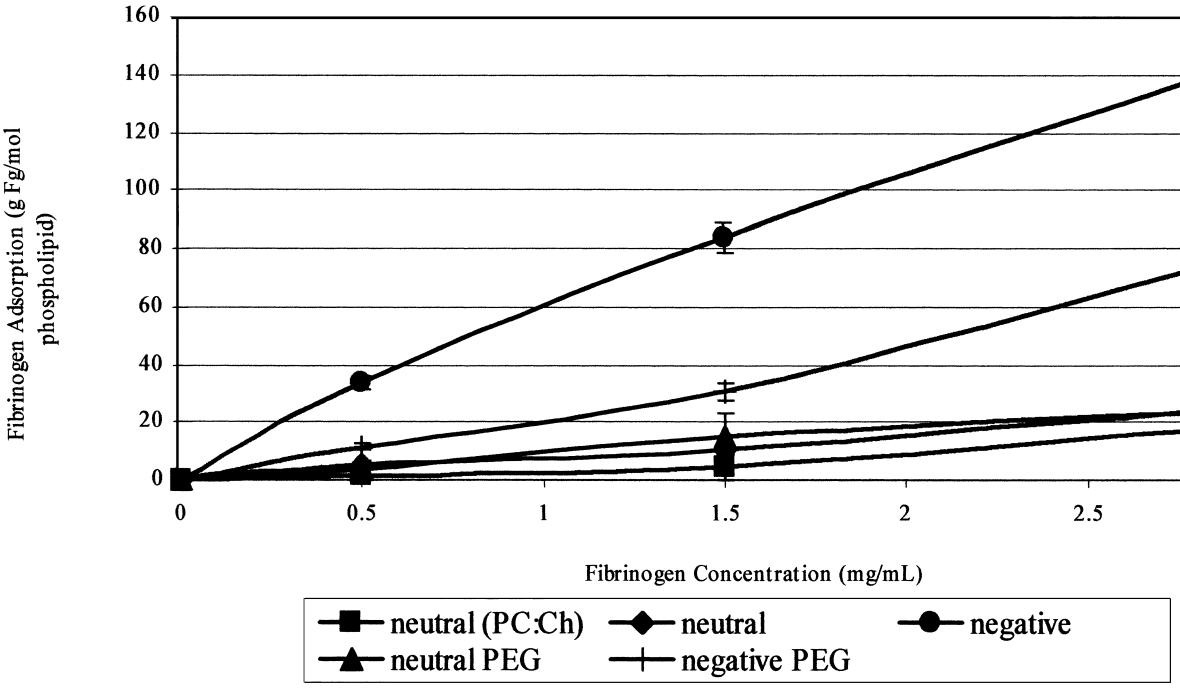

Fig. 1. Fibrinogen adsorption to neutral and negative liposomes. Each curve represents a single experiment with replicate measurements at each concentration. PEG 2000 is included at 5 mol% of total phospholipid in the PEG liposomes. Error bars = ±1 S.D., $n = 3$.

bility was checked by measuring the size distribution of the liposomes before and after centrifugation/resuspension. It was found that the distribution remained constant (data not shown). Pellet removal from the centrifuge tubes was necessary prior to radioactivity measurement because significant radioactivity was associated with the walls of the centrifuge tubes.

The effect of PEG molecular weight, PEG loading, and surface charge were investigated. The adsorption isotherms for neutral and negatively charged liposomes are shown in Fig. 1. Following previous practice in reporting this kind of data [4] adsorption is shown as mass of protein per mol of lipid (measured by phosphate assay). Adsorption increases monotonically, with no indication of saturation over the fibrinogen concentration range investigated. Using data on the area per mol of phospholipid in a bilayer, it can be estimated that surface concentrations range from about 0.01 (neutral) to 0.1 μg/cm^2 (negative). Monolayer adsorption of fibrinogen, expected to range from 0.14 to 1.8 μg/cm^2 depending on orientation [28], is thus not reached up to a concentration of 3 mg/ml, where typical biomaterial surfaces, eg polymeric materials, adsorb dense monolayers of the order of 1 μg/cm^2. These observations support the point of view that phospholipids are somewhat 'resistant' to protein adsorption [29]. The low adsorbed amounts and lack of a plateau at relatively high concentrations indicate low binding affinity.

It is seen in Fig. 1 that there is no significant difference between neutral and PEG-modified neutral liposomes. Any decrease could probably not have been measured since adsorption to neutral liposomes was already very low (approximately 1% of a typical monolayer). Fig. 1 also shows that the neutral, and neutral-PEG liposomes adsorb less than the negative or negative-PEG liposomes. It is of note in this connection that strongly negatively charged liposomes are rapidly eliminated from the circulation [1,6,30], and that such liposomes have shown increased uptake by macrophage-like cells [31]. The present data demonstrate that fibrinogen adsorption is strongly influenced by electrostatic interactions. Adsorption is presumably enhanced by interactions of the positive domains in the protein with the negative liposomes. Our results are in agreement with those from a study of adsorption to spread lipid bilayers re-

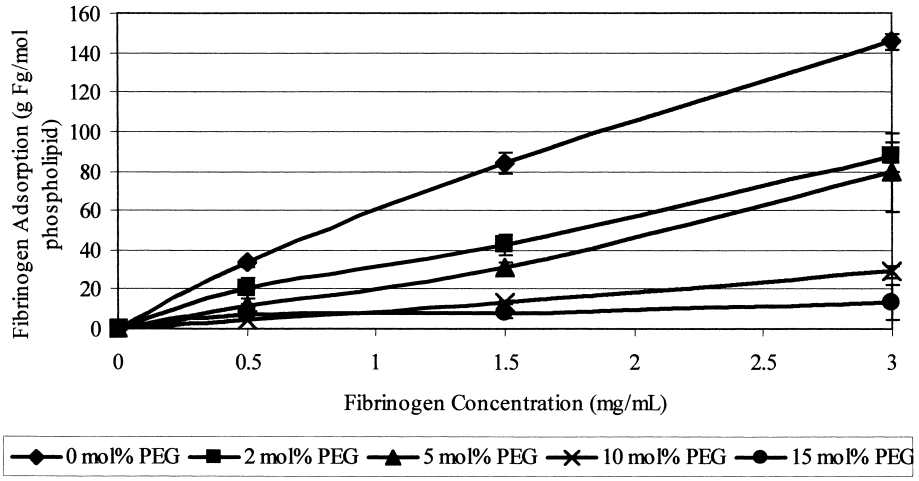

Fig. 2. Effect of PEG loading on the adsorption of fibrinogen to negatively charged liposomes (PA:PE-mPEG 2000:Ch). Each curve represents a single experiment with replicates at each concentration. Error bars = ± 1 S.D., $n = 3$.

ported by Malmsten [27]. Fibrinogen adsorption was found to be 100-fold greater on negative (PA) than on neutral (PC) phospholipids. Adsorption of IgG and albumin to negative surfaces was also found to be high.

From the data in Fig. 1, it is clear that there would have been little point in studying the effect of PEG molecular weight and loading on adsorption to neutral liposomes. Therefore these studies were done only for the negative liposomes. As seen in Fig. 2, adsorption decreases as PEG (molecular weight 2000) loading increases. As little as 2 mol% PEG reduced fibrinogen adsorption significantly. This result is in agreement with data from other types of studies. Calcium induced fusion and hence close approach of PS:PEG 2000 liposomes was shown to be inhibited by only 2 mol% PEG [32]. Also, Du et al. [18] reported that a significant reduction in protein adsorption to lipid bilayers occurred at low PEG loadings (less than 1 mol% DSPE-PEG 5000). Maruyama et al. [12] also found that neutral liposomes containing only 3 mol% of DSPE-PEG 2000 showed increased residence times in the circulation.

From Fig. 2 it is also seen that at loadings of 10–15 mol% PEG, adsorption levels on the negative liposomes were reduced to those on the neutral or

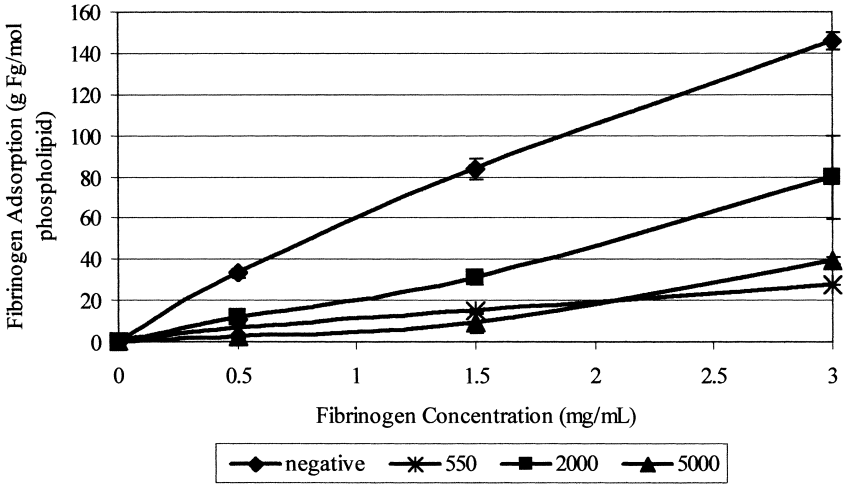

Fig. 3. Effect of PEG molecular weight on the adsorption of fibrinogen to negatively charged PEG liposomes (PEG loadings at 5 mol% of total phospholipid). PEG molecular weights of 550, 2000 and 5000 were studied. Each curve represents a single experiment with replicates at each concentration. Error bars = ± 1 S.D., $n = 3$.

Table 2
Liposome pelleting efficiencies (average of two samples) in experiments to measure protein adsorption from 10% plasma

Liposome type	Pelleting efficiency (%)
Neutral	29.5
Neutral PEG	6
Negative	92.5
Negative PEG	64

The initial lipid mass per sample was 1.5 mg.

neutral PEG liposomes (Fig. 1). This suggests that at this level, where the polymer chains are expected to be in a brush, i.e., fully extended, configuration [33], PEG 2000 is capable of completely shielding the negative charge of phosphatidic acid and eliminating electrostatic interactions.

Data on the effect of PEG molecular weight on fibrinogen adsorption are shown in Fig. 3. A significant reduction in fibrinogen adsorption occurs for all PEG molecular weights compared to the unmodified negative liposomes. Molecular weights of 550 and 5000 showed the lowest adsorption. The strong effect of PEG 550 was unexpected, since PEG in this molecular weight range has been found to be relatively ineffective in reducing protein adsorption on other surfaces [34,35]. In addition, a minimum molecular weight of 750 has been found to be necessary to increase the lifetime of liposomes in the circulation [9,12,13].

3.3. Adsorption from plasma

The plasma adsorption protocol involved extensive washing of the liposomes which resulted in low pelleting efficiencies. Table 2 shows that PEG modification of both neutral and negative liposomes caused the pelleting efficiency to decrease. This is most likely a result of the steric repulsion effect of PEG and the enhanced stability of PEG liposomes. The negative liposomes (both unmodified and PEG-modified) also demonstrated significantly higher pelleting efficiencies than the neutral liposomes.

3.3.1. Total protein adsorption

Typical data are shown in Table 3. The adsorption levels on the neutral and negative liposomes are of a similar order of magnitude. For the negative liposomes, there appears to be little effect of PEG modification, or if anything a slight increase in adsorption. For the neutral liposomes it appears that PEG modification causes an increase in adsorption, i.e., the opposite of the expected effect. It is possible that the low pelleting efficiencies of the PEG liposomes, resulting in relatively small samples, lead to less accurate values for phospholipid concentration as assessed by phosphate assay. The data certainly do not indicate a decrease in adsorption as a result of PEG modification.

The adsorbed quantities are mostly in the region of 300–400 g protein/mol phospholipid. Compared to the amounts adsorbed in the fibrinogen experiments, these are considerably higher. The differences may be due in part to the higher protein concentrations in the plasma (of the order of 5 mg/ml in 10% plasma versus 3 mg/ml in the highest concentration fibrinogen solution). However, the adsorption levels are still rather small relative to full monolayer coverage, again suggesting the low affinity of these surfaces for protein binding.

The present numerical adsorption data may be compared to those of Oja et al. [4] and Semple et al. [5] from in vivo experiments in which liposomes were injected into mice. These authors reported levels up to 80 g protein/mol of lipid, i.e., a factor of 4 or 5 lower. In addition the adsorbed amounts were found to be greater on negative than on neutral liposomes.

3.3.2. SDS–PAGE

To examine the specific interactions of a range of proteins with the liposomes, adsorption from plasma was studied using SDS–PAGE and Western blotting. For the gels it should be noted that loading on each lane was on an equal volume basis, and since total protein adsorption was essentially the same on the

Table 3
Typical data on total protein adsorption to liposomes after exposure to plasma for 2 h

Liposome type	Protein adsorption (g protein per mol phospholipid, mean ± S.D., $n = 6$)
Neutral	405 ± 40
Neutral PEG	1046 ± 57
Negative	305 ± 25
Negative PEG	364 ± 29

Plasma was diluted to 10% normal strength with TBS.

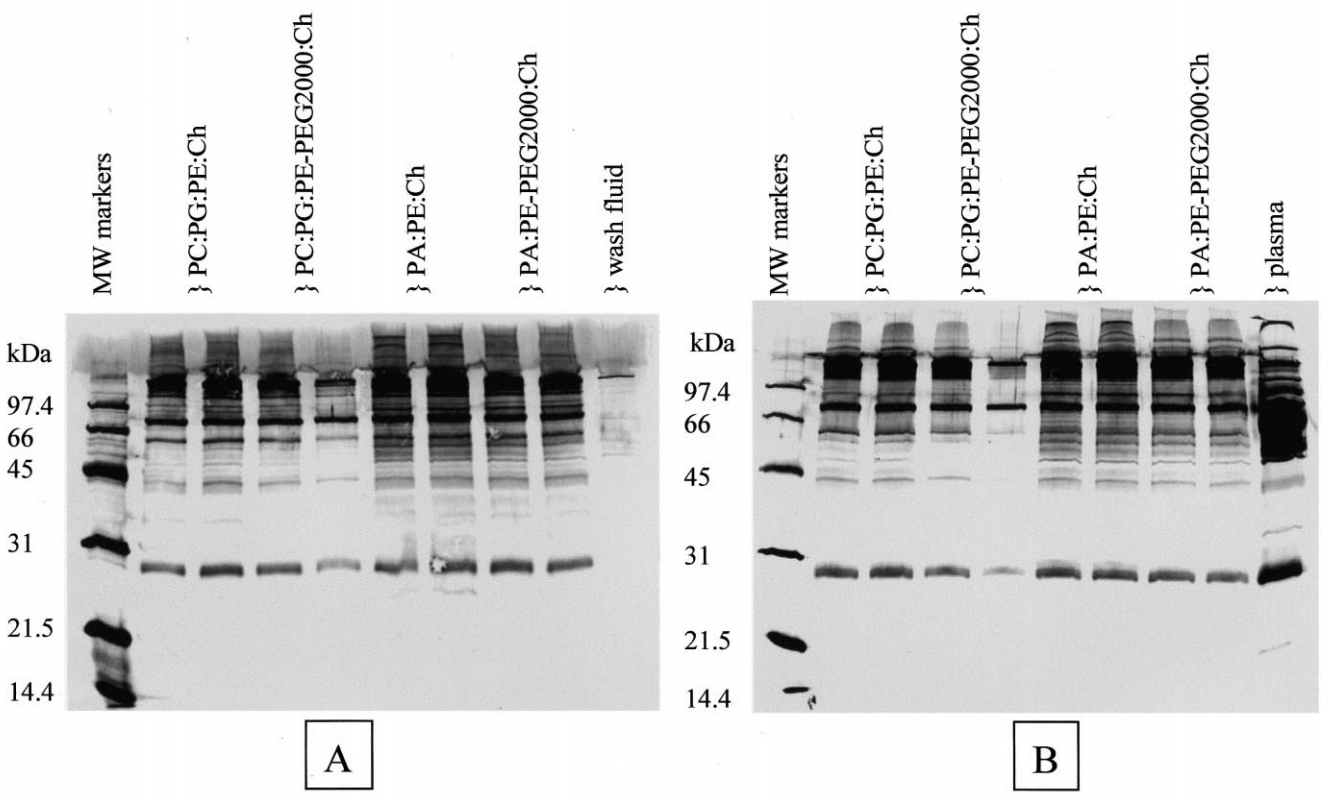

Fig. 4. Reduced SDS–PAGE data for plasma exposed liposomes. (A) One μl of sample was loaded onto each lane. In lane 10, 15 μl of the final wash fluid was loaded. (B) Replicate of A. One μl of sample was loaded onto each lane. In lane 10, 5 μl of 1:100 diluted plasma was loaded.

different types, the mass of total protein loaded was also similar.

Fig. 4 shows gels of the proteins adsorbed to liposomes from 10% plasma. The lane corresponding to the final wash fluid in Fig. 4A, shows essentially no protein except for a faint band at high molecular weight (estimated at about 130 kDa). This observation confirms that the wash protocol reduced unbound protein to an insignificant level. Clearly a substantial amount of protein is associated with the liposomes after plasma contact, indicating that significant nonspecific adsorption occurred. However, no significant differences in the extent or pattern of protein binding among the different liposome types is discernable. This result is in contrast to data reported by Oja et al. [4], showing that the gel patterns of proteins adsorbed to negative and neutral liposomes are very different. These were in vivo experiments in a mouse model.

It can be seen by comparing the liposome gels to the gel of the plasma (rightmost lane of Fig. 4B), that the liposome surfaces cause partitioning, with enrichment of some proteins and impoverishment of others relative to the plasma. All the liposome types appear to adsorb a number of proteins with bands in the high molecular weight range above 97 kDa. Adsorption of high molecular weight proteins has been seen by others [36,37]. A band at about 75 kDa is more intense in the liposome than in the plasma gels. Also the absence of bands in the range of 66–45 kDa, prominent in plasma, and due at least in part to albumin, should be noted. This is surprising since albumin has been shown to bind extensively to liposomes [36,37].

3.3.3. *Western blotting*

The immunoblot data are shown in Figs. 5–9. Table 4 indicates the proteins corresponding to the lane numbers in these figures. It should be noted that the bands cannot be compared quantitatively among proteins within a given blot because of differences in the responses of the staining reactions. Also, it is

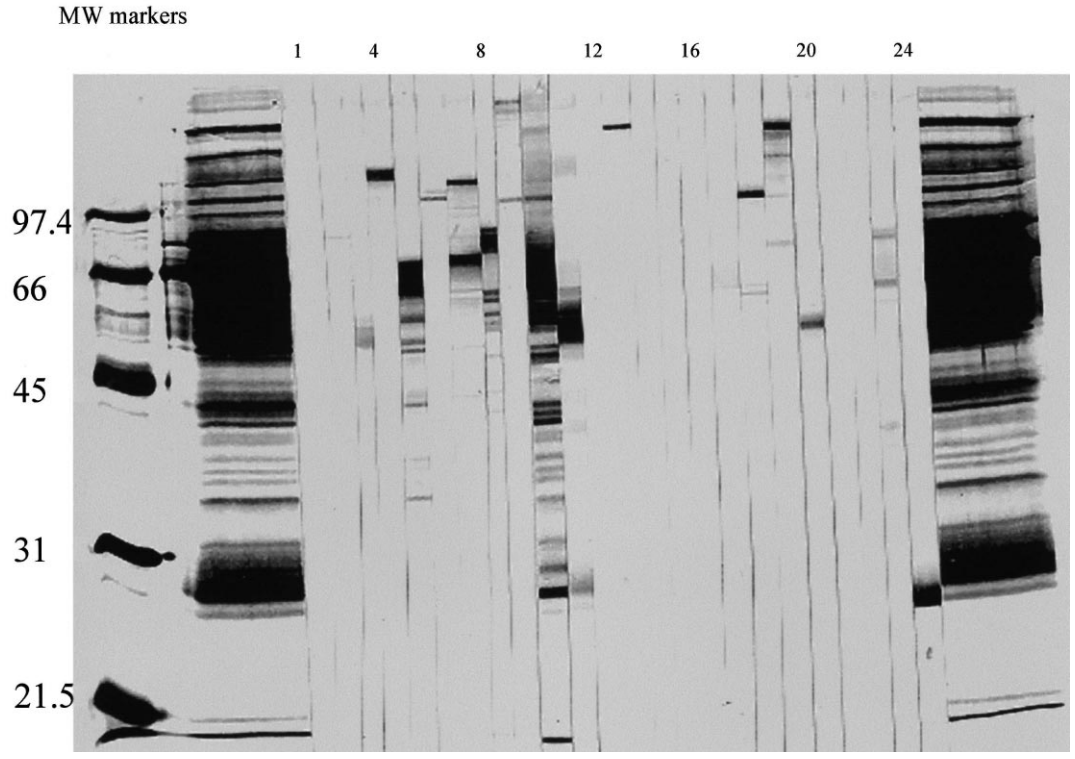

Fig. 5. Western blot of plasma using antibodies to 24 plasma proteins. Five μl of 1:10 diluted plasma was loaded along with 95 μl tracking dye. Gels are shown to the immediate right and left of the blot lanes. See Table 4 for explanation of lane numbers.

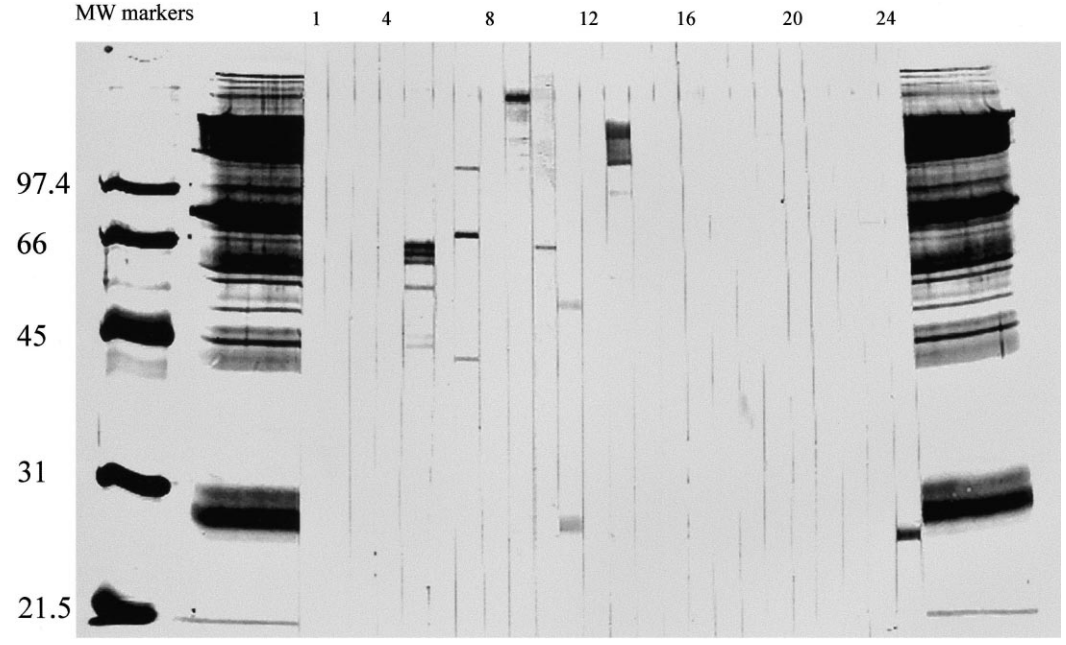

Fig. 6. Western blot of plasma proteins adsorbed to neutral liposomes at room temperature. Adsorption time, 2 h in 10% plasma. Gels are shown to the immediate right and left of the blot lanes.

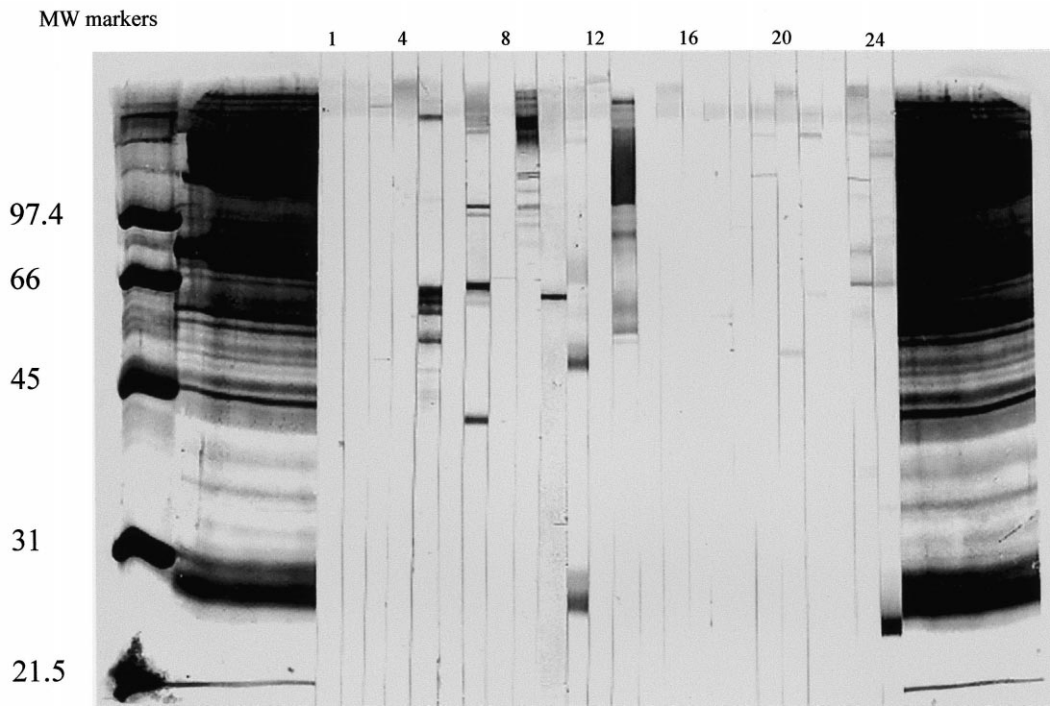

Fig. 7. Western blot of plasma proteins adsorbed to neutral PEG liposomes at room temperature. Adsorption time, 2 h in 10% plasma. Gels are shown to the immediate right and left of the blot lanes.

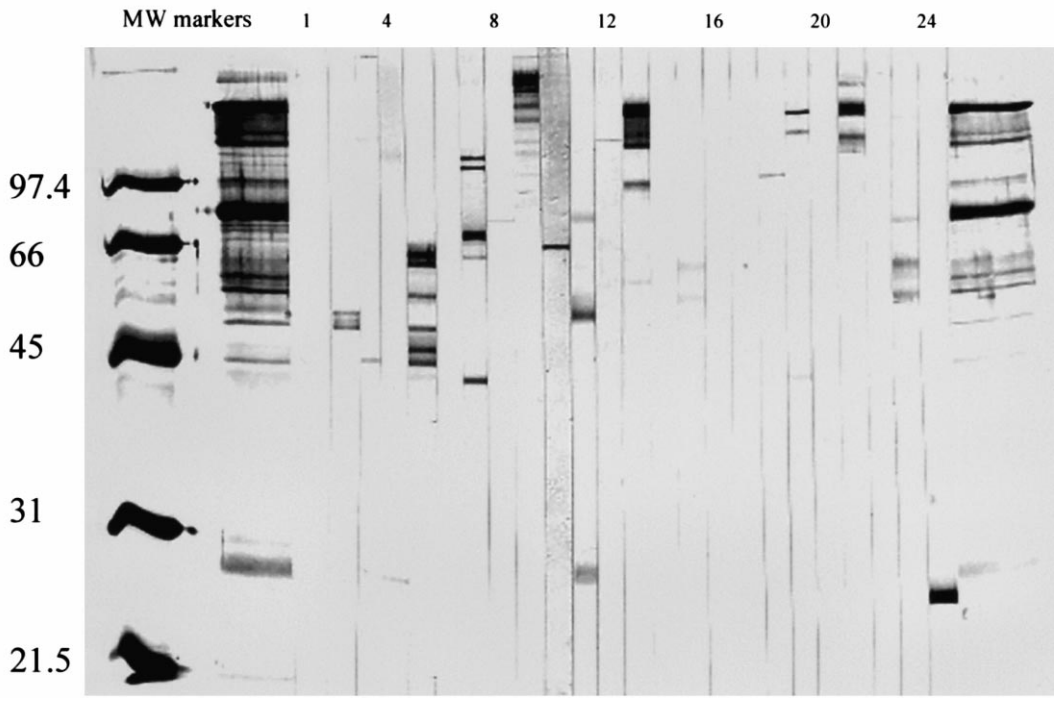

Fig. 8. Western blot of plasma proteins adsorbed to negative liposomes at room temperature. Adsorption time, 2 h in 10% plasma. Gels are shown to the immediate right and left of the blot lanes.

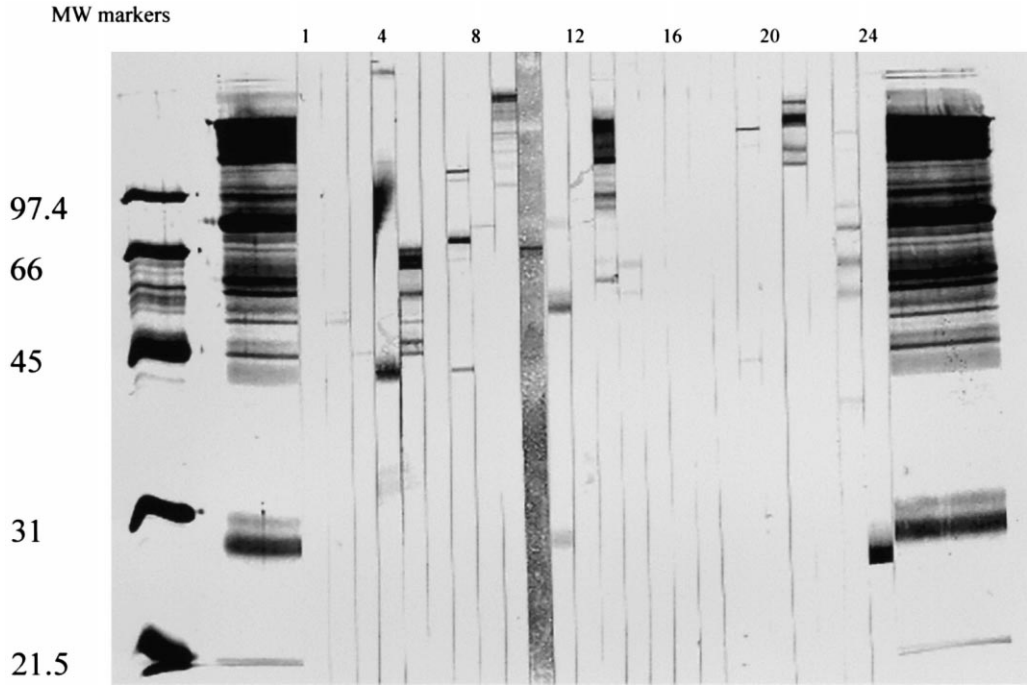

Fig. 9. Western blot of plasma proteins adsorbed to negative PEG liposomes at room temperature. Adsorption time, 2 h in 10% plasma. Gels are shown to the immediate right and left of the blot lanes.

not valid to attribute quantitative significance to band intensity differences among the different liposome types. However, a number of qualitative differences are clear.

Only weak responses were seen for the coagulation contact activation factors (factor XI, factor XII, prekallikrein, and HMWK) in the plasma blot, reflecting their low concentrations. Factor XII was evident on the negative and negative PEG liposomes, as expected based on the known affinity of this protein for negatively charged surfaces [38]. On the other hand HMWK, also a highly surface active protein, appeared to be depleted on the liposomes relative to the plasma.

Other proteins associated with coagulation (factor V, fibrinogen, protein S, protein C, and plasminogen) showed varying adsorption tendencies. Protein C, and protein S were either undetectable or were present in small amounts. The plasminogen blot in the plasma sample showed bands at molecular weights of 110 and 106 kDa. Although these bands are slightly higher than the reported values, they most likely correspond to the α and β forms of intact plasminogen. This protein was not detected in any of the liposome blots. Although fibrinogen was clearly adsorbed to the liposomes, it appeared to be depleted relative to the plasma, and showed evidence of degradation (bands in the 30 kDa range). Factor V appeared to be present in greater amounts on the negative and negative PEG liposomes compared to the neutral liposomes. It was also enriched on the negative liposomes compared to plasma.

The complement proteins investigated included complement C3, factor B, factor H, and factor I. C3 interactions with liposomes have been studied by others [1,3,39]. Liposomes can in principle activate complement by either the classical or the alternative pathway. Negatively charged liposomes have been shown to activate the classical pathway in the absence of immunoglobulins, which are normally required for classical pathway activation [40]. Based on the generally accepted mechanism for the protective effect of PEG, a reduction in C3 would be expected on the PEG modified liposomes. As seen in the immunoblots, this was not the case: there were no differences among the liposome types. However, the C3 blots of the plasma and liposome samples were quite distinct. C3 was largely intact in the plasma sample

with bands at 115 and 72 kDa corresponding to the α and β chains, whereas evidence of complement activation in the liposome samples was indicated by an additional band at 42 kDa, most likely an α-chain fragment. The other complement proteins investigated, i.e., factors B, H, and I were all depleted relative to plasma. This finding argues against significant complement activation since factor B is known to bind to C3 when activation occurs [41] so that factor B would be expected to be enriched on the liposome surface.

The cell-adhesive proteins fibronectin and vitronectin were also investigated. Fibronectin is known to bind to plasma components such as IgG and C3, and has been shown to adsorb to liposomes [1]. It is considered to be a nonimmune plasma opsonin. As seen in the immunoblots, fibronectin was found to bind significantly to all liposome types. The blots for vitronectin showed strong bands for the negative and negative-PEG liposomes.

The albumin blots of all the liposome types showed that this protein was strongly depleted relative to plasma. In the plasma sample (Fig. 5), numerous bands were present at molecular weights higher and lower than that of the intact molecule, indicating cleaved, dimeric and other complexed forms. In the liposome blots albumin appears as a single band of modest intensity at the expected molecular weight of 66 kDa. Low adsorption of albumin was unexpected since in previous work, we found that albumin binding to DPPC–cholesterol liposomes was extensive [22]. Reports in the literature are mixed, in some cases showing significant albumin binding [2,16, 18,36,37], and in others only minimal interactions [42,43]. Low adsorption may be due to replacement of initially adsorbed albumin by proteins of higher affinity, in accordance with observations on phospholipid surfaces by Malmsten [44].

IgG is a known opsonin which has been reported to bind to liposomes and phospholipid surfaces [27,45]. The data presented here suggest that IgG binding to all liposome types was minimal. However, we have observed that IgG stains only lightly in this type of blot, so its concentration may be underestimated.

Other proteins investigated included α2-macroglobulin, apolipoprotein AI, transferrin, β-lipoprotein, β2-microglobulin, and hemoglobin. α2-Macroglobulin is a nonspecific serum protease inhibitor which regulates enzyme activity in the coagulation, fibrinolytic, and complement systems. It is prominent in all the liposome blots and is strongly enhanced in the liposomes compared to the plasma. A single band of mass greater than 120 kDa, presumably due to the intact protein, was seen on the plasma blot, whereas strong bands in the range from greater than 120 to 57 kDa, presumably due to degradation products, were seen in all liposome samples. This result is in contrast to previous data from our laboratory which showed that very little α2-macroglobulin was bound to DPPC-cholesterol liposomes modified with *N*-substituted polyacrylamides [22]. α2-Macroglobulin has not received much attention in previous liposome studies, although Black and Gregoriadis [46] found that it is adsorbed to various charged liposome preparations. It has also been identified as an opsonic factor [47], and a modified form was shown to be the main protein which promotes liposome-related macrophage activation [48].

The binding of apolipoprotein AI is of interest

Table 4
List of proteins probed for in each lane of the immunoblots

Lane	Protein
1	Factor XI
2	Factor XII
3	Prekallikrein
4	HMWK
5	Fibrinogen
6	Plasminogen
7	C3
8	Transferrin
9	Fibronectin
10	Albumin
11	IgG
12	β-Lipoprotein
13	α2-Macroglobulin
14	Vitronectin
15	Protein C
16	β2-Microglobulin
17	Hemoglobin
18	Factor B
19	Factor H
20	Factor I
21	Factor V
22	Thrombin
23	Protein S
24	Apolipoprotein AI

because this protein plays an important role in the destabilization of lipid vesicles in the blood. Adsorption of this protein has been observed on liposomes having a range of compositions [22,42,43]. As can be seen in Figs. 6–9, it was adsorbed extensively to all the liposome types, including the PEG modified liposomes, giving one of the strongest responses at a molecular weight of approximately 27 kDa.

A marked difference in the transferrin blots was observed for the plasma and liposome samples. Transferrin was seen in intact and cleaved forms in plasma (80–42 kDa), whereas in the liposome samples it was barely detectable with a very weak band at the expected molecular weight of about 80 kDa.

β-Lipoprotein, β2-microglobulin, and hemoglobin were undetectable in both the plasma and liposome samples.

The intense band at 75 kDa seen on the gels of the liposome samples (Fig. 4) could not be attributed to any of the proteins investigated in the blotting experiments. It is unlikely that it represents transferrin given the very weak response of this protein for all liposome types. Additionally, the possibility that it could be attributed to the β chain of C3 does not hold up since this component was seen at a mass of about 68 kDa.

Essentially no differences were observed between the negatively charged and neutral liposomes. The similarity in the blots of the liposome types, independent of their overall charge, is in contrast to the fibrinogen adsorption data which showed that adsorption to neutral and neutral-PEG liposomes was lower than to negative and negative-PEG liposomes.

Perhaps most significantly, no differences were seen between the unmodified and PEG modified liposomes, whether neutral or negative. It could be concluded that on neutral liposomes, the barrier provided by PEG of molecular weight 2000 at a loading of 5 mol% of the total phospholipid is not capable of reducing protein binding in contact with blood. However, in vivo studies have shown prolonged circulation of such liposomes [11,49]. Thus it may be that the prolonged lifetimes of PEG liposomes in the circulation are not directly related to reduced protein adsorption. Other possible mechanisms are inhibition of lipases or direct steric repulsion between PEG-liposomes and macrophages [17].

4. Conclusions

The aim of this study was to investigate the effects of PEG modification and surface charge of liposomes on protein adsorption to the liposome surface. The data on fibrinogen adsorption from buffer to negatively charged liposomes support the widely held view that PEG provides a steric barrier which reduces protein adsorption. However, no such effect of PEG was seen for neutral liposomes. In addition, there was no evidence that adsorption of proteins from plasma, as evaluated by total protein assay and gel-immunoblotting methods, was reduced by PEG modification. The results of this study suggest that mechanisms other than repulsion of opsonizing proteins may be responsible for the prolonged lifetime of PEG modified liposomes in the circulation. One can only speculate as to other possible mechanisms. It may be that PEG acts directly as a steric barrier in the interaction of the liposomes with macrophages. Clearly, further investigation will be required to clarify the mechanisms involved.

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