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Modification of liposomes with *N*-substituted polyacrylamides: identification of proteins adsorbed from plasma

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Abstract

Liposomes prepared from DMPC (80%) and cholesterol (20%) were modified with a series of hydrophobically modified *N*-substituted polyacrylamides, namely, poly[*N*-isopropylacrylamide] (PNIPAM), poly[*N,N*-bis(2-methoxyethyl) acrylamide] (PMEAM), and poly[(3-methoxypropyl)acrylamide] (PMPAM). The hydrophobic group, *N*-[4-(1-pyrenylbutyl)-*N*-*n*-octadecylamine was attached to one end of the polymer chains to serve as an anchor for incorporation into the liposome bilayer. Liposome–polymer interactions were confirmed using fluorescence spectroscopy and chemical analysis. Microscopy revealed differences in aggregation tendency between unmodified and polymer-modified liposomes. Proteins adsorbed to liposome surfaces during exposure to human plasma were identified by immunoblot analysis. It was found that both unmodified and polymer-modified liposomes adsorb a wide variety of plasma proteins. Contact phase coagulation proteins, complement proteins, cell-adhesive proteins, serine protease inhibitors, plasminogen, antithrombin III, prothrombin, transferrin, α_2-microglobulin, hemoglobin, haptoglobin and β-lipoprotein as well as the major plasma proteins were all detected. Some differences were found between the unmodified and polymer-modified liposomes. The unmodified liposomes adsorbed plasminogen mainly as the intact protein, whereas on the modified liposomes plasminogen was present in degraded form. Also, the liposomes modified with PNIPAM in its extended conformation (below the lower critical solution temperature) appeared to adsorb less protein than those containing the 'collapsed' form of PNIPAM (above the LCST).

Keywords: Plasma protein; Adsorption; Liposome; Immunoblot; Amphiphilic polymer; Fluorescence

1. Introduction

The tendency of liposomes to be eliminated rapidly from the blood circulation is a major limitation on their use as drug delivery vehicles. Liposomes tend to be taken up by monophagocytes in various tissues of the reticuloendothelial system (RES) [1]. The mechanisms responsible for the recognition and clearance of liposomes are not well understood. Clearance is thought to be related to the binding of opsonins which consequently promote phagocytosis [2–4]. An understanding of the interactions of blood components with liposome surfaces is therefore needed to develop liposomes with improved blood compatibility and longer circulation lifetimes. Several investigators have attempted to identify the plasma proteins that adsorb to liposome surfaces [5–8]. Among the

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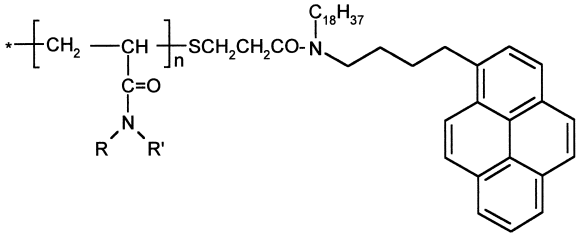
proteins believed to bind to liposomes are IgG [6], albumin [5–7], α_2-macroglobulin [6,7], apolipoprotein A-1 [6,7], fibrinogen [7], α_1-antitrypsin [7] and fibronectin [2]. Most of these studies have relied on gel electrophoresis for characterization of adsorbed proteins. Thus although previous work has shown that liposomes do adsorb plasma proteins, there is no information on the identities and relative amounts of the various proteins. There is also no definitive demonstration of the involvement of these adsorbed proteins in liposome clearance. In addition, there have been very few attempts to correlate protein binding with lipid composition. Chonn et al. [8] have attempted to study the relationship between the circulation lifetimes of liposomes and the association of blood proteins. They observed that liposomes which bound greater amounts of protein had shorter circulation half-lives. Liu and coworkers investigated the role of complement components in the uptake of negatively-charged liposomes by liver cells [9], and concluded that both recognition and uptake of liposomes are determined by adsorption of complement proteins.

Modification of liposome surfaces with hydrophilic polymers such as poly(ethyleneglycol) (PEG) has been shown to increase their circulation times up to 10-fold [10,11]. Important properties of protective polymers such as PEG are their solubility in water [12], hydrophilicity, biocompatibility, osmotic and entropic interactions [13] and chain flexibility [14–16]. Poly(*N*-alkyl acrylamides) [14,17–19] have also been recognized as promising candidates for liposome protection [19]. In the present work, liposome surfaces were modified with three different water soluble hydrophobically-modified polyacrylamides: poly[*N*-isopropylacrylamide] (PNIPAM), poly[*N,N*-bis(2-methoxyethyl)acrylamide] (PNMEAM) and poly[(*N*-3-methoxypropyl)acrylamide] (PMPAM). All the polymers were labeled with pyrene, a fluorescent dye, attached to one end, in close proximity to an octadecyl chain (Fig. 1). Both the pyrene and octadecyl groups act as anchors for the polymer in the liposome membrane. Aqueous solutions of these polymers exhibit lower critical solution temperatures (LCST) [20] above which the individual macromolecular chains collapse on themselves, and aggregate, causing the polymer to precipitate. The LCSTs for PNIPAM, PNMEAM and PMPAM are reported to be 32°C, 42°C and 44°C, respectively [21]. Liposomes modified with each of these polyacrylamides were prepared and their protein adsorption behavior in plasma was compared to that of the unmodified liposomes. Protein interactions were investigated by elution of adsorbed proteins and analysis by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)/immunoblotting methods.



PNIPAM: R=H, R'=$CH(CH_3)_2$

PNMEAM: R=$(CH_2)_2$-O-CH_3, R'=$(CH_2)_2$-O-CH_3

PMPAM: R=H, R'=$(CH_2)_3$-O-CH_3

Fig. 1. Chemical structure and composition of the polymers used for liposome modification.

2. Materials and methods

2.1. Materials

Pyrene-labeled, hydrophobically-modified poly[*N*-isopropylacrylamide] (PNIPAM, $M_n = 2700$), poly[*N,N*-bis(2-methoxyethyl) acrylamide] (PMEAM, $M_n = 2400$) and poly[(3-methoxypropyl)acrylamide] (PMPAM, $M_n = 2500$) samples were prepared as described previously [22]. 1,2-Dimyristoyl-*sn*-glycero-3-phosphatidylcholine (DMPC) was purchased from Avanti Polar Lipids, and purity was confirmed by thin-layer chromatography ($CHCl_3$/MeOH/H_2O 65:25:4, v/v, development with iodine vapor). Cholesterol (>99%) was obtained from Sigma. Pooled human platelet-poor plasma was prepared from citrated blood from four healthy donors (courtesy Canadian Red Cross). The plasma was stored at −70°C until required. 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT) were obtained from Bio-Rad. Goat antisera to human factor XI, factor XII, antithrombin III (ATIII), albumin, and high-molecular-weight kininogen (HMWK), and sheep antisera to human prekallikrein

and prothrombin were from Cedarlane Laboratories (Hornby, ON, Canada). Goat antisera to human transferrin, α_2-macroglobulin, β-lipoprotein, IgG, plasminogen, and fibrinogen, and rabbit antisera to human β_2-microglobulin, hemoglobin, haptoglobin and complement C3c were from Sigma. Goat antisera to human complement C3, factor B, factor H and factor I, as well as rabbit antisera to human fibronectin, protein C and vitronectin were from Calbiochem Corp. (La Jolla, CA, USA). Goat antiserum to human α_1-antitrypsin was obtained from Enzyme Research Laboratories (South Bend, IN, USA). Affinity-purified goat anti-rabbit IgG-alkaline phosphatase conjugate was from Bio-Rad, affinity-purified rabbit anti-goat IgG-alkaline phosphatase conjugate was from Sigma, and rabbit anti-sheep IgG-alkaline phosphatase conjugate was from Bethyl Laboratories (Montgomery, TX, USA).

2.2. Instrumentation

Fluorescence spectra were recorded on a SPEX Fluorolog 212 spectrometer equipped with a DM3000F data system. The temperature of the water-jacketed cell holder was controlled with a Neslab circulating bath. The temperature of the sample fluid was measured with a thermocouple immersed in the sample. Emission spectra were recorded with an excitation wavelength of 346 nm. Excitation and emission slit widths were 2 mm for PNIPAM and PMPAM, and 1 mm for PNMEAM solutions and mixtures, unless otherwise indicated. The fluorescence of the pyrene-labeled poly(*N*-alkyl acrylamides) consists of two contributions: a broad featureless signal centered at 483 nm due to pyrene excimer (intensity I_e) and a well-resolved spectrum with the [0,0] band at 378.5 nm attributed to isolated excited pyrenes (monomer emission, intensity I_m). The ratio of excimer to monomer intensity was taken as the ratio of the intensity at 483 nm to half the sum of the intensities at 378.5 and 398 nm. Excimer emission requires that an excited pyrene (Py*) and a pyrene in the ground state come into close proximity within the Py* lifetime. The process is predominant in concentrated pyrene solutions or under circumstances where microdomains of high local pyrene concentration form, even though the macroscopic pyrene concentration is low. In aqueous solutions of the pyrene-labeled poly(alkyl acrylamides) the excimer emission is strong, indicating the formation of polymeric micelles, as described in detail elsewhere [22]. Light microscopy was performed on a Leitz Laborlux S microscope equipped with a Javelin UltrachipJ Hi Res CCTV camera. Images were captured using Confocal Assistant software version 3.10. Gel electrophoresis was performed using a Bio-Rad Mini-ProteanJ II dual slab cell, and a Bio-Rad Mini Transblot cell was used for electrophoretic transfer. Blots were scanned with a Howtek Scanmaster 3+, and processed using Biomage Whole Band Analyzer software (Millipore) operating on a Sparc Workstation.

2.3. Preparation of liposomes

Large unilamellar liposomes (LUVs) were prepared using the reverse-phase evaporation technique. DMPC (40 mg) and cholesterol (9.78 mg) were dissolved in an ether/chloroform mixture (1:1, v/v; 20 ml). Tris-buffered saline, pH 7.4 (2 ml) was rapidly added using a 5-ml syringe with a 22.5-gauge needle. The resulting mixture was sonicated for 2 min. In vacuo solvent removal at 37°C caused formation of a gel which eventually collapsed to give a milky suspension of LUVs. This suspension was dialyzed overnight, against Tris-buffered saline (membrane molecular mass cutoff: 6000–8000). Lipid concentrations were determined by phosphate assay [23]. Microscopy (1000× magnification) revealed predominantly unilamellar liposomes with a size distribution in the range of 3–10 μm. Generally, a drop of liposome suspension was placed on a glass slide, and a glass cover slip was placed on top. This preparation was immediately observed under 1000× magnification with white light (oil immersion).

2.4. Polymer–liposome interactions

Fluorescence emission spectra of polymer solutions in buffer (0.05 g l^{-1}) and liposome–polymer suspensions in buffer (lipid and polymer concentrations, 0.05 g l^{-1}) were taken at 25°C. Liposome–polymer mixtures were incubated for 24 h at 25°C before use. Emission spectra of liposome–polymer mixtures were measured at increasing temperatures (25–50°C). The polymer and lipid concentrations

were 0.01 and 0.05 g l^{-1}, respectively. The heating rate was 0.5°C per minute. The excitation and emission slit widths for all mixtures were 1 mm and 2 mm, respectively.

In order to determine a suitable level of polymer to insert into the lipid bilayer without destroying the liposomes, polymer–liposome suspensions were centrifuged and the distribution of polymer between the aqueous and lipid phases was determined [24,25]. Briefly, the polymer–liposome suspension was centrifuged and the distribution of polymer between aqueous and lipid phases was determined to establish if there is a preference for the polymers to incorporate into the liposome. Polymer-coated liposomes were also incubated in fresh buffer and centrifuged. The pellet and supernatant fractions were analyzed for the presence of polymer to determine whether the polymers can also desorb from the liposome surface.

2.5. Preparation of polymer-modified liposomes

To prepare liposomes for adsorption experiments, suspensions of LUVs (1 mg) were introduced into 0.5-ml Eppendorf tubes and the buffer was removed after centrifugation at $4000\times g$ for 10 min at 25°C. The liposome pellet was resuspended in one of the polymer solutions (0.5 ml, 1.1×10^{-4} M, 3.6×10^{-4} M, 3.1×10^{-4} M for PNIPAM, PNMEAM and PMPAM, respectively where M is mol l^{-1} of pyrene) and incubated overnight at 25°C. This suspension was centrifuged and the supernatant was carefully removed before use in adsorption experiments. All unmodified and polymer-coated liposomes were prepared from the same batch of LUVs.

2.6. Adsorption of plasma proteins

Liposomes (1 mg) were incubated with human plasma (0.5 ml, diluted to 25% in TBS) for 2 h at 37°C in 0.5-ml Eppendorf tubes. The liposomes were centrifuged and the plasma was removed. The liposome pellet was washed three times with fresh TBS and resuspended in 2% SDS (40 μl). The proteins were separated by polyacrylamide gel electrophoresis (4% stacking gel, 12% separating gel, reducing conditions with mercaptoethanol) and identified by immunoblotting [26]. Briefly, after gel electrophoresis, the proteins were electrophoretically transferred to an Immobilon PVDF membrane. The blots were cut into 3-mm strips and blocked with 5% nonfat dry milk. The strips were incubated with antisera to different proteins and then with the appropriate alkaline phosphatase-conjugated second antibody. 5-Bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium were used as a chromogenic substrate system for alkaline phosphatase.

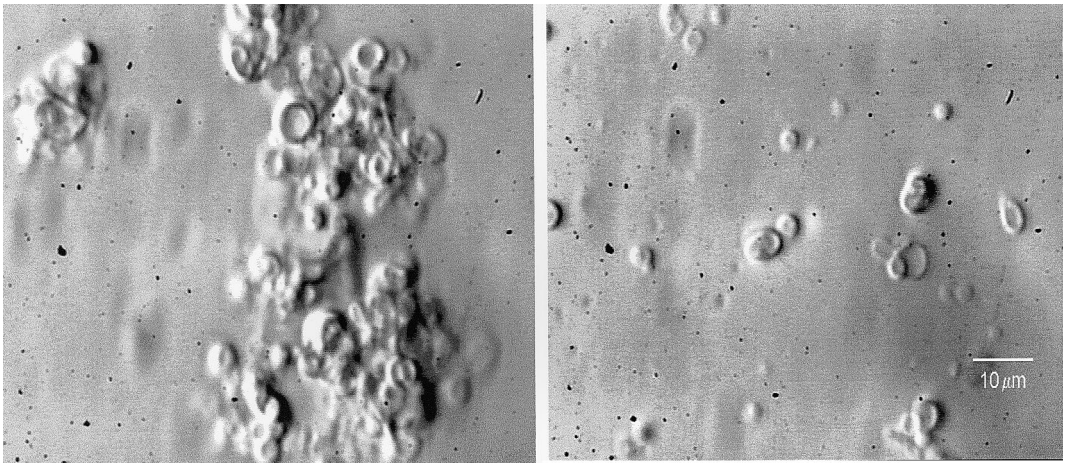


Fig. 2. Light microscopy images of: (left) unmodified, and (right) PNIPAM-modified LUVs in TBS. For the micrograph on the right, liposomes (concentration approximately 1×10^{6} μl^{-1}) were incubated with hydrophobically modified PNIPAM overnight.

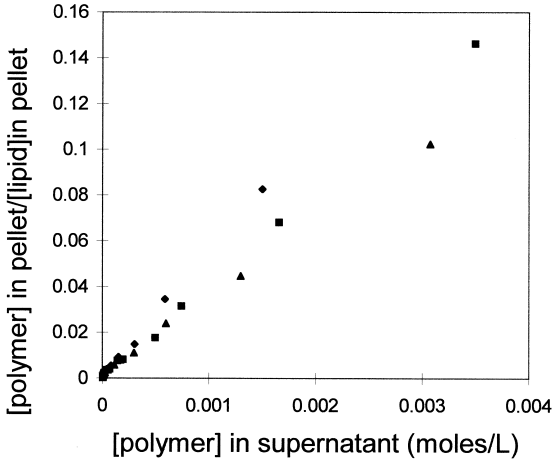


Fig. 3. Binding of hydrophobically-modified polymers to liposomes at 25°C. P_b, bound polymer; P_f, free polymer in solution; L, lipid. The slope indicates the binding constant, K ($K = [P_b]/([P_f][lipid])$). Polymer concentrations are corrected to represent polymer-C_{18}Py concentrations. (♦) PNIPAM–liposome mixture; (■) PNMEAM–liposome mixture; (▲) PMPAM–liposome mixture.

3. Results and discussion

3.1. Polymer-modified liposomes in water

The unmodified liposomes appeared microscopically as large aggregates whether in buffer, in albumin solution (10 g l^{-1}), or in plasma (Fig. 2). In contrast, the polymer-modified liposomes did not aggregate, indicating that the surface properties had been significantly altered. This stabilization effect was observed for all three polymers. Similar behavior has been reported for polyethylene oxide (PEO)-modified liposomes [27]. The polymer-modified liposomes were also observed to be present as individual vesicles in plasma solutions. Both unmodified and polymer-modified liposomes appeared to be spherical, and ranged between 3 and 10 μm in diameter, independent of the medium in which they were suspended. These qualitative observations provide strong evidence of polymer–liposome interactions.

Table 1
Change in the ratio I_e/I_m as a function of time for mixtures of liposomes and poly(*N*-alkyl acrylamides)

Time (h)	PNIPAM	PNMEAM	PMPAM
0	1.810	2.056	3.368
0.02	0.185	0.628	0.904
0.5	0.138	0.456	0.613
1.0	0.108	0.362	0.454
1.5	0.087	0.328	0.404
2.0	0.069	0.303	0.375
2.5	0.062	0.289	0.362
3.0	0.058	0.268	0.345
16	0.035	0.200	0.279

Polymer concentration, 0.01 g l^{-1}. Emission spectra were taken at 25°C. Excitation wavelength, 346 nm.

3.2. Fluorescence measurements

Evidence of polymer–liposome interactions was also obtained from fluorescence measurements. We showed previously that in water, the hydrophobically-modified (HM) polyacrylamides readily form micelles [22], and that the water–polymer systems show a high ratio of pyrene excimer to monomer intensity ($I_e/I_m > 1$). In the presence of liposomes, the excimer emission decreases significantly (from 1.8 to 0.03 for PNIPAM, 2.1 to 0.2 for PNMEAM, 3.4 to 0.3 for MPAM), suggesting a disruption of the polymeric micelles upon interaction with liposomes. It is concluded that the pyrene chromophores become separated as the polymers are embedded in the liposome bilayer, although some polymer micelles remain as indicated by the presence of a small excimer peak.

Experiments were conducted to obtain an indication of the time required for the hydrophobically

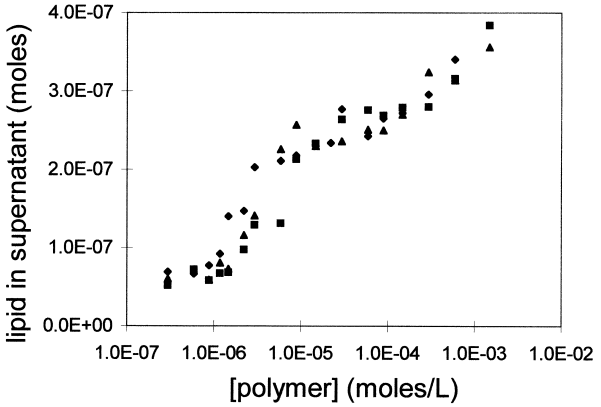


Fig. 4. Phosphate content in supernatant (per 0.5 ml) after centrifugation of polymer-modified liposomes. The polymer concentrations are corrected for polymer-C_{18}Py. (♦) PNIPAM–liposome mixture; (■) PNMEAM–liposome mixture; (▲) PMPAM–liposome mixture.

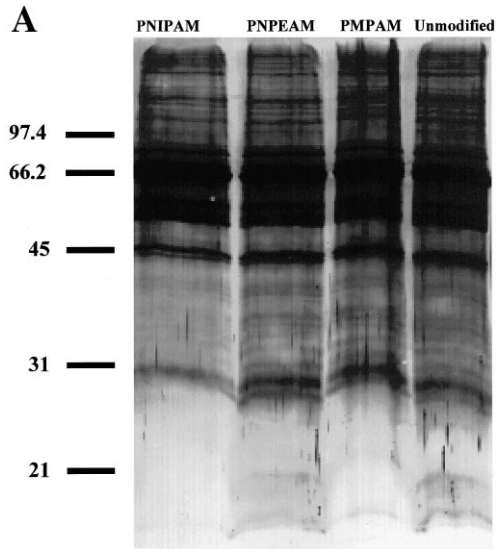


Fig. 5. (A) Gold-stained SDS–polyacrylamide gel (12%) of proteins adsorbed to various liposome surfaces. LUVs (1 mg) were incubated in 25% plasma (0.5 ml) for 2 h at 37°C. (B) Western blots of plasma proteins adsorbed to unmodified DMPC liposomes at 37°C. Adsorption time 2 h in 25% plasma. (C) Western blots of plasma proteins adsorbed to PNIPAM-modified liposomes at 25°C. Adsorption time 2 h in 25% plasma. (D) Western blots of plasma proteins adsorbed to PNIPAM-modified liposomes at 37°C. Adsorption time 2 h in 25% plasma. (E) Western blots of plasma proteins adsorbed to PNMEAM-modified liposomes at 37°C. Adsorption time 2 h in 25% plasma. (F) Western blots of plasma proteins adsorbed to PMPAM-modified liposomes at 37°C. Adsorption time 2 h in 25% plasma.

modified polymers to be incorporated into the liposomes. Emission spectra were taken at 30-min intervals. For all three polyacrylamides, a decrease in I_e/I_m occurred upon the addition of liposomes to the polymer solutions (Table 1). Most of the effect occurred within the first 30 min, but I_e/I_m continued to decrease for approximately 5 h before becoming constant. Modified liposomes were therefore prepared by maintaining the polymer–liposome suspensions at room temperature for at least 5 h.

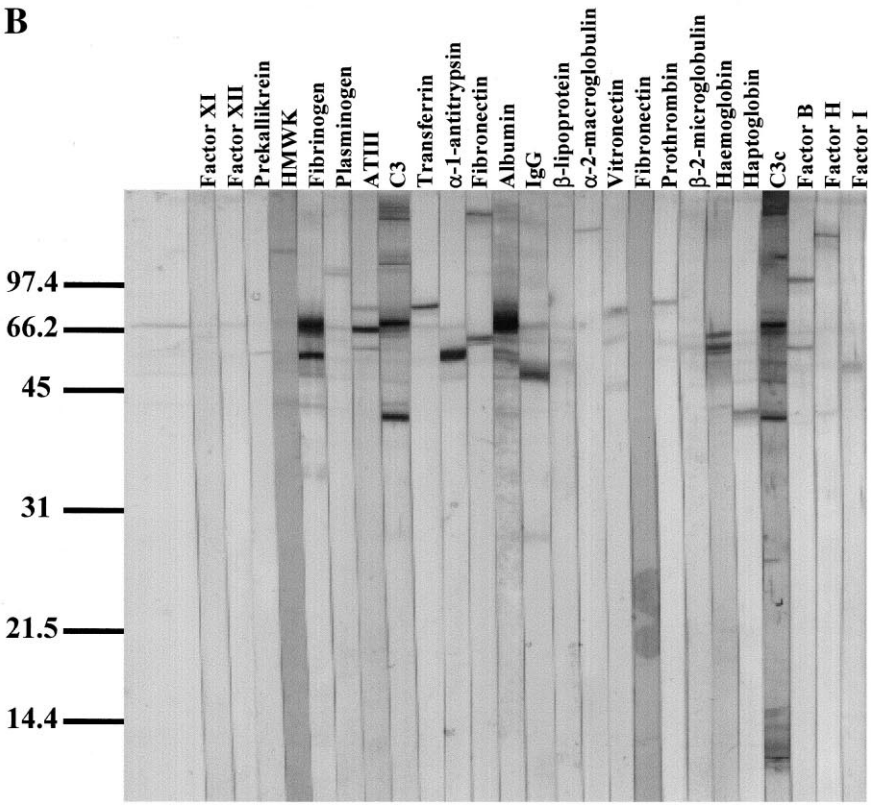
3.3. Temperature effects

Since the polymer-modified liposomes were to be used in protein adsorption experiments at 37°C, fluorescence emission spectra were taken at several temperatures in this vicinity (25–50°C). Liposomes modified with PNMEAM-C_{18}Py and PMPAM-C_{18}Py showed no loss of polymer upon heating to 50°C as suggested by the fact that in their emission spectra I_e/I_m did not change significantly. Spectra of the PNIPAM-C_{18}Py liposome suspensions revealed a very small increase in I_e/I_m beginning at about 37°C, suggesting the formation of micelles, presumably due to desorption of a small amount of polymer from the liposomes.

3.4. Binding of polymers to liposomes

The distribution of hydrophobically-modified polymer between the aqueous and the lipid phases in polymer–liposome suspensions was investigated by determining the polymer concentration in the supernatant and pellet fractions after centrifugation [24]. For all three polymers the concentration was higher in the pellet than in the supernatant, suggesting strongly that the polymer was bound to the liposomes.

Binding curves for the liposome–polymer systems were derived from these equilibration experiments (Fig. 3). The linear character of the data shows that the interaction of the hydrophobically modified polymers with the lipid bilayer is most probably a simple partitioning between the water and lipid phases. The binding appears to be non-cooperative, and the binding constants estimated from the slopes of the regression lines are 55.7 M^{-1} for PNIPAM, 41.6 M^{-1} for PNMEAM and 33.8 M^{-1} for



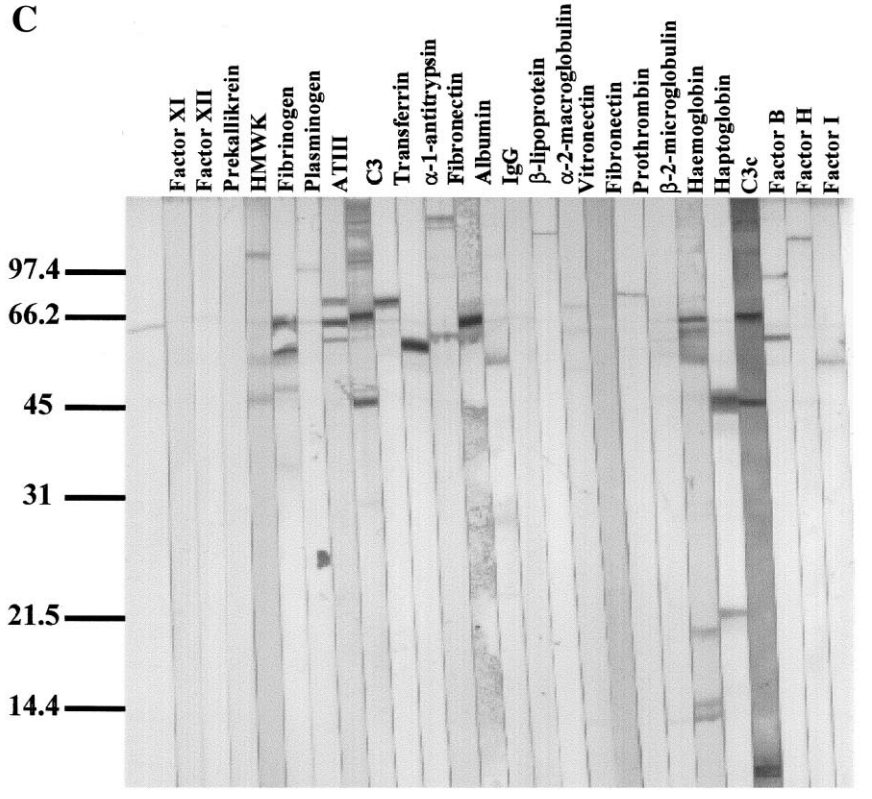
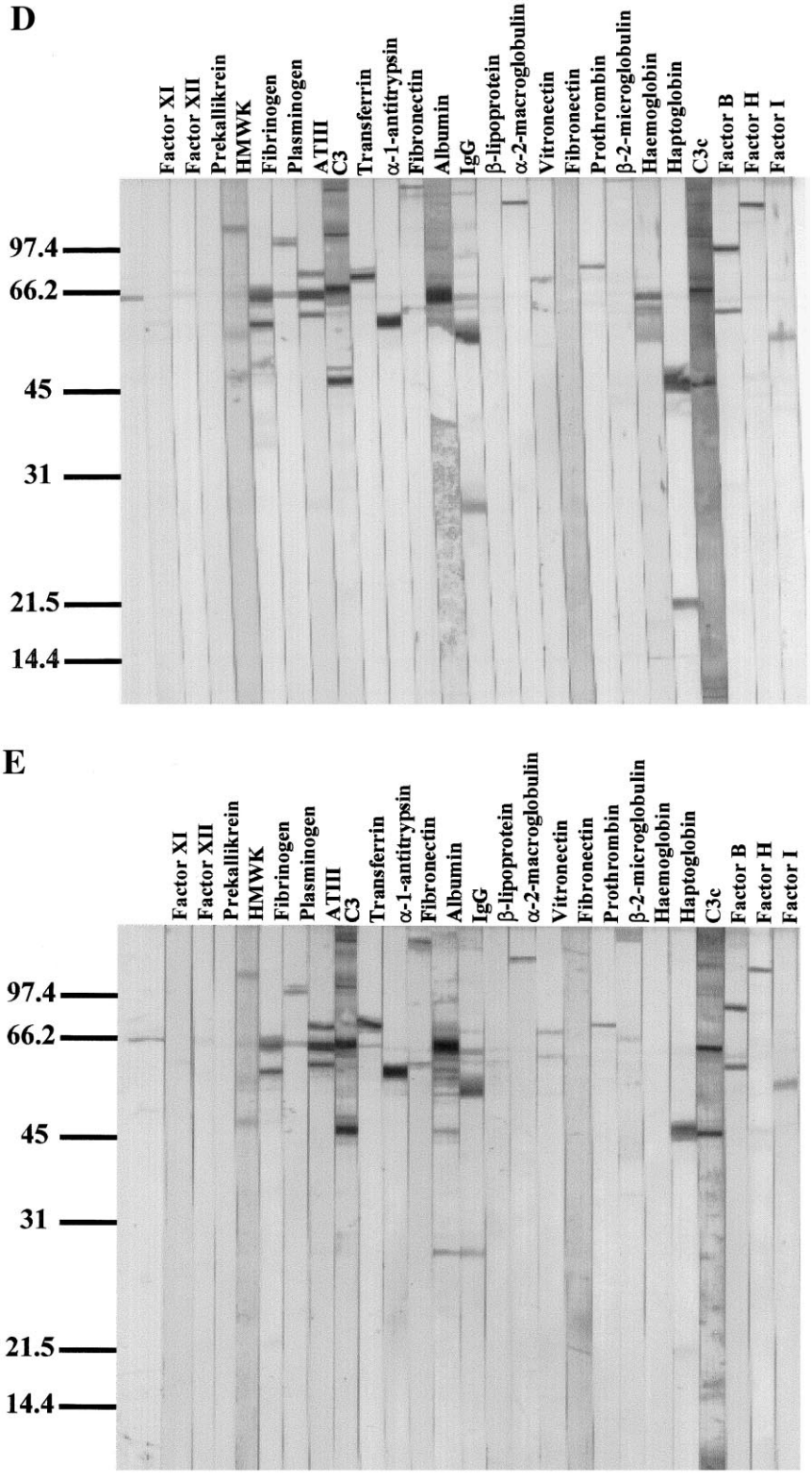


Fig. 5 (*continued*).



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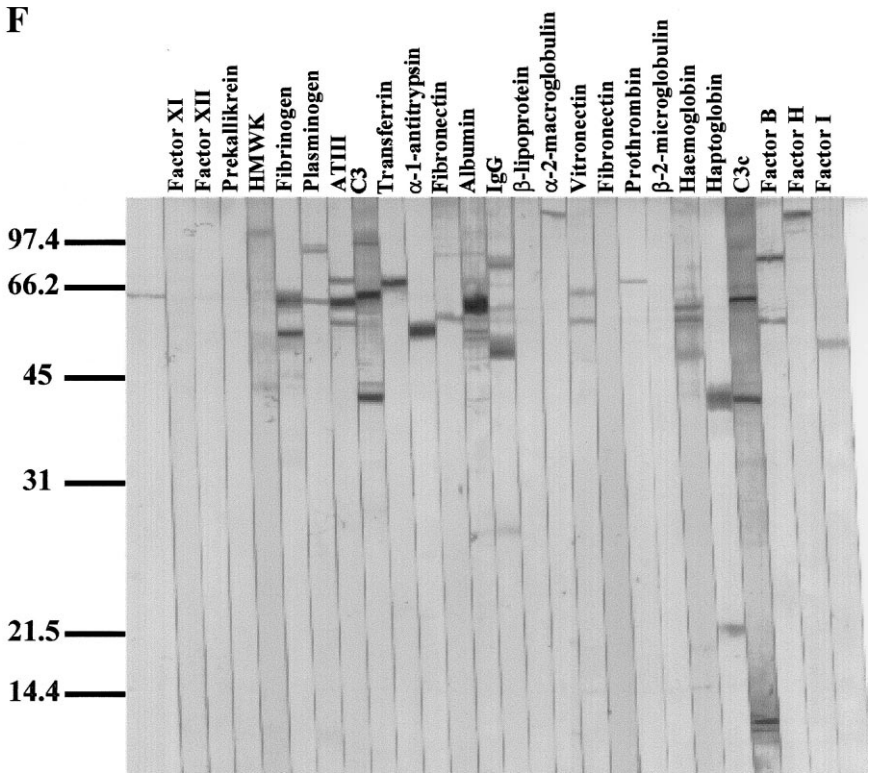

Fig. 5 (*continued*).

PMPAM, implying relatively weak binding in all three cases.

Polozova and coworkers [24] have performed similar polymer–liposome binding studies with a random copolymer of NIPAM and *N*-[4-(1-pyrenyl)butyl]-*N*-*n*-octadecylacrylamide. They reported the formation of small (150–200 nm) lipid–polymer aggregates that remained in the supernatant after centrifugation, and were thought to arise from a partial extraction of lipids and polymer from the liposomes. In the present experiments, analysis of the supernatant for phosphate revealed low concentrations of lipid, dependent on the initial polymer concentration (Fig. 4). The amount of lipid lost from the pellet increases with increasing polymer concentration. Size analysis by dynamic light scattering showed that small polymer–lipid aggregates approximately 400 nm in size (i.e., larger than those reported by Polozova et al. [24]) were present. These are most likely liposomes that are difficult to centrifuge, since they do not readily aggregate. The proportion of such liposomes remaining in the supernatant was never more than 25% of the total liposome preparation. At polymer concentrations greater than 1.4×10^{-4} M (PNIPAM), 3.7×10^{-4} M (PNMEAM) and 3.2×10^{-4} M (PMPAM), the amount of phosphate detected in the supernatant increased considerably (Fig. 4); hence above these concentrations significant destabilization of the liposomes occurred. Thus, slightly lower polymer concentrations (1.1×10^{-4} M, 3.6×10^{-4} M and 3.1×10^{-4} M for PNIPAM, PNMEAM, and PMPAM, respectively) were used to prepare liposomes for protein adsorption studies.

To determine whether polymer binding to liposomes is reversible, the polymer-modified liposomes were suspended in fresh TBS, and the distribution of polymer between the aqueous and lipid phases was determined. Incubation times of 5, 30, and 120 min and 16 h were investigated. Changes in polymer concentration in the pellet and supernatant fractions were negligible in this time range, indicating that the polymer–liposome interaction is irreversible. This is in agreement with gel filtration chromatography data on PNIPAM-modified liposomes [25] which showed that the polymer remained anchored to the

liposomes during the separation and contact with buffer.

The hydrophobically-modified polymers were investigated with regard to their possible interaction with plasma and with HSA in buffer. Using fluorescence, simple aqueous solutions of the polymers (no liposomes) exhibited I_e/I_m values greater than 1.5. In the presence of HSA (10 g l^{-1}), the I_e/I_m values decreased to approximately 0.1, and were even lower in 25% plasma. Presumably, the plasma proteins interact with the polymer in such a way as to prevent interaction of the pyrene chromophores. In the presence of liposomes, the I_e/I_m values did not change significantly whether in TBS, HSA or plasma.

These results leave open the possibility that the polymers may dissociate from the liposomes and associate with the proteins in solution. Therefore incubation–centrifugation experiments were repeated to determine whether the polymer associates with the lipid bilayer in the presence of protein. In human serum albumin (10 g l^{-1}) or human plasma (25%), the polymers partitioned into the pellet fraction during a 2 h incubation at 25°C or 37°C, thus indicating that in protein adsorption experiments, the modified liposomes should retain the different polymers in the bilayer.

3.5. Interactions of liposomes with plasma proteins

For each of the liposome types, adsorption experiments were done using the same mass of liposomes, the same volume of plasma, the same volume of SDS as eluate, and the same volume of eluate for loading onto the gels. On this basis the band intensities on the gels should be comparable from one liposome type to another. With respect to the blots, the band intensities for a given protein should be comparable from one liposome type to another, but comparison of different proteins for a given liposome type or for different liposome types is only approximate since the intensity of the bands undoubtedly depends on the characteristics of the different antigen–antibody and color reactions.

Gels from initial experiments using gold staining show many intense bands (Fig. 5A) indicating the presence of many different proteins. The banding patterns are similar for all four liposome types, indicating similar protein adsorption behavior. The PNIPAM, and to a lesser extent the PNMEAM liposomes, show a relative absence of bands in the 45 to 30 kDa region. The overall stain density of the gels also appears similar suggesting that similar amounts of the various proteins are adsorbed to the different liposomes.

The immunoblots allow for the positive identification of specific plasma proteins (Fig. 5B–F). Most of the adsorption experiments were done at 37°C. For the PNIPAM-modified liposomes, experiments were also done at 25°C to determine whether protein adsorption is affected by the polymer conformation (fully extended at 25°C versus collapsed at 37°C). The PNIPAM-coated liposomes incubated at 25°C are referred to as PNIPAM25 and the immunoblot is shown in Fig. 5F.

As seen in Fig. 5, the blots for the unmodified and polymer-modified liposomes are similar from an overall perspective. Most of the proteins tested for were present, thus indicating that extensive nonspecific adsorption to the liposome surfaces occurs. For a given protein the blots all show similar patterns. For some proteins, small differences are seen in band intensity and with respect to the presence or absence of low molecular mass fragments. For the most part, proteins present in the plasma at high concentration show the highest band intensities, again suggesting that adsorption to the liposome surfaces may (to a first approximation) be concentration dependent and non-specific. For example, on all of the liposome types the band intensity for albumin, the most abundant protein in plasma, is the highest. Fibrinogen, IgG, ATIII, C3, α_1-antitrypsin, and haptoglobin are also adsorbed to the liposome surfaces in relatively large amounts. Transferrin, α_2-macroglobulin and β-lipoprotein, which are present in plasma at concentrations comparable to fibrinogen and α_1-antitrypsin, show lower band intensities. The liposomes presumably have lower affinities for these proteins.

Of the contact phase coagulation proteins, only HMWK adsorbs to the liposome surfaces in significant amounts. Factors XI and XII, and prekallikrein are either absent, or present only in trace quantities. This observation adds to the considerable body of evidence suggesting that HMWK is a highly surface active protein [28]. The PNIPAM25 and PNMEAM liposomes adsorb relatively small amounts of HMWK, and the PNIPAM liposomes appear to ad-

sorb more HMWK than the PNIPAM25 (band intensity ratio ~3:1), suggesting that protein interactions are reduced when the polymer is in its more extended conformation. Similar effects have been noted with respect to whether the lipid bilayer is in the gel or liquid crystalline state [29]. The gel state has been shown to adsorb more protein than the liquid crystalline state. Only the PNMEAM liposomes show bands for both factor XI and factor XII, although the amounts appear to be small.

It has been reported [4] that liposomes interact extensively with proteins of the complement system, and that binding of these proteins is important for opsonization. Therefore immunoblots were run using antibodies directed against C3, C3c, factor B, factor H and factor I. Positive responses were obtained for all liposome types. C3, the most abundant of the complement proteins, showed the highest band intensities. Moreover, complement activation appears to have occurred in all cases as suggested by the presence of a strong band at about 40 kDa in the C3 blot, probably due to iC3b [26]. Of the various liposome types, the PNIPAM and PNIPAM25 liposomes adsorb the smallest amounts of C3, and show the lowest level of activation. The PNIPAM25 liposomes adsorb smaller amounts of factors B, H and I than the PNIPAM liposomes, again possibly due to the more extended conformation of the PNIPAM at 25°C versus 37°C. The PNIPAM25 liposomes also adsorb lesser amounts of factor B, factor H and factor I than any of the other liposomes. If liposome elimination from the circulation is linked to the binding of complement components, the PNIPAM liposomes may be of interest for further study.

With regard to the cell adhesion proteins fibronectin and vitronectin, the intact forms of both are observed in the blots for all the liposome types. All the surfaces appear to adsorb similar amounts of fibronectin. The unmodified liposomes clearly adsorb more vitronectin than any of the modified ones.

The abundant plasma proteins, albumin, fibrinogen and IgG, are adsorbed to the liposomes mainly in intact form. The PNIPAM25 liposomes adsorb smaller amounts of albumin than any of the other types. In the case of fibrinogen, the PNIPAM25 and PNMEAM liposomes adsorb similar, relatively small quantities. The greatest amounts are seen on the unmodified and PNIPAM liposomes. These liposomes show three to four times greater intensity of the 66 kDa band than any of the others. For IgG, the PNIPAM25 liposomes adsorb very small amounts compared to the others (ratios of the order of 0.03:1). Thus, as for other protein groups, it appears that PNIPAM25-modified liposomes have relatively weak interactions with the major plasma proteins.

Intact and degraded plasminogen appear on the blots for all liposome types, although the relative amounts of the two are variable. The unmodified liposomes adsorb plasminogen mainly in its intact form, whereas on the modified liposomes degraded, or possibly activated plasminogen predominates. We have no explanation for this observation at present: it may be that binding to the NIPAM moieties is similar to binding to L-lysine which induces a conformational change resulting in facilitated activation of plasminogen to plasmin [30].

The objectives of this work were to prepare liposomes with various NIPAM polymers incorporated into the membranes, and to investigate their interactions with plasma proteins to determine whether the polymers would exert a protein repellent effect. It appears that all of the liposome compositions adsorb proteins from plasma in a similar fashion. The only exception are the PNIPAM-modified liposomes at 25°C, i.e., below the lower critical solution temperature, which appear to adsorb less protein than the other types including the unmodified controls. They show the lowest intensities for albumin (~50% of the other liposomes), IgG, transferrin and hemoglobin. The data therefore suggest that PNIPAM-liposomes at 25°C and 37°C interact differently with plasma proteins, and this may be due to the different conformations of the polymer above and below the critical solution temperature. This is analogous to the effect of polyethylene glycol on protein adsorption which has been attributed to chain flexibility and hydrophilicity resulting in steric repulsion of approaching proteins [31]. Also in the case of PEO-grafted surfaces, others have noted, in agreement with the results of this work, that protein adsorption decreases with increasing quality of the bathing fluid as a solvent for PEO [32,33]. For example Tiberg et al. [32] showed that on a surface with immobilized PEO–PPO copolymer, protein adsorption decreased significantly as the temperature was lowered away from the copolymer cloud point.

Both PNMEAM and PMPAM were chosen for this study because at 37°C they are below their LCST and thus should be in the extended conformation, and because their side chains contain an ether linkage. By analogy with polyethylene glycol they might thus be expected to exhibit steric repulsion. However, when incorporated into the liposomes they appear to have no inhibitory effect on protein interactions in plasma. This is in contrast to PNIPAM, which had a similar molecular mass and the same terminal group on the side chains as the PNMEAM and PMPAM. It appears that in this series of polymers the substituents on the N atom are important in these phenomena. It may be that substituents which are intermediate in hydrophilicity between isopropyl and methoxyethyl or methoxypropyl to give a LCST just above 37°C would provide steric repulsion and thus extend lifetime in the circulation for drug delivery applications.

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