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Interaction of human serum albumin with membranes containing polymer-grafted lipids: spin-label ESR studies in the mushroom and brush regimes

Rosa Bartucci [a], Manuela Pantusa [a], Derek Marsh [b], Luigi Sportelli [a,*]

[a] *Lab. di Biofisica Molecolare, Dipartimento di Fisica and Unità INFM, Università della Calabria, Ponte P. Bucci, Cubo 31 C, I-87036 Arcavacata di Rende (CS), Italy*

[b] *Max-Planck-Institut fur Biophysikaliche Chemie, Abteilung Spektroskopie, D-37070 Gottingen, Germany*

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Abstract

The adsorption of human serum albumin (HSA) to dipalmitoyl phosphatidylcholine (DPPC) bilayer membranes containing poly(ethylene glycol)-grafted dipalmitoyl phosphatidylethanolamine (PEG-DPPE) was studied as a function of content and headgroup size of the polymer lipid. In the absence of protein, conversion from the low-density mushroom regime to the high-density brush regime of polymer-lipid content is detected by the change in ESR outer hyperfine splitting, $2A_{max}$, of chain spin-labelled phosphatidylcholine in gel-phase membranes. The values of $2A_{max}$ remain constant in the mushroom regime, but decrease on entering the brush regime. Conversion between the two regimes occurs at mole fractions $X_{PEG}(m \rightarrow b) \approx 0.04$, 0.01–0.02 and 0.005–0.01 for PEG-DPPE with mean PEG molecular masses of 350, 2000 and 5000 Da, respectively, as expected theoretically. Adsorption of HSA to DPPC membranes is detected as a decrease of the spin label $2A_{max}$ hyperfine splitting in the gel phase. Saturation is obtained at a protein/lipid ratio of ca. 1:1 w/w. In the presence of polymer-grafted lipids, HSA adsorbs to DPPC membranes only in the mushroom regime, irrespective of polymer length. In the brush regime, the spin-label values of $2A_{max}$ are unchanged in the presence of protein. Even in the mushroom regime, protein adsorption progressively becomes strongly attenuated as a result of the steric stabilization exerted by the polymer lipid. These results are in agreement with theoretical estimates of the lateral pressure exerted by the grafted polymer in the brush and mushroom regimes, respectively.

Keywords: Polymer lipid; PEG-lipid; Serum albumin; Steric protection; Opsonization; Liposome; Lipid–protein interaction; Spin label; Electron spin resonance

1. Introduction

Opsonization, i.e., the adsorption of serum proteins from plasma, and subsequent recognition by components of the immune system lead to the clearance of intravenously administered liposomes from the blood circulation. The lifetime of liposome-based drug carriers can be increased substantially by inclusion of lipids with hydrophilic polymer headgroups in the vesicle membrane [1]. Phosphatidylethanolamines *N*-derivatised by poly(ethylene glycol) (PEG-PEs) have proved to be particularly useful grafted lipids for producing such sterically stabilised liposomes.

The nature of the interaction of polymer-grafted liposomes with serum proteins is therefore fundamental to their effective interaction (see, e.g. Ref. [2]). Previous biophysical studies have concentrated much on the theoretical aspects of protein interactions with polymer lipids [3–6]. In the present work, we have studied the interaction of human serum albumin (HSA) with dipalmitoyl phosphatidylcholine (DPPC) bilayer lipid membranes containing varying amounts of PEG-DPPE polymer-lipids of different polymer sizes. This was done by using electron spin resonance (ESR) spectroscopy of spin-labelled phosphatidylcholine at the C-5 position in the *sn*-2 acyl chain (5-PCSL). Adsorption of HSA to the DPPC membranes increases the mobility registered by ESR spectra of the spin-labelled lipid chains in the gel phase. The spin-label mobility measurements also reflect the changes in lipid packing density (cf. Ref. [7]) on transition from the mushroom to brush regime of polymer-lipid concentration. Addition of poly(ethylene glycol)-lipids (PEG-lipids) abolishes

* Corresponding author. Tel.: +39-984-496076; fax: +39-984-494401.
E-mail address: sportelli@fis.unical.it (L. Sportelli).

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the protein-induced changes in the brush regime at high polymer grafting density, and also progressively attenuates the primary adsorption of HSA in the mushroom regime of low polymer grafting density.

2. Materials and methods

2.1. Materials

HSA and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) were from Sigma (St. Louis, MO). High purity (>99%) PEG-lipids, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-poly(ethylene glycol), with average polymer molecular weights 350, 2000 or 5000 Da (PEG:350-DPPE, PEG:2000-DPPE, and PEG:5000-DPPE, respectively), and spin-labelled phosphatidylcholine 1-palmitoyl-2-(5-(4,4-dimethyl-oxazolidine-*N*-oxyl)stearoyl)-*sn*-glycero-3-phosphocholine (5-PCSL), were from Avanti Polar Lipids (Birmingham, AL). Reagent grade salts for the 10 mM phosphate buffer solution (PBS) at pH = 7.2 were from Merck (Darmstadt, Germany).

2.2. Spin-label ESR measurements

Samples for ESR measurement were prepared by dissolving the required amounts of DPPC and PEG-DPPE, together with 1% by weight of the spin-labelled lipid (5-PCSL), in chloroform–methanol. The solvent was evaporated in a nitrogen gas stream and then kept under vacuum overnight. The dried lipid samples were hydrated either with PBS at pH 7.2 or with the desired concentration of HSA in the same buffer (final lipid concentration 50 mM), by heating and vortexing for ca. 30 min at 45 °C. The hydrated lipid dispersions were concentrated by centrifugation, sealed in 1-mm (i.d.) 100-μl glass capillaries and then incubated at 4 °C before measurement. ESR spectra were recorded on a 9-GHz Bruker (Karlsruhe, Germany) spectrometer, model ER 200D-SRC. Sample capillaries were inserted in a standard 4-mm (i.d.) quartz ESR tube containing light silicone oil for increased thermal stability, and were centered in a TE_{102} rectangular ESR cavity (ER 4201, Bruker). Sample temperature was controlled with a Bruker ER 4111VT variable temperature control unit (accuracy ± 0.5 °C).

At certain temperatures in the gel phase and for a given DPPC/PEG-DPPE composition, the conventional ESR spectra of chain spin-labelled phosphatidylcholine showed two distinct spectral components [7,8]. One component corresponds to the lamellar gel phase environment that is characteristic of the mixtures of lipids and low content of PEG-lipids. The second sharper component represents the quasi-isotropic environment of the micelles of PEG-lipids. The bilayer–micelle conversion with increasing PEG-DPPE content in DPPC/PEG-DPPE mixtures was studied resolving and quantifying the micellar population by means of spectral subtractions as described in Ref. [9]. The mole fractions of PEG-lipid at the onset of micellisation have been determined as: $X_{PEG}^{on} = 0.22 \pm 0.07$, 0.07 ± 0.03 and 0.03 ± 0.01 for mixtures of DPPC with PEG:350-DPPE, PEG:2000-DPPE and PEG:5000-DPPE, respectively [7,8]. The spin-label ESR measurements for the present study were all confined to the lamellar phase of DPPC/PEG-DPPE mixtures, i.e., to contents of polymer lipid below X_{PEG}^{on}.

2.3. Turbidimetric measurements

Aqueous dispersions of DPPC/PEG-DPPE for spectrophotometric measurement were prepared as described above, but with omission of the spin label. The lipid suspensions were transferred to 3-ml quartz cells of 1-cm optical path, and incubated at 4 °C before measurement. The final lipid concentration was ca. 2 mg/ml. Optical density measurements were made at 400 nm with a Jasco 7850 spectrophotometer, equipped with a Peltier thermostatted cell holder (model EHC-441) at 20 ± 0.1 °C.

3. Results

3.1. Adsorption of HSA to DPPC

The ESR spectra at 20 °C of the 5-PCSL spin label in gel-phase DPPC membranes, in the presence and absence of HSA (1:1 w/w), are given in the inset to Fig. 1. Adsorption of the protein to the membranes causes a reduction of ca. 1.8 G in the outer hyperfine splitting, $2A_{max}$, in the ESR spectrum of the spin-labelled lipid chains. The size of this decrease remains approximately constant throughout the gel phase, up to the start of the DPPC pretransition at ca. 32 °C (see Fig. 2). In the fluid phase, above 42 °C, the value of $2A_{max}$ is only slightly smaller (by ca. 0.6 gauss) in the presence of HSA than in its absence (see Fig. 2). The dependence of $2A_{max}$ at 20 °C on the protein/lipid ratio is given in the main body of Fig. 1. Initially, the outer hyperfine splitting decreases rapidly with added protein, and reaches a minimum, saturation value at an HSA/DPPC ratio of ca. 1:1 w/w. At the same protein/lipid ratio, a saturation value is also obtained for the optical density at 400 nm, OD_{400}, of the dispersions by using turbidimetry (see Fig. 3). All subsequent measurements were therefore performed at this protein/lipid ratio of 1:1 w/w. At protein/lipid ratios higher than 1:1 w/w, the value of $2A_{max}$ begins to increase (see Fig. 1), achieving a final saturation value of ca. 62.7 G at an HSA/lipid ratio of 3:1 w/w. This biphasic response to protein binding will be discussed later.

3.2. Dependence of ESR spectra from 5-PCSL on polymer-lipid content

The dependence of the outer hyperfine splitting of 5-PCSL on PEG-lipid content of DPPC membranes in the gel

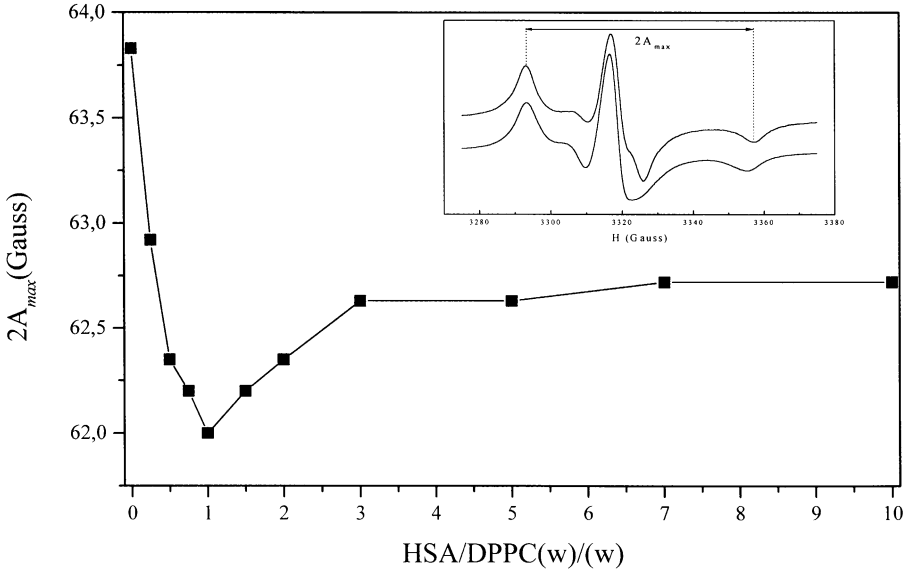

Fig. 1. Dependence of the outer ESR hyperfine splitting, $2A_{max}$, from the 5-PCSL phosphatidylcholine spin label in DPPC membranes at 20 °C on the weight ratio of human serum albumin added to the lipid dispersion. Inset: ESR spectra of 5-PCSL in DPPC membranes at 20 °C, in the presence (lower) and absence (upper) of 1:1 w/w HSA.

phase at 20 °C is given by the open square symbols in Fig. 4. Panels A, B and C correspond to the PEG:350, PEG:2000 and PEG:5000 polymer lipids, respectively. The dependence is seen with best resolution for PEG:350-DPPE because the changes in $2A_{max}$ extend over the largest range of mole fractions with the short polymer lipid. After an initial constant region, the value of $2A_{max}$ drops steeply at 3–5 mol% PEG:350-DPPE (see Fig. 4A). Beyond this, the outer hyperfine splitting varies only slowly from 7 to 23 mol% PEG-lipid, after which PEG:350-DPPE begins to induce micelle formation in mixtures with DPPC [8]. A similar overall pattern is observed with the longer polymer lipids PEG:2000 and PEG:5000-DPPE, except that the whole dependence is shifted progressively to lower mole fractions, such that the initial constant region is less well resolved (Fig. 4B,C). The onset and completion of the sharp drop in $2A_{max}$ occur at ca. 0.5 and 3 mol%, respectively, for PEG:2000-DPPE, and at ca. 0.25 and 1 mol% for PEG:5000-DPPE. As elaborated further in Discussion, the initial constant region of high and constant $2A_{max}$ corresponds to noninteracting polymer headgroups in the mushroom regime of low grafting density. The subsequent decrease in $2A_{max}$ results from interactions between the polymer headgroups in the brush regime of high grafting density.

3.3. Adsorption of HSA to DPPC/PEG-DPPE mixed membranes

Adsorption of HSA added at a 1:1 w/w ratio with respect to DPPC gives rise to a pronounced decrease in $2A_{max}$ of 5-

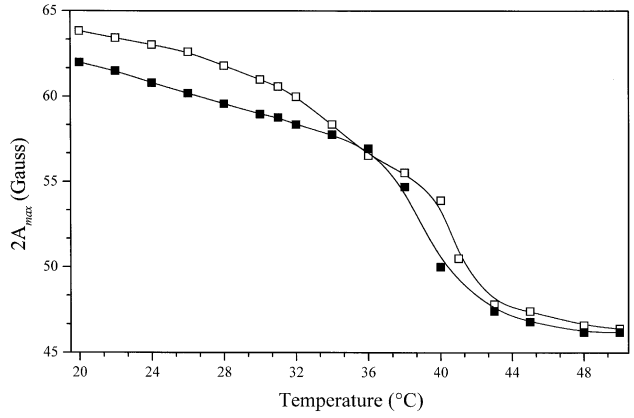

Fig. 2. Temperature dependence of the outer ESR hyperfine splitting, $2A_{max}$, from the 5-PCSL phosphatidylcholine spin label in DPPC membranes in the absence (open squares) and in the presence (solid squares) of 1:1 w/w of human serum albumin.

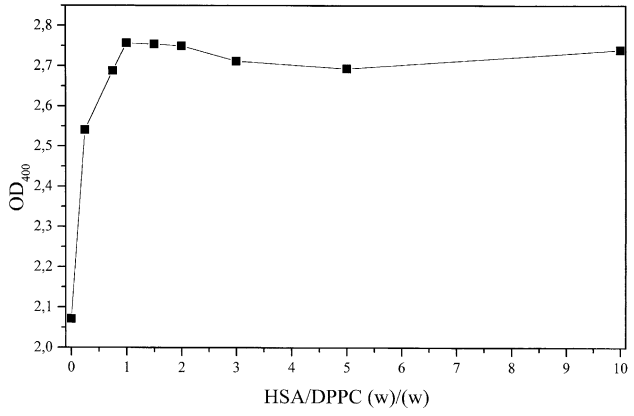

Fig. 3. Dependence of the optical density at 400 nm of DPPC bilayer dispersions at 20 °C on the weight ratio of HSA added to the lipid dispersion.

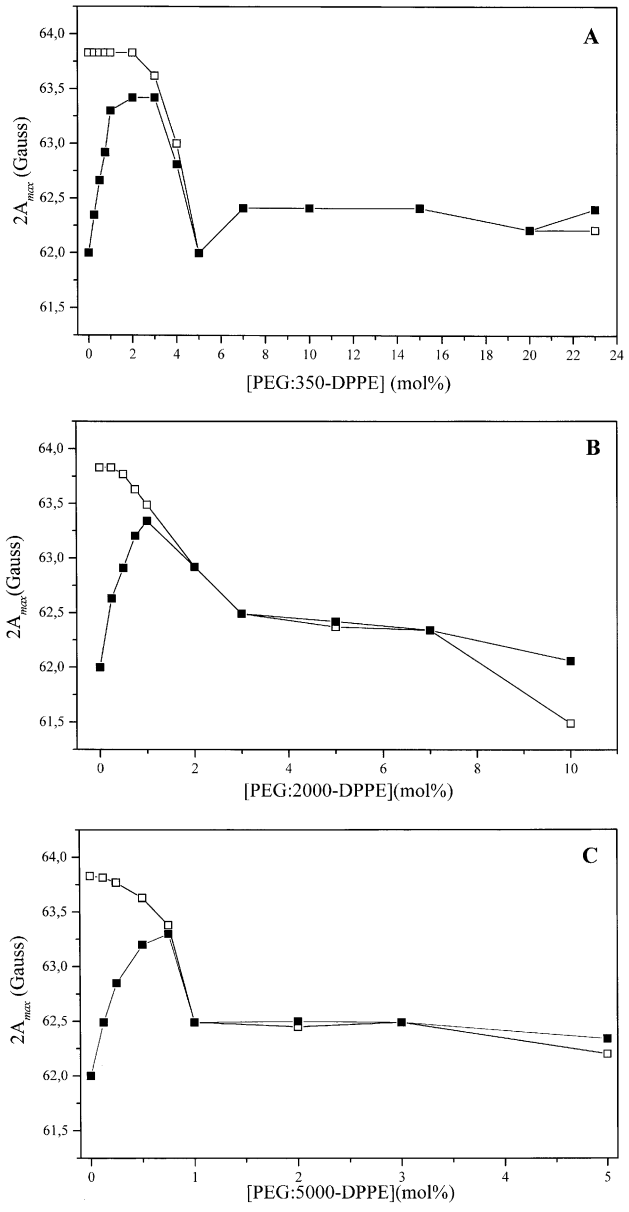

Fig. 4. Dependence of the outer ESR hyperfine splitting, $2A_{max}$, from the 5-PCSL phosphatidylcholine spin label on content of PEG-DPPE polymer lipid in DPPC membranes at 20 °C, in the absence (open squares) and in the presence (solid squares) of 1:1 w/w of human serum albumin. Mixtures of DPPC are with: (A) PEG:350-DPPE, (B) PEG:2000-DPPE, and (C) PEG:5000-DPPE.

PCSL in the gel phase, as already noted (see Figs. 1 and 2). Admixture of up to 1 mol% PEG:350-DPPE to DPPC progressively diminishes, but does not totally abolish, the protein-induced increase in spin-labelled lipid chain mobility (see Fig. 4A). This more limited adsorption of HSA at 2 mol% PEG:350-DPPE is characterised by a reduction in $2A_{max}$ of ca. 0.4 G. It persists, at least to an attenuated extent, throughout the mushroom and brush transitional regimes of polymer lipid content, up to 4 mol% PEG:350-DPPE. Above this level of PEG-lipid admixture, addition of HSA has no effect on the lipid chain mobility; primary adsorption of HSA to the lipid membranes no longer takes place. Examination of Fig. 4B,C reveals a similar pattern for the adsorption of HSA to DPPC membranes containing the longer PEG-lipids. Adsorption of HSA is decreased progressively by addition of polymer-lipid in the mushroom regime and is abolished in the brush regime. The mole fractions at which this occurs are, however, correspondingly reduced for the longer polymer lipids.

4. Discussion

This study yields information not only on the interaction of HSA with sterically stabilised liposomes, but also on the conversion between the mushroom and brush regimes of polymer grafting density. The latter is discussed first, before going on to consider interactions of HSA with polymer lipids.

4.1. Mushroom and brush regimes

In the mushroom regime at low grafting densities, the polymer headgroups do not interact. The surface polymer chains assume a random configuration, the characteristic dimensions of which are determined by the Flory radius, R_F. n**T**his scales with polymer length, n_p, according to Ref. [10]:

$$R_F \approx a_m n_p^{3/5} \quad (1)$$

where a_m is the effective size of a monomer unit that depends upon the relative contributions of stretching and excluded volume to the free energy. An experimental value of $a_m = 0.43$ nm was obtained for PEG-lipids by Evans et al. [11] from fitting adhesion measurements. Adopting this value gives $R_F \approx 1.5$, 4.2 and 7.4 nm for PEG:350, PEG:2000 and PEG:5000, respectively. The transition to the high-density brush regime occurs at a content of grafted lipid for which the surface-associated polymer mushrooms first begin to overlap. This occurs at a mole fraction of PEG-lipid given by:

$$X_{PEG}^{m\to b} \gtrsim A_l/\pi R_F^2 \quad (2)$$

where A_l is the membrane area per lipid molecule. For DPPC in the gel phase: $A_l \approx 0.48$ nm^2 [12], thus Eq. (2) predicts that the conversion from mushroom to brush configurations takes place at $X_{PEG}(m \to b) \geq 0.07$, 0.009 and 0.003 for PEG:350, PEG:2000 and PEG:5000, respectively.

The mushroom regime is delineated in the ESR experiments by the region of approximately constant $2A_{max}$ at low PEG-lipid contents in the absence of protein (see Fig. 4). This is followed by a steep drop corresponding to the transition to the brush regime. The latter is characterised by an increased mobility of the lipid chains that results from the expansion in membrane area induced by the lateral

pressure in the polymer brush [7]. The midpoint at which the sharp decrease occurs in $2A_{max}$ (i.e., $X_{PEG} \approx 0.04$, 0.015 and 0.0075, for PEG:350-DPPE, PEG:2000-DPPE and PEG:5000-DPPE, respectively) agrees reasonably well with the theoretical estimates for the conversion from mushroom to brush regimes given immediately above. The midpoint of the transition in Fig. 4 scales according to polymer length with an exponent of ca. -0.8, which is less steep than the value of ca. $-6/5$ predicted from Eqs. (1) and (2) but of a similar magnitude.

4.2. Adsorption of HSA

In the absence of PEG-lipid, HSA adsorbs to DPPC membranes and reduces the outer hyperfine splitting, $2A_{max}$, of the 5-PCSL spin label by 1.8 G at 20 °C. This corresponds to a loosening of the lipid packing in the gel phase, which is opposite to the effects of binding basic proteins to fluid-phase anionic membranes [13,14]. In the latter cases, the binding is primarily of direct electrostatic origin. Also, the adsorption of HSA to phosphatidylcholine causes only rather small changes of lipid mobility in the fluid phase.

Extrapolating the initial slopes in Figs. 1 and 3 to the quasi-saturation values attained at a protein/lipid ratio of ca. 1:1 w/w and assuming a molecular mass of ~68 kDa for HSA, it can be estimated that there are ca. 150 DPPC lipids per HSA molecule. The HSA monomer is an approximately heart-shaped molecule with overall dimensions $8 \times 8 \times 3$ nm [15]. The larger cross-sectional area is therefore 64 nm^2, as compared with a membrane surface area of $\sim$ 72 nm^2 provided by 150 DPPC molecules in the gel phase. This comparison suggests that, at a 1:1 w/w ratio of HSA to DPPC, protein absorption approaches complete surface coverage of lipid membrane. The biphasic response in Fig. 1 that is characterised by a subsequent limited increase in the values of $2A_{max}$ at protein/lipid ratios >1:1 w/w may correspond to a rearrangement in orientation of the adsorbed HSA such that the smaller protein cross-section of 24 nm^2 is preferentially presented to the membrane surface. Notably, the protein/lipid ratio of 1:3 w/w is close to that of the minimum to maximum protein cross section.

4.3. Suppression of protein absorption by PEG-lipids

Because HSA is a relatively large protein ($\sim 8 \times 8 \times 3$ nm), primary adsorption, i.e., adsorption directly at the lipid surface, occurs only at low grafting densities. It is seen from Fig. 4 that, in practice, this means only in the "mushroom" regime for lamellar membranes. If it is assumed that the membrane surface area occupied by the polymer mushrooms is inaccessible to HSA, protein adsorption, P, will decrease linearly with mole fraction, X_{PEG}, of polymer lipid according to:

$$P/P_o = 1 - X_{PEG}/X_{PEG}^{m \to b} \quad (3)$$

where P_o is the protein adsorption in the absence of polymer lipid and $X_{PEG}^{m \to b}$ is the mole fraction of polymer lipid corresponding to complete surface coverage. The latter is given by Eq. (4) taken as an equality. Although the outer hyperfine splitting is not necessarily a linear measure of protein adsorption, the results of Fig. 4 are qualitatively in agreement with Eq. (3), in the mushroom regime. The form of Eq. (3) automatically implies that protein adsorption is abolished in the brush regime. A nonlinear approach to zero adsorption might be expected close to the mushroom–brush transition, however, if the protein compresses the polymer mushrooms somewhat. Such a deviation from the simple predictions of Eq. (3) is observed, at least for PEG:350-DPPC (Fig. 4A).

Admixture of low concentrations of PEG-lipid in the mushroom regime decreases the protein-induced reduction in $2A_{max}$ by 50% at 0.75 mol% PEG:350-DPPE, 0.5 mol% PEG:2000-DPPE and 0.25 mol% PEG:5000-DPPE, respectively (Fig. 4A–C). For comparison, binding of HSA to DSPE is reduced to $\sim$ 50% by admixture of 1.4 mol% of PEG:2000-DSPE [2]. The latter corresponds to total protein adsorption, both primary adsorption to the lipid surface and secondary adsorption at the polymer headgroup, to which the spin-label measurements are not sensitive. Note also that HSA is an acidic protein (pI=4.7) and therefore its adsorption is disfavoured by the electrostatic membrane surface potential introduced by admixture of negatively charged PEG-lipids.

In the brush regime, the values of $2A_{max}$ are identical in the presence and absence of protein. The most likely explanation for this result is that the protein no longer binds to the polymer-grafted membranes, in the brush regime. For comparison, direct binding experiments reveal that almost no HSA adsorbs to DSPE membranes that contain 4.7 mol% of PEG:2000-DSPE [2]. This is in agreement with the present results for ≥ 3 mol% of PEG:2000-DPPE in DPPC (Fig. 4B), and would tend to support our interpretation. Secondary adsorption at the periphery of the polymer brush cannot be excluded by the ESR experiments, because this location may be too remote to affect the mobility of the spin-labelled lipid. The small increase in $2A_{max}$ in the presence of protein at 23 mol% PEG:350-DPPE (Fig. 4A), or 10 mol% PEG:2000-DPPE (Fig. 4B), or 5 mol% PEG:5000-DPPE (Fig. 4C) might be the result of secondary adsorption. These polymer-lipid contents correspond to the onset of micelle formation [7,8]. Therefore, the nature of the secondary adsorption may change at this point.

Complete exclusion of the protein from the liposome surface in the brush regime, relative to only partial exclusion in the mushroom regime, can be understood at least qualitatively in terms of the lateral pressure exerted in the polymer brush (cf. Ref. [7]). An expression for the latter obtained from the standard theories of polymer physics is (see, e.g., Ref. [16]):

$$\Pi_{PEG}^{brush} = m_F k_B T n_p a_m^{2m_F} (X_{PEG}/A_l)^{m_F+1} \quad (4)$$

where X_{PEG} is the mole fraction of polymer lipid, k_B is Boltzmann's constant and T is the absolute temperature. The exponent m_F is 5/6 in the Alexander-de Gennes scaling theory, and is 2/3 in the mean-field theory. The mole fraction of PEG-lipid at which the effect on HSA adsorption in the mushroom regime is already appreciable (~ 50% reduction) has a ratio of ca. 0.15–0.2, relative to that at the beginning of the brush regime, for both PEG:350-DPPE and PEG:2000-PPE (see Fig. 4). From the scaling according to ${X_{PEG}}^{m+1}$ in Eq. (4), this implies that the lateral pressure is increased by a factor of ca. 20 × in the brush regime. This should certainly be sufficient to suppress primary adsorption completely, given that it is already reduced substantially in the mushroom regime.

Extrapolation with Eq. (4) may tend to underestimate the lateral pressure in the mushroom regime, because partial compression of the polymer chains is assumed. An upper estimate for the mushroom regime can be obtained from the Volmer equation of state with an excluded area per polymer lipid of $\pi {R_F}^2$:

$$\Pi_{PEG}^{mush} = \frac{k_B T \cdot X_{PEG}}{A_l - \pi R_F^2 \cdot X_{PEG}} \quad (5)$$

At $X_{PEG} = 0.01$ for PEG:350-DPPE, this amounts to ${\Pi_{PEG}}^{mush} \approx 0.1$ mN m^{-1}. The repulsive free energy for adsorption of HSA would then correspond to ${\Pi_{PEG}}^{mush}$. $N_A A_p \approx +1.5$–4 kJ mol^{-1}. The larger value would result in a reduction in the extent of binding to ca. 20% of that in the absence of the polymer lipid, i.e., comparable to the value suggested from the changes in $2A_{max}$ for the 5 PCSL spin label.

5. Conclusions

The transition from mushroom to brush regimes of polymer-grafted lipid density scales in the expected manner with polymer length. HSA adsorbs strongly to the surface of phosphatidylcholine membranes, reducing the lipid packing density in the gel phase. Primary adsorption of the protein is strongly reduced by polymer-grafted lipids, already in the low-density mushroom regime, and is completely eliminated in the high-density brush regime. These results hold quite generally for the different PEG-lipids, irrespective of the polymer length, and are in accord with estimates made by using the theories of polymer physics.

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