Poly(ethylene glycol) amphiphile adsorption and liposome partition

James M. Van Alstine[a,*], Martin Malmsten[b], Donald E. Brooks[c]

[a]Department of Chemistry, University of Alabama in Huntsville, Huntsville, AL 35899, USA
[b]Institute for Surface Chemistry, P.O. Box 5607, S-114 86, Stockholm, Sweden
[c]Departments of Pathology and Chemistry, University of British Columbia, Vancouver, B.C. V6T 1B5, Canada

Abstract

Surface localized poly(ethylene glycol) (PEG) amphiphiles of type $C_{16:0}-EO_{151}$ and $C_{18:2}-EO_{151}$ were studied via ellipsometry at macroscopic, flat methylated silica (MeSi), phosphatidic acid (PA), and phosphatidylcholine (PC) surfaces. At these surfaces the amphiphiles adsorb similarly, in a non-cooperative manner, achieving a plateau (≈ 0.1 PEG chains/nm^2) well below amphiphile critical micelle concentration (CMC). The resultant PEG-enriched layers were 10–15 nm thick, with a polymer concentration (≈ 0.07 g/cm^3) greater than the PEG-enriched phase of many dextran, PEG aqueous two-phase systems. PEG-amphiphile adsorption (mg/m^2) at hydrophobic and phospholipid flat surfaces correlated with changes in the partition (log K) of PC liposomes in such two-phase systems. PEG-amphiphile adsorption at macroscopic surfaces appears to represent a balance between hydrophobic attraction and repulsive intra-chain interactions which promote chain elongation normal to the surface.

Keywords: Adsorption; Partitioning; Critical micelle concentration ; Ellipsometry; Liposomes; Poly(ethylene glycol) (PEG)

1. Introduction

Modification with poly(ethylene glycol) (PEG) and other linear water-soluble polymers significantly modifies surfaces and the in vitro and in vivo behavior of drugs carrying colloids, macromolecules, and particles [1,2]. Pharmaceutical applications of PEG include polymer derivatization of drugs, proteins, and colloidal drug carriers such as liposomes. PEG-derivatized proteins have a variety of applications, from PEG antibodies for immunoaffinity separation to industrial enzymes with enhanced thermal or solvent stability, to therapeutic enzymes with prolonged serum circulation times [1–6]. PEG may be localized on liposomal drug carrier surfaces via inclusion of a hydrophobic PEG amphiphile, e.g.

PEG phosphatidyl-ethanolamine, into their lipid membranes. The resultant enhancement of circulation half-life, which appears related to reduced adsorption of serum proteins, has generated interest in PEG-coated colloids as parenteral drug carriers [1,7–10]. In addition to these uses, surface-localized PEG chains reduce non-specific protein adsorption and false positive results in solid-phase diagnostics [11] and related assays [12]. They improve the biocompatibility of materials in contact with biological fluids in implant and extracorporeal therapies [1], control phase wall wetting [13] and reduce variable and pH-sensitive phenomena, e.g. electroosmosis and adsorption, which negatively affect free solution electrophoretic separation methods [14,15].

Ellipsometry is often used to provide quantitative information on the thickness, grafting density, and protein rejection capabilities of polymer coatings on

---

*Corresponding author.

flat surfaces [18–22]. Data obtained with ellipsometry agree well with those obtained with other methods for proteins, polymers and surfactants at various model surfaces [16,17,23–25]. Therefore, ellipsometry aids investigations related to grafting density, molecular shape and surface layer thickness of polymer coatings, as well as the multitude of phenomena influenced by such coatings [1,15–17].

Partition in aqueous polymer two-phase systems readily provides information related to a variety of changes in the surface features of macromolecules and particles such as liposomes, cells and other colloids [26,27]. The technique involves measuring the equilibrium distribution of material between the two phases which form when neutral polymers, such as dextran and PEG, are mixed together at low concentration in aqueous solution. Such phase systems contain a PEG-enriched upper phase and a denser dextran-rich lower phase. Systems prepared with buffered NaCl solutions possess negligible interfacial bulk phase potential [28–30]. The partition of particles between the phases in such systems is primarily influenced by interfacial tension acting to localize them at the phase interface and interactions with phase polymers, e.g. adsorption, lowering their interfacial free energy in the corresponding phase [6,27–31]. The latter appears to correlate with membrane lipid features such as phosphatidylcholine/sphingomyelin (PC/S) ratio, or the related unsaturated fatty acid (UFA) ratio of poly- to mono-unsaturated acyl groups esterified to phospholipids [30].

Covalent coupling of hydrophobic "tails" to PEG significantly enhances its interaction with many substances. Micromolar concentrations of such PEG amphiphiles appear to negligibly affect interfacial tension or other phase system properties [6,31] but often dramatically increase particle partition in a concentration dependent and surface discriminating manner [6,28,32,33]. Sharp and coworkers showed that increases in particle partition correlated with the amount of adsorbed [^{14}C]PEG 6000 ester [6,29,31]. Eriksson and coworkers [28,32] and Walter and coworkers [27,33] have shown that ester-induced partition of different species erythrocytes or liposomes also correlates with the membrane PC/S ratio and UFA ratios. Van Alstine and coworkers used hydrophobic affinity partition to investigate disease-

related changes in bacteria [34], cancer cells [35], and blood cells of patients with multiple sclerosis [36].

Ellipsometry and partition are complementary in terms of target surfaces (e.g. planar versus particulate, and simple/model versus complex/biological) as well as the information obtained (e.g. surface localized polymer layer thickness and grafting density versus surface localized polymer effect on partition). They are good candidates for comparative investigation of the adsorption of purified, well-characterized PEG amphiphiles. Use of such amphiphiles is required to express results in molar concentrations, relate them to the surfactant nature, e.g. CMC, of the amphiphile and eliminate contaminants which can alter phospholipid-containing surfaces [38]. In the present investigation, ellipsometry was used to study the in situ adsorption of palmitate ($C_{16:0}$) and linoleate ($C_{18:2}$) esters of PEG of 6650 molecular mass, i.e. 151 EO units, at model hydrophobic (methylated silica) and phospholipid (PC and PA) surfaces. The information obtained was compared with partition data for PEG esters adsorbed at the surfaces of PC liposomes. Ellipsometry provided insight to partition results and how they reflect the ability of terminally anchored PEG chains to control the interfacial behavior of surfaces and particles.

2. Materials and methods

2.1. Materials

Water for ellipsometry was purified by a Milli-RO 10PLUS unit (Millipore Corp., USA), including depth filtration, carbon adsorption and decalcification preceding reverse osmosis. Subsequently, it was led through a Milli-Q PLUS185 unit (UV light; 185 nm and 254 nm) and a Q-PAK unit consisting of an active carbon unit, a mixed bed ion exchanger, an Organex cartridge, and a final 0.22-μm Millipak 40 filter. Water for partitioning was double distilled in quartz and subjected to a similar column filtration/ion-exchange regime.

Poly(ethylene glycol)-fatty acid monoesters were synthesized, purified and analyzed as described in detail elsewhere [39,40]. In the ester preparations

used, 15 mol% or less of the PEG hydroxyl end-groups were esterified, reducing the di-esterified PEG content of the preparation to 3% or less. Ester purification involved repeated solvent extraction and Sephadex LH-20 size-exclusion chromatography in methanol–water (4:1, v/v) to remove unreacted fatty acids and limit the PEG chain length polydispersity. PEG head group molecular mass was determined by size-exclusion high-pressure liquid chromatography to be 6650 ± 200, representing an average of 151 ± 5 ethylene oxide (EO) units per molecule. For simplicity, the esters are referred to as $C_{i:j}$–EO_{151} (see Table 1). They were stored at $-60°C$ under nitrogen until used. PEG fatty acid ester critical micellization concentration (CMC) estimates in water containing 1.5% (w/w) PEG 8000 were obtained as described previously using fluorescence enhancement of 8-anilino-1-naphthalene sulfonate [41] and are given in Table 1. Also included are statistical estimates of the ratio of mono- to di-esterified molecules based on analysis of total esterified end-groups [39,40].

Phosphatidylcholine (PC) of 99+% purity for use in ellipsometry was from Scotia LipidTeknik AB, Sweden. The fatty acid composition (wt%) for PC was: $C_{16:0}$ 14.8, $C_{18:0}$ 3.2, $C_{18:1}$ 9.1, $C_{18:2}$ 62.6, $C_{18:3}$ 7.6, and others 2.7. Phosphatidic acid (PA) from egg yolk (Avanti Polar-Lipids) for use in ellipsometry had a composition (wt%) of: $C_{16:0}$ 34, $C_{16:1}$ 1, $C_{18:0}$ 11, $C_{18:1}$ 31, $C_{18:2}$ 18, $C_{20:4}$ 3, and $C_{22:6}$ 2. The total distribution of acyl groups in the mixtures of molecular species was determined by the manufacturers via capillary gas chromatography of methyl esters. The lipids were used without further purification, as were chemicals for the buffer preparation and the phospholipid layer deposition (analytical grade). Dimyristoyl-PC (DMPC, 99% pure) for liposome preparation was obtained from Sigma. Custom [^{14}C]DMPC (95% pure, 50 μCi/ml in benzene, 55 mCi/mmol) was obtained from Applied Science Labs. [^{3}H]Palmitic acid (99% pure, 10 mCi/ml in benzene, 12 Ci/mmol) was from New England Nuclear.

mally in pure and saturated oxygen, followed by annealing and cooling in argon flow to generate an oxide layer thickness of about 30 nm. The slides were then cleaned in a mixture of 25% NH_4OH, 30% H_2O_2 and H_2O (1:1:5, v/v) at 80°C for 5 min, followed by cleaning in a mixture of 32% HCl, 30% H_2O_2 and H_2O (1:1:5, v/v) at 80°C for 5 min. Methylated silica surfaces were prepared from the silica surfaces by double rinsing subsequently with water, ethanol, and trichloroethylene (pro Analysi, Merck), followed by treatment with a 0.1 wt% solution of $Cl_2(CH_3)_2Si$ (Merck) in trichloroethylene for 90 min [19]. Finally, they were rinsed four times in trichloroethylene and ethanol. This procedure rendered the slides hydrophobic, with an advancing and receding contact angle of 95° and 88°, respectively. They were stored in ethanol until use.

Phospholipid-coated surfaces were prepared by spin-coating onto methylated silica surfaces [20]. This was performed by dissolving the lipid (0.5 wt%) in a solvent containing hexane (40 wt%), 2-propanol (20 wt%), acetone (20 wt%) and decane (20 wt%). The solution (1.0 ml) was added dropwise to the rotating surface (300 rpm), followed by spinning at 3000 rpm for 40 s. This procedure resulted in lipid films with an approximate thickness of about 10–20 nm, as obtained from ellipsometry. The phospholipid-coated surfaces displayed a good water wettability. ESCA analysis indicated essentially pinhole free lipid films. The deposition technique was previously shown by the authors to result in phospholipid layers identical to those prepared by Langmuir–Blodgett deposition as regards protein adsorption, indicating a preferential orientation of the outermost molecules with the head groups towards the aqueous solution [20]. The flat plates used in the present investigation have the advantage over emulsion droplet and liposome surfaces in the avoidance of artifacts related to size polydispersity affecting surface area estimates, as well as adsorption-induced flocculation and coalescence.

2.2. Ellipsometry surfaces

Silica surfaces were prepared from polished silicon slides (p-type, boron-doped, resistivity 7–13 Ω/cm; Okmetic, Finland). They were oxidized ther-

2.3. Ellipsometry

Ellipsometry measurements were performed by means of null ellipsometry [24] in 0.01 M phosphate buffer, 0.15 M NaCl, pH 7.5, at 25°C, using an

automated thin-film ellipsometer (Rudolph, Type 436) controlled by a personal computer. A xenon lamp filtered to 4015 Å was used as the light source. A thorough description of a similar experimental setup has been given by Landgren and Jönsson [42]. Prior to adsorption ellipsometry requires determination of the complex refractive index of the substrate. In the case of a layered substrate such as oxidized silicon a correct determination of the adsorbed layer thickness and mean refractive index requires determination of the silicon bulk complex refractive index ($N_2 = n_2 - ik_2$) as well as of the thickness (d_1) and the refractive index (n_1) of the oxide layer. This is done by measuring the ellipsometric parameters Ψ and Δ in two different media, e.g. air and buffer. From the two sets of Ψ and Δ, n_2, k_2, d_1 and n_1 can be determined separately [25,42].

Phospholipid surface measurements of the bare substrate were performed in the buffer solution treating the surfaces as being homogeneous, since the five layer optical evaluation required for these surfaces, as well as the possibility of interfacial fractionation [43] and insertion into the lipid layer, preclude detailed analysis of the adsorbed layer structure. The procedure employed allows the adsorbed amount to be determined, whereas effective values are obtained for the adsorbed layer thickness and mean refractive index [20]. All measurements were performed by four-zone null ellipsometry in order to reduce effects of optical component imperfections [24]. After optical analysis of the bare substrate surface, the PEG–fatty acid ester solution was added to the cuvette, and the values of Ψ and Δ recorded. (The adsorption was monitored in one zone, since the four-zone procedure is time-consuming and corrections for component imperfections had already been performed.) The maximal time-resolution between two measurements is 3–4 s. Stirring was performed by a magnetic stirrer at about 300 rpm. From Ψ and Δ, the mean refractive index (n_f) and average thickness (δ_{el}) of the adsorbed layer were calculated numerically according to an optical three- and four-layer model for the phospholipid surfaces and methylated silica, respectively [20]. The adsorbed amount (Γ) was calculated according to de Feijter [44], using a refractive index increment (dn/dc) of 0.15 cm^3/g for the PEG fatty acid esters [45].

2.4. Two-phase system

Two-phase systems were prepared as described previously [6,34] by mixing appropriate weights of the following aqueous stock solutions; 20% (w/w) dextran T500 (M_r 500 000 from light scattering data, Pharmacia Fine Chemicals, Uppsala, Sweden: lot 7830), 30% (w/w) PEG 6000 (M_r 6650 from high pressure liquid chromatography [40], Union Carbide, New York, NY, USA: lot B-529-9104), 0.6 M NaCl, and 0.22 M Na$_2$HPO$_4$, 0.07 M NaH$_2$PO$_4$, pH 7.2 buffer. (PEG 6000 has been redesignated 8000 by Union Carbide.) Dextran concentrations were determined polarimetrically while PEG stock concentrations were determined from refractive index measurements as described previously [34,35]. Once compounded the phase system was allowed to settle overnight at room temperature in a cylindrical separatory funnel. The phases were then separated and used immediately. A one-phase system of 5.0% (w/w) dextran, 4.0% (w/w) PEG, 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.2, was used throughout and, in keeping with convention it was designated "(5,4) V". The system possessed a bulk phase potential of 0.28 ± 0.14 mV (mean ± S.D., top phase positive) measured as described previously [30] except that open ended microcapillary glass electrodes were used [31,39]. System interfacial tension was 4.99 ± 0.06 μN/m and (w/w) phase compositions [26,27] were top phase 0.71% dextran, 5.95% PEG and bottom phase 9.95% dextran, 1.50% PEG. The above values are for 22°C but are not expected to differ significantly at 26°C [6,39].

2.5. Liposome preparation and partition

Dimyristoyl-phosphatidylcholine (DMPC) small unilamellar vesicle (SUV) liposomes of approximately 20 nm diameter and 2000 kDa molecular mass [46] were prepared according to the method of Huang and Thompson [47], except that the lipid mixture was lyophilized as opposed to being dried under nitrogen [48]. A 250–300 mg sample of DMPC containing 17 μCi of [^{14}C]DMPC was dissolved in 3 ml of benzene in a 20 ml vial at 37°C and lyophilized to remove the benzene; 8 ml of pH 7 buffer [10 mM Tris–HCl, 0.1 M KCl, 0.2 mM EDTA, 0.3% (w/w) NaN$_3$] were added and the

suspension was mixed by vortexing followed by sonication to clarity over a 1 h period using a Model 150 Sonic Dismembrator (Fisher Scientific) equipped with a 12.7 mm diameter titanium probe at 90% power output. Sonication was performed in 3-min intervals with cooling to keep the vial temperature a few degrees above 21°C. The liposome preparation was concentrated to 2 ml by 60-min centrifugal ultrafiltration using CF-25 membrane filter cones (Amicon) and subjected to 4°C chromatography in the above buffer on a K26/76 column (50 × 2.5 cm I.D.) of Sepharose 4B (Pharmacia) gel at a flow-rate of 0.5 ml/min. Eluent fractions of 3 ml were monitored by turbidity at 300 nm and liquid scintillation counting to 0.2% error of a 100-μl sample in 10 ml of Atomlight (New England Nuclear) in a Beckman LS-233 liquid scintillation counter. Different fractions containing either SUV liposomes or larger multilamellar liposomes [47] were isolated and stored under nitrogen to minimize oxidation. Control differential scanning calorimetry measurements (Perkin Elmer DSC-1B) were performed in order to verify the gel to liquid crystalline phase transition temperature (T_m) of the samples and the lack of a significant effect of adsorbed ester on liposome T_m [6].

Partition studies involved pooling two peak SUV column fractions (6 ml) and adding them to 140 ml of PEG-rich upper phase to form a "load mix". A 100-μl sample volume of load mix was added to 15 ml Atomlight and counted (see above) to determine net counts per min (cpm). Equal 2.5-ml aliquots of load mix were added to 2.5-ml aliquots of dextran-rich phase in 100 mm × 13 mm glass or plastic test tubes which were then capped, mixed 20 times by inversion, and equilibrated in a waterbath (Braun Thermomix 1440) for 0.5 h at 26 ± 0.5 °C. This temperature was predetermined to be high enough above the 21.5°C lipid phase transition temperature of the DMPC liposomes to ensure the lipid was in the "fluid" state, as was the PC and PA used in ellipsometric studies, and eliminate partition effects related to T_m [6,34]. The tubes were remixed and equilibrated for another hour before PEG ester was added in a 10–50 μl aliquot from a 4°C stock solution, followed by remixing and further equilibration for 0.5 h. Finally, the tubes were mixed and after 30 min settling 250 μl of upper or lower phase

(sampled by piercing plastic tubes with a syringe) were taken into 15 ml of Atomlight and counted to an error of 2% or less. Ten samples were counted per load mix, and up to three samples were taken per experiment tube with four separate tubes averaged per experimental condition, i.e. ester concentration. Appropriate controls were used to determine "net" cpm values from accurate estimates of background cpm, tube wall adsorption cpm, etc. Results were expressed as percent of the net load mix cpm repartitioning back into the upper phase (% partition) or as a partition coefficient (K) equivalent to the net cpm repartitioning into the upper phase divided by the net remaining cpm. The data expressed here are part of a more comprehensive study involving the effect of various factors such as T_m, PEG-ester type and purity, surfactants, liposome size, and phase system properties on liposome partition [6,39]. Similar results were seen if [^{14}C]DMPC was replaced with 15 μCi of [^{3}H]palmitic acid (not shown).

3. Results and discussion

3.1. Ellipsometry

Table 1 CMC data were reported and discussed previously [6,41] except for the $C_{18:0}$–EO_{151} CMC of 4 ± 3 μM [39] which encompasses a previous estimate of 1.1 μM [41].

Adsorption isotherms for $C_{16:0}$–EO_{151} and $C_{18:2}$–EO_{151} at methylated silica, phosphatidic acid (PA), and phosphatidyl-choline (PC) are shown in Fig. 1 and Fig. 2 with concentration data normalized (Ceq/CMC) in regard to surfactant hydrophobicity per CMC values (Table 1). On all three surfaces the saturated and unsaturated esters (of similar CMC, i.e. hydrophobicity) exhibit similar normalized adsorption curves, and reach saturation adsorption well below CMC. The marginally greater (mg/m^2) adsorption for $C_{18:2}$–EO_{151} may reflect a slightly greater PEG chain length resulting from the chromatography used to remove lytic unesterified fatty acids from the preparation [39,40].

PEG-derivatized fatty acids adsorb rapidly and apparently non-cooperatively at PA or PC surfaces (not shown) as well as at MeSi (Fig. 3). Similar adsorption plateau were also seen for both esters

Table 1
EO_{151}-amphiphile CMC values and adsorption characteristics

Acyl group[a]	CMC[b] (μM) (mean ± S.D.)	Ratio of mono- to di-esterified PEG molecules[c]	Saturation adsorption at methylated silica[d]	
			Amount (Γ, mg/m^2)	Thickness (δ_{el}, nm)
Hexadecanoate ($C_{16:0}$)	42 ± 4	17.9	0.90 ± 0.05	11.0 ± 2.0
Octadecanoate ($C_{18:0}$)	4 ± 3	10.6	N.D.	N.D.
cis-9,*cis*-12-Octadecadienoate ($C_{18:2}$)	40 ± 5	30.5	1.15 ± 0.05	13.0 ± 2.0

[a]Fatty acyl group designation ($C_{i:j}$) where *i* represents the number of carbon atoms and *j* the number of double bonds in the acyl group.
[b]CMC in water containing 1% (w/w) PEG 8000 [6,41].
[c]Mono- to di-esterified ratio from statistical analysis of substitution data [39,40].
[d]Adsorption at phosphatidic acid and phosphatidylcholine surfaces appears similar to adsorption at methylated silica (see Fig. 1 and Fig. 2, plus text).
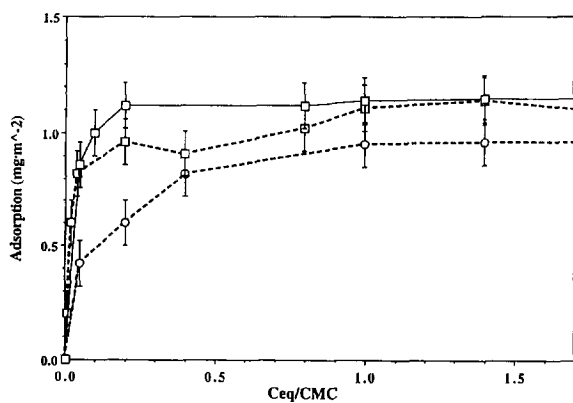

Fig. 1. Comparative adsorption isotherms for EO_{151}–$C_{16:0}$ (○) and EO_{151}–$C_{18:2}$ (□) at PC (- - -) and MeSi (———) from 0.01 *M* phosphate buffer, 0.15 *M* NaCl, pH 7.5, 25°C.
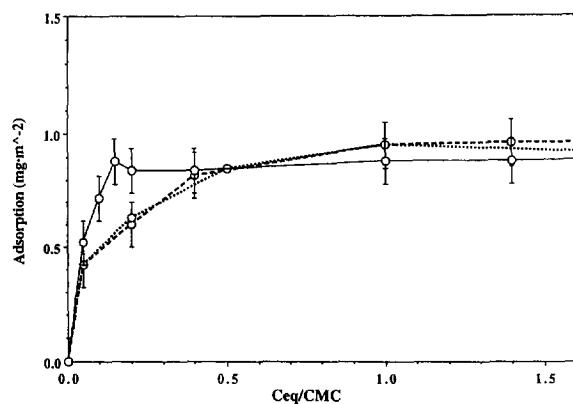

Fig. 2. Adsorption isotherms for EO_{151}–$C_{16:0}$ at methylated silica (———), PA (· · ·) and PC (- - - dashed line) from 0.01 *M* phosphate buffer, 0.15 *M* NaCl, pH 7.5, 25°C.
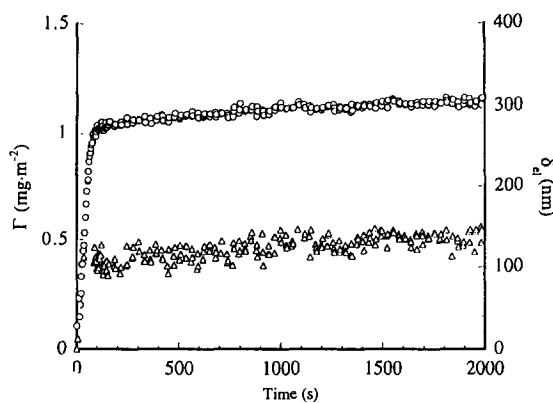

Fig. 3. Adsorbed amount (Γ, ○) and adsorbed layer thickness (δ_{el}, △) versus time for EO_{151}–$C_{18:2}$ at methylated silica from 0.01 *M* phosphate buffer, 0.15 *M* NaCl, pH 7.5, 25°C.

(Table 1). Native (unmodified) PEG exhibits negligible adsorption at the surfaces studied (not shown) [2,20,21]. The PEG chains of adsorbed esters should therefore be oriented toward the solution, reducing any correlation between adsorption and bulk micellization. An adsorbed layer of appreciable (≈ 10 nm) thickness appears to form early in the adsorption process. Approximately 90% of adsorption saturation is reached after 3 min, however saturation still takes some time to achieve. This may be due to the PEG chains already localized at the surface, as much as to a reduction in the hydrophobic surface area for adsorption.

The PEG-ester adsorbed layer refractive index is about 1.355 [2]. Assuming that refractivity of the esters in the adsorbed layer is similar to bulk

solution, and applying the bulk refractive index increment, the average water content in the adsorbed layers is 0.93 g/cm^3. The adsorbed ester layer thickness of 10–15 nm is not expected from the $\approx$3.5 nm radius of gyration (R_g) expected for PEG chains in a random coil under these conditions [49]. The conformation of the PEG chains and their apparent elongation normal to the surface could be due to inter-chain lateral pressure (vide infra). Their adsorbed amount of 0.9–1.1 mg/m^2, corresponds to a PEG (cross-sectional) surface area of approximately 10 nm^2 per chain. When compared with the solution R_g repulsive inter-chain interactions seem to be a plausible cause for coil elongation, and may be the prime determinant of adsorption saturation.

Previous results indicate that the inner hydrophobic layer is 1–2 nm thick [19–21]. This indicates that the PEG layer thickness is 11 $\pm$ 3 nm, which compares favorably with other estimates for layers formed with PEG chains of similar mass [2]. Given PEG molecular mass, surface concentration, and layer thickness, surface density can be estimated as 0.1 (i.e. 0.07 $\pm$ 0.02) chains/nm^2. This corresponds to estimates of 0.1–0.4 chains/nm^2 for covalently attached, terminally anchored PEG coatings of similar surface properties [1,15–17,23].

Fig. 1 and Fig. 2 also suggest a reproducible tendency for the PEG esters to adsorb with slightly greater affinity for the MeSi surface, which is underscored by the equivalency of adsorption at PA and PC (Fig. 2). In all three cases adsorption saturates below CMC. This is in keeping with ellipsometry studies related to the adsorption of other hydrophobe modified PEG chains, i.e. PEG–PPG–(PEG) block copolymers, at hydrophobic surfaces [2,51–53].

PEG-amphiphile saturation at MeSi, PA or PC appears to involve ready alteration of PEG chain solution structure with significant elongation normal to the surface. Such molecular freedom of movement was previously hypothesized as contributing to the low CMC values associated with these PEG-ester amphiphiles [41]. Lim and Herron recently reported modeling experiments where decreasing the separation distance of terminally anchored EO$_{40}$ chains from 24 Å to 12 Å resulted in significantly increased PEG layer thickness. They concluded that under the conditions modeled, PEG conformation was a function of both chain packing density and solvency conditions [23]. The ability to use adsorption to generate surfaces of controlled PEG grafting density may benefit a range of studies which have previously relied on more complicated model systems [15–17,23].

3.2. Partition studies

Fig. 4 indicates the effect of micromolar concentrations of C$_{18:0}$- and C$_{18:2}$–EO$_{151}$-fatty acid esters on the partition of PC liposomes in the (5,4) V system. Given a slight but reproducible ($\approx$15%) dependency of ester induced partition on DMPC lipid phase state [6,39] the data are shown for liposomes at a temperature ($T > T_m$) where their PC fluidity is in keeping with that of the PC used in ellipsometry (see Section 2.3. In Fig. 4, PEG-ester bulk concentration data are expressed in moles per liter (Fig. 4A) or normalized against CMC data from Table 1 (Fig. 4B and Fig. 4C).

Fig. 4A shows log K versus ester concentration (μM) for DMPC small unilamellar vesicle (SUV) liposomes. In this phase system human erythrocytes partition between the interface and the upper phase, with approximately 2% upper phase partition in the absence of ester and a saturation partition of 85% (log $K = 0.6 \pm 0.1$) [6,34,39]. At 26°C DMPC SUV liposomes partition between the upper phase (18$\pm$6%) and the interface (81$\pm$10%) with $\leq$1% lower phase partition at 0 μM ester [39]. Liposome ester-induced partition plateaus at higher values (95$\pm$5%, log $K = 1.28$) (Fig. 4). In keeping with the observations of Eriksson and Albertsson [32], Tilcock et al. [37] and Van Alstine [39], samples of larger (multilamellar) liposomes from the same liposome preparation (see Section 2.5) exhibited greater interfacial attraction and partition curves more similar to those of erythrocytes (not shown). Particle partition is expected to vary inversely with interfacial tension and particle size [6,50].

The ability of a PEG ester to induce particle partition into the PEG-rich phase is expected to relate to its ability to interact hydrophobically with the particle. It is generally expected to vary directly with tail length and saturation [6,28,32] or inversely with CMC (Table 1) [6,41]. This may explain the slight difference in ester molar effectiveness at
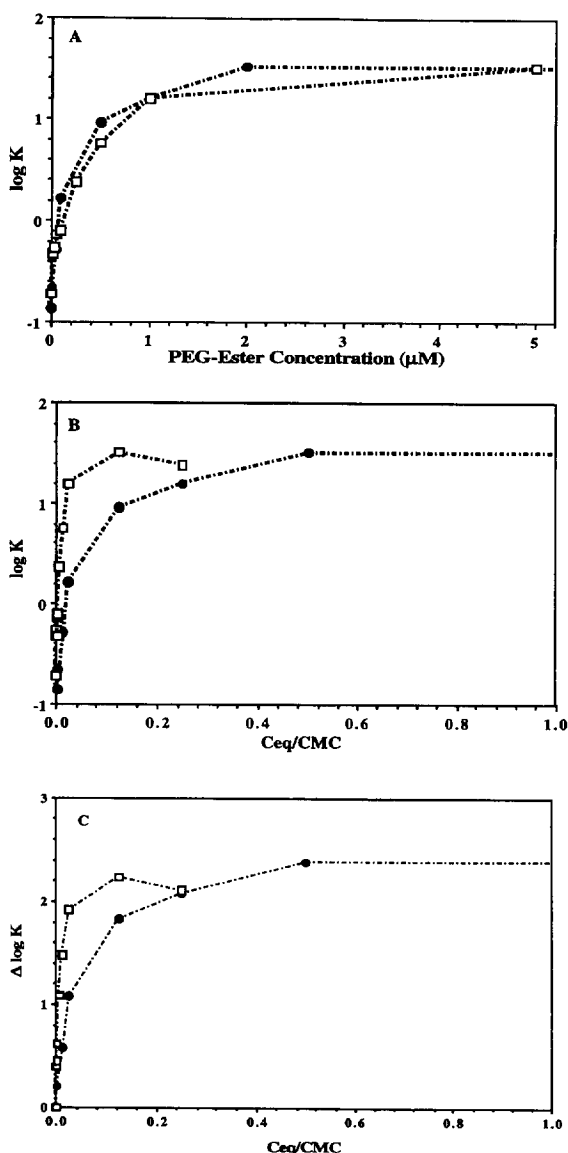
Fig. 4. PEG-rich phase partition versus (bulk system) PEG-ester concentration for DMPC liposomes at 26°C ($T > T_m$) in (5,4) V two-phase system containing EO_{151}–$C_{18:0}$ (●) or EO_{151}–$C_{18:2}$ ester (□). (A) Log K partition versus ester concentration (μM); (B) log K versus ester concentration (Ceq/CMC); (C) Δlog K versus ester concentration (Ceq/CMC).

inducing partition seen in Fig. 4A. When the data are normalized (Ceq/CMC) the ester partition curves reach a similar plateau level well below CMC. In this they mimic the ellipsometry adsorption data for $C_{16:0}$- and $C_{18:2}$–EO_{151} (see above). However, the

$C_{18:0}$-ester appears less slightly less effective than the $C_{18:2}$-ester at altering liposome partition. This may be an artifact of the larger error in estimating the CMC, as opposed to molar concentration, of the $C_{18:0}$ ester (Table 1). The $C_{18:2}$-ester data are believed to be more accurate in the normalized form.

Given a direct relationship (at first approximation, for the same system) between ester adsorption and log K [6,29,31] expressing the Fig. 4 data in terms of Δlog K versus Ceq/CMC should help isolate the partition contribution of ester adsorption from ester-free particle partition [50] and allow a more direct comparison of ellipsometry adsorption isotherms and "partition isotherms". Over a wide range of Ceq/ CMC (only a portion of which is shown) the resulting increases in PC liposome Δlog K due to adsorption of $C_{18:0}$ or $C_{18:2}$ esters is quite comparable. The data suggest that PEG-amphiphile adsorption to these particle surfaces shares many features with their adsorption to MeSi, PA or PC coated slides detected via ellipsometry. The adsorption and Δlog K versus Ceq/CMC curves are relatively independent of hydrophobic moiety, do not suggest cooperativity, exhibit an inhibitory effect of surface localization on further adsorption, and suggest that saturation adsorption is reached well before micelle formation.

Fig. 4C invites direct comparison between the adsorption of the esters at flat surfaces (determined via ellipsometry) and at particles (inferred from changes in Δlog K partition). Such a comparison is shown in Fig. 5. The data shown in Fig. 5 (but not Fig. 4) take into account the effect of the phase system on ester hydrophobicity, i.e. CMC. Sharp [31] noted that the apparent CMC of the EO_{151}–$C_{16:0}$ ester was decreased by half in the upper phase of a system similar to that used here. This effect appeared to be due to the presence of dextran, since in water containing 13% (w/w) PEG 8000 the CMC of the ester was increased to 62 μM [31]. A correction of 0.5 × CMC was therefore applied to the Fig. 5 data for the $C_{18:2}$ ester, whose CMC is similar to that of the $C_{16:0}$ ester.

Fig. 5 does not take into account the concentration-dependent partition of PEG ester between the phases. Sharp reported that the partition of EO_{151}–$C_{16:0}$ in a system similar to that used here, is approximately 65% at < 1 μM and increases with
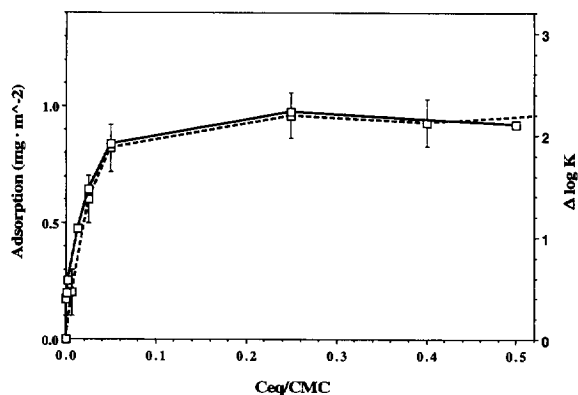
Fig. 5. Double axis plot of PEG-ester adsorption (mg/m^2) and net partition ($\Delta \log K$) versus ester concentration (Ceq/CMC) for EO_{151}–$C_{18:2}$ at PC coated slide (- - -) and PC liposome (———).

concentration [31]. More detailed information for other esters is not available. Due to the poorer solvent conditions, saturation adsorption should occur at a lower concentration in the partition environment than in the ellipsometry buffer. However, other phenomena such as adsorption of underivatized polymers will also affect ester adsorption. Such effects will modify but not eliminate the correlation noted between PEG-amphiphile adsorption detected at flat surfaces via ellipsometry and at microparticle surfaces via partition. A correlation which appears to hold for other particle types [54].

Expressing partition data as $\log K$ (which should vary with the particle surface features responsible for the partition [26,31,50] suggests that ester-induced partition of liposomes (at $T > T_m$) is not appreciably cooperative. Terminally anchored PEGs appear to enjoy conformational freedom of movement as evidenced by their ability to reduce protein adsorption [1,2,16,22,55]. Such layers may have PEG concentrations (≈ 0.07 g/cm^3) and properties similar to the PEG-enriched phases of aqueous polymer systems further removed from the critical point than the (typical) system used in the present study [26,27]. The free energy differences driving ester-induced partition [50] may therefore relate to the net interaction of the resulting PEG layers (not individually adsorbed ester molecules) with the phases. This may explain why the partition contribution of individual ester molecules reduces as their surface concentration increases. It also explains why similarly sized

particles coated with adsorbed PEG may exhibit similar partition plateau with K values greater than those of native PEG or PEG-ester monomers in solution.

Differences between the partition effects of adsorbed PEG ester and underivatized PEG are most likely due to the nature of the adsorbed polymer layer [31]. Although low molecular mass PEGs are known to cross phospholipid bilayers [56] and the terminal end regions of larger PEGs may intercalate to some extent into bilayer membranes, native PEG 6000 molecules are expected to primarily adsorb weakly and multifocally (at low surface concentration) with a very different adsorbed structure than that noted above for the PEG esters used in the present study.

PEG esters adsorb at MeSi coated surfaces via hydrophobic interaction with surface methyl groups. Adsorption at phospholipid coated slides appears to involve the intercalation of PEG-ester acyl tails between phospholipid head groups and their insertion into the underlying hydrocarbon matrix. Similarities in the adsorption isotherms at these different surfaces (Fig. 1 and Fig. 2) suggest that any free energy advantage offered by intercalation into the matrix is offset by steric and other similar hindrances posed by the PA or PC head groups. Repulsive interactions between ester PEG head groups and phospholipid phosphate groups [30,57] might combine with other phospholipid head group related hindrances, e.g. inter-group H-bonding [58,59], to reduce intercalation. If this is true, the effectiveness of PEG esters at increasing particle partition, or intercalation of underivatized PEG into the bilayer, should be significantly influenced by membrane phospholipid head group type including PC/S ratios [28,32,37,58,59]. As noted previously, it might be less affected by the nature of the covalent bond joining the PEG amphiphile to its alkyl tail [60], by substituting PA for PC ([32] and Fig. 2) or by changes in the "fluidity" of the PC matrix [6,39]. The relationship between (ester-induced) partition and UFA ratios in erythrocytes may reflect related variations of phospholipid head groups and head-group interactions [54]. Other research also suggests that phospholipid head groups are a primary determinant of liposome and cell partition [30,33,61–64].

The above studies indicate that ellipsometry can

provide insight into surface phenomena which affect partition. They also show that partition can rapidly and cost effectively evaluate, under biocompatible conditions, colloid surface interactions that cannot be readily studied by ellipsometry or other methods. The weak dependence of PEG-fatty acid ester adsorption on the underlying surface, as regards MeSi, PC and PA, implies that such amphiphiles may be used to modify a number of biomedically important surfaces. The ability to vary PEG surface concentration and molecular size should benefit a range of studies including those related to colloidal drug carriers.

5. Notation

$C_{i:j}$	i is no. carbon and j is no. methylene groups
CMC	critical micelle concentration
Ceq/CMC	bulk equilibrium concentration normalized in regard to CMC
DMPC	dimyristoyl-PC
EO	ethylene oxide unit
LMV	large multilamellar vesicle
PA	phosphatidic acid
PC	phosphatidylcholine
PEG	poly(ethylene glycol)
PEG	poly(ethylene glycol)
PPG	poly(propylene glycol)
MeSi	methylated silica
S	sphingomyelin
SUV	small unilammellar vesicle
UFA	phospholipid esterified fatty acid ratio of poly- to mono-unsaturated acyl groups

Acknowledgments

References

[1] J.M. Harris (Editor), Poly(Ethylene Glycol) Chemistry — Biotechnical and Biomedical Applications, Plenum Press, New York, 1992.

[2] M. Malmsten and J.M. Van Alstine, Colloids Surf. B, (1996) submitted.

[3] B. Lassen and M. Malmsten, J. Mater. Sci. Mater. Med., 5 (1994) 662.

[4] C. Delgado, G.E. Francis and D. Fisher, Crit. Rev. Ther. Drug Carrier Syst., 9 (1992) 249.

[5] L.J. Karr, J.M. Harris, S.G. Shafer, J.M. Van Alstine, and R.S. Snyder, J. Chromatogr., 442 (1988) 219.

[6] D.E. Brooks, J.M. Van Alstine, S.J. Stocks, and K.A. Sharp, in J.M. Harris (Editor), Poly(Ethylene Glycol) Chemistry — Biotechnical and Biomedical Applications, Plenum Press, New York, 1992, pp. 57–71.

[7] L. Illum, P. West, C. Washington and S.S. Davis, Int. J. Pharm., 54 (1989) 41.

[8] A. Mori, A.L. Klibanov, V.P. Torchilin and L. Huang, Fed. Eur. Biochem. Soc. Lett., 284 (1991) 263.

[9] H.M. Patel, Crit. Rev. Ther. Drug Carrier Syst., 9 (1992) 39.

[10] A. Chonn, S.C. Semple and P.R. Cullis, J. Biol. Chem., 267 (1992) 18759.

[11] V. Thomas, K. Bergström, G. Quash and K. Holmberg, Colloids Surf. A, 77 (1993) 125.

[12] E. Österberg, K. Bergström, K. Holmberg, J.A. Riggs, J.M. Van Alstine, T.P. Schuman, N.L. Burns and J.M. Harris, Colloids Surf. A, 77 (1993) 159.

[13] J.F. Boyce, B.A. Hovanes, J.M. Harris, J.M. Van Alstine and D.E. Brooks, J. Colloid Interface Sci., 149 (1992) 153.

[14] J.M. Van Alstine, N.L. Burns, J.A. Riggs, K. Holmberg and J.M. Harris, Colloids Surf. A, 77 (1993) 149.

[15] N.L. Burns, J.M. Van Alstine and J.M. Harris, Langmuir, 11 (1995) 2768–2776.

[16] K.L. Prime and G.M. Whitesides, J. Am. Chem. Soc., 115 (1993) 10714.

[17] Y.S. Lin, V. Hlady, C.-G. Gölander, Colloids Surf. B, 3 (1994) 49.

[18] S.W. Kim and J. Feijen, Crit. Rev. Biocomp., 1 (1986) 229.

[19] U. Jönsson, B. Ivarsson, I. Lundström and L. Berghem, J. Colloid Interface Sci., 90 (1982) 148.

[20] M. Malmsten, J. Colloid Interface Sci., 166 (1994) 333.

[21] M. Malmsten and B. Lassen, J. Colloid Interface Sci., 166 (1994) 490.

[22] M. Malmsten and J.M. Van Alstine, J. Colloid Interface Sci., (1996) in press.

[23] K. Lim and J.N. Herron, in J.M. Harris (Editor), Poly(Ethylene Glycol) Chemistry — Biotechnical and Biomedical Applications, Plenum Press, New York, 1992, pp. 29–55.

[24] R.M.A. Azzam and N.M. Bashara, Ellipsometry and Polarized Light, North-Holland Press, Amsterdam, 1989.

[25] F. Tiberg and M. Landgren, Langmuir, 9 (1993) 927.

[26] P.-Å Albertsson, Partition of Cell Particles and Macromolecules, Wiley-Interscience, New York, 1986

[27] H. Walter and G. Johansson (Editors), Aqueous Two-phase Systems, Methods in Enzymology, Vol. 228, Academic Press, New York, 1994.

[28] E. Eriksson, P.-Å Albertsson and G. Johansson, Mol. Cell. Biochem., 256 (1986) 123.

[29] K.A. Sharp, Biophys. J., 41 (1983) 353a.

[30] H. Walter, H., E.J. Krob and D.E. Brooks, Biochemistry, 15 (1976) 2959.

[31] K.A. Sharp, Theoretical and Experimental Studies on Erythrocyte Partition in Aqueous Polymer Two-Phase Systems, Ph.D. Thesis, The University of British Columbia, Vancouver, Canada, 1985.

[32] E. Eriksson and P.-Å. Albertsson, Biochim. Biophys. Acta, 507 (1978) 425.

[33] H. Walter, E.J. Krob and R. Tung, Exp. Cell Res., 102 (1976) 14.

[34] J.M. Van Alstine, P.B. Sorrenson, T.J. Webber, R.G. Grieg, G. Poste and D.E. Brooks, Exp. Cell Res., 164 (1986) 366.

[35] J.M. Van Alstine, T.J. Trust and D.E. Brooks, Applied Environ. Microbiol., 51 (1986) 1309.

[36] J.M. Van Alstine and D.E. Brooks, Clin. Chem., 30 (1984) 441.

[37] C. Tilcock, P.R. Cullis, T. Dempsey, B. Youens and D. Fisher, Biochim. Biophys Acta, 979 (1989) 208.

[38] B. Deuticke, Biochim. Biophys Acta, 163 (1968) 494.

[39] J.M. Van Alstine, Cell Partition in Aqueous Polymer Two-Phase Systems, Ph.D. Thesis, The University of British Columbia, Vancouver, Canada, 1984.

[40] J.M. Harris, E.C. Struck, M.G. Case, S.G., Paley, M. Yalpani, J.M. Van Alstine and D.E. Brooks, J. Polym. Sci., Polym. Chem. Ed., 22 (1984) 341.

[41] J.M. Van Alstine, K.A. Sharp and D.E. Brooks, Colloids Surf., 17 (1986) 115.

[42] M. Landgren and B. Jönsson, J. Phys. Chem., 97 (1993) 1656.

[43] G.J. Fleer, M.A. Cohen Stuart, J.M.H.M. Scheutjens, T. Cosgrove and B. Vincent, Polymers at Interfaces, Chapman and Hall, London, 1993.

[44] J.A. de Feijter, J. Benjamins and F.A. Veer, Biopolymers, 17 (1978) 1759.

[45] A. Tontisakis, R. Hilfiker and B. Chu, J. Colloid Interface Sci., 135 (1990) 427.

[46] A. Watts, D. Marsh and P.F. Knowles, Biochemistry, 17 (1978) 1792.

[47] C. Huang and T.E. Thompson, in N.O. Kaplan (Editor), Methods in Enzymology, Vol. 32, Academic Press, New York, 1974, pp. 485–489.

[48] B. DeKruijff, P.R. Cullis and G.K. Radda, Biochim. Biophys. Acta, 406 (1975) 6.

[49] K. Devanand and J.C. Selser, Macromolecules, 24 (1991) 5943.

[50] D.E. Brooks, K.A. Sharp and D. Fisher, in H. Walter, D.E. Brooks and D. Fisher (Editors), Partitioning in Aqueous Two-Phase Systems, Academic Press, New York, 1985, pp. 11–85.

[51] F. Tiberg, B. Jönsson and B. Lindman, Langmuir, 10 (1994) 3714.

[52] M.R. Böhmer and L.K. Koopal, Langmuir, 6 (1990) 1478.

[53] M. Malmsten, P. Linse and T. Cosgrove, Macromolecules, 25 (1992) 2474.

[54] J.M. Van Alstine and M. Malmsten, J. Biol. Chem., (1996) submitted.

[55] S.I. Jeon and J.D. Andrade, J. Colloid Interface Sci., 142 (1991) 159.

[56] L.L.M. Van Deenen, in D. MacN. Surgenor (Editor), The Red Blood Cell, Vol. 1, Academic Press, New York, 1974, pp. 147–211.

[57] S. Bamberger, G.V.F. Seaman, K.A. Sharp and D.E. Brooks, J. Colloid Interface Sci., 99 (1984) 194.

[58] E. Eriksson, J. Chromatogr., 205 (1981) 189.

[59] E. Eriksson, Partition of Membranes in Aqueous Two-Phase Systems, Ph.D. Thesis, Lund University, Sweden, 1981.

[60] J.M. Harris, M.G. Case, B.A. Hovanes, J.M. Van Alstine and D.E. Brooks, Ind. Eng. Prod. Res. Dev., 23 (1984) 86.

[61] P.T. Sharpe and G.S. Warren, Biochim. Biophys. Acta, 772 (1984) 176.

[62] P.T. Sharpe, Mol. Cell. Biochem., 68 (1985) 151.

[63] C. Tilcock, R. Chin, J. Veiro, P.R. Cullis and D. Fisher, Biochim. Biophys Acta, 986 (1989) 167.

[64] L. McEvoy, P. Williamson and R.A. Schlegel, Proc. Natl. Acad. Sci. USA, 83 (1986) 3311.