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Transfer of stearic acids from albumin to polymer-grafted lipid containing membranes probed by spin-label electron spin resonance

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Abstract

Human serum albumin (HSA) has been spin-labelled with stearic acids having the nitroxide moiety attached to the hydrocarbon chain either at the 5th or at the 16th carbon atom (n-SASL, n=5 and 16, respectively) with respect to the carboxyl groups. Its interaction with sterically stabilized liposomes (SSL) composed of dipalmitoylphosphatidylcholine (DPPC) mixed with submicellar content of poly(ethylene glycol:2000)-grafted dipalmitoyl phosphatidylethanolamine (PEG:2000-DPPE) has been studied by conventional electron spin resonance (ESR) spectroscopy. In the absence of bilayer membranes, the ESR spectra of nitroxide stearic acids non-covalently bound to HSA are single component powder patterns, indicative of spin labels undergoing temperature dependent anisotropic motion in the slow motional regime on the conventional ESR timescale. The adsorption of HSA to DPPC bilayers results in two component ESR spectra. Indeed, superimposed to an anisotropic protein-signal appears a more isotropic signal due to the labels in the lipid environment. This accounts for the transfer of fatty acids from the protein to DPPC bilayers. Two spectral components with different rotational mobility are also singled out in the spectra of n-SASL bound to HSA when DPPC/PEG:2000-DPPE mixtures are present in the dispersion medium. The fraction, $f_L(16\text{-}SASL)$, of spin labels transferred from the protein to lipid/polymer-lipid lamellar membranes has been quantified performing spectral subtraction. It is found that $f_L(16\text{-}SASL)$ decreases on increasing the content of the polymer-lipid mixed with DPPC. It is strongly reduced in the low-density mushroom regime and levels off in the high-density brush regime of the polymer-lipid content as a result of the steric stabilization exerted by the PEG-lipids. Moreover, the fraction of transferred fatty acids from HSA to SSL is dependent on the physical state of the lipid bilayers. It progressively increases with increasing the temperature from the gel to the liquid-crystalline lamellar phases of the mixed lipid/polymer-lipid membranes, although such a dependence is much weaker in the brush regime.
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Keywords: Human serum albumin; PEG-lipids; Sterically stabilized liposomes; Stearic acids spin labels; Electron spin resonance; Fatty acids transfer

1. Introduction

Human serum albumin (HSA) is the most abundant protein in the blood plasma. It is a multifunctional protein that binds, stores and transports a variety of drugs, ligands, as well as hydrophobic and amphipathic molecules, combining high affinity and broad specificity [1,2]. In particular, HSA shows a strong affinity to bind reversibly and non-covalently fatty acids, and it is the major transport protein for fatty acids.

Recently, it has been shown, both theoretically and experimentally, that the adsorption of plasma proteins to bilayer membranes is attenuated and, in some cases, totally inhibited when polymer-grafted lipids are mixed with lipids to form long circulating sterically stabilized liposomes (SSL) [3–9]. In particular, by using a chain labelled phospholipid and conventional electron spin resonance (ESR) spectroscopy, it has been shown that HSA adsorbs to the surface of dipalmitoylphosphatidylcholine (DPPC) membranes in the gel phase [8]. The primary adsorption of HSA is first reduced and then abolished when the content of poly(ethylene glycol)s-grafted dipalmitoyl phosphatidylethanolamine (PEGs-DPPE) mixed with DPPC is increased

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from the low-density mushroom regime to the high-density brush regime of polymer-lipids content [8].

Further insights into the interaction of HSA with SSL can be gained by ESR spin labelling the protein HSA instead of the lipid component of the samples. Exploiting its affinity to bind fatty acids, the protein labelling can be properly carried out using spin-labelled fatty acids. Indeed, stearic acids with the nitroxide moiety at different position, n, along the hydrocarbon chain (n-SASL) have been used to investigate not only a variety of molecular aspects occurring in Albumin (see, e.g., Refs. [10–12]), but also physical properties of phospholipid membrane model systems [13–16].

In this paper, we present conventional ESR results on HSA spin-labelled with stearic acids having the nitroxide reporter group either at the C-5 carbon atom (5-SASL), close to the lipid polar head, or at the C-16 position (16-SASL), toward the terminal methyl end of the chain. The interaction of the spin-labelled protein with sterically stabilized liposomes of DPPC/PEG:2000-DPPE is studied as a function of temperature and polymer-lipid concentration up to 4 mol% in the submicellar regime [17]. In this range of concentration of the polymer-lipid, the conversion from the mushroom to the brush regime was detected experimentally to occur at mole fraction $X_{\mathrm{PEG:2000\text{-}DPPE}}$ ($m \rightarrow b$) ≈ 1.5 mol% [8]. This value agrees reasonably with the theoretical estimates of ≈ 1 mol%, when gel phase lipids are considered [8].

The results show the transfer of fatty acids from HSA to polymer-grafted membranes. This conclusion relies on the experimental evidence of composite ESR spectra consisting of two label populations: one in the protein and an other in the lipid membrane phase. Because the protein ESR signal is more anisotropic whereas the lipid component is more isotropic, the two components are readily resolved in the conventional spin-label ESR spectra and the relative populations of n-SASL in the protein and bilayer environments quantified by ESR difference spectroscopy. In this way, it is found that the transfer of stearic acids from the protein to the membranes is affected, in a temperature-dependent manner, by the content of the PEG-lipid in the lipid dispersions, especially in the mushroom regime at low concentration of the PEG-lipid.

2. Materials and methods

2.1. Chemicals

Essentially fatty acid-free HSA and the synthetic lipid 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC) were from Sigma. High-purity (>99%) PEG-lipid 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-poly (ethylene glycol) with PEG of average molecular mass 2000 Da (PEG:2000-DPPE) was obtained from Avanti Polar Lipids. The stearic acid spin labels 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxy (5-SASL) and 2-(14-carboxytetradecyl)-4,4-dimethyl-2-ethyl-3-oxazolidinyloxy (16-SASL) were from Aldrich. The reagent grade salts for the 10 mM phosphate buffer solution (PBS) at pH 7.2 were from Merck. All materials were used as purchased with no further purification. Distilled water was used throughout.

2.2. Sample preparation

2.2.1. Spin-labelled protein

Dispersions of HSA spin-labelled with either 5- or 16-SASL were prepared as follows. A volume of methanol containing the spin label was first evaporated under a flow of nitrogen gas and any residual solvent removed under vacuum. A buffered HSA solution (10 mM PBS at pH 7.2) was then added to the dried spin label. The final concentration of both the stearic acid and the protein was 1 mM. Finally, the dispersions were heated at 45 °C and periodically vortexed for 30 min. Samples for measurements on the spin-labelled protein in the presence of the PEG-grafted lipid dispersions were prepared hydrating dried lipid mixtures of the required amounts of DPPC and PEG:2000-DPPE with the spin-labelled HSA buffered solution. Complete dispersion was achieved by heating at 45 °C and periodically vortexing for 30 min. In any dispersion, the final protein/lipid ratio was 1:1 wt/wt. This experimental condition ensures that the binding sites were in excess with respect to the spin label, since there are five fatty acid binding sites for HSA molecule [18].

2.2.2. Spin-labelled lipid dispersions

Spin-labelled lipid dispersions were prepared by dissolving the required amounts of DPPC and PEG:2000-DPPE, together with 1% by weight of the nitroxide stearic acid spin labels, in chloroform–methanol.

The solvent was evaporated in a nitrogen gas stream and then kept under vacuum overnight. The dried lipid samples were fully hydrated with PBS at pH 7.2 (the final lipid concentration was 25 mM), by heating above the chain-melting transition temperature of the dispersions (i.e., 45 °C) and periodically vortexing for 30 min.

All the samples were transferred in a 1-mm (i.d.), 100 μl ESR glass capillaries, flame sealed and stored overnight at 4 °C before the measurements.

2.3. Electron spin resonance

2.3.1. ESR measurements

Conventional ESR spectra were acquired using a Bruker spectrometer model ESP 300 operating at 9 GHz. The sample capillary was inserted in a standard 4 mm diameter ESR quartz tube containing light silicon oil for thermal stability and centred 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). Conventional, in-phase, absorption

ESR spectra were recorded well below saturation at a microwave power of 10 mW using a 100 kHz field modulation frequency for phase sensitive detection and 1 G_{p-p} as amplitude of the magnetic field modulation signal.

2.3.2. Spectral subtraction

The experimental findings show that the spin-labelled HSA/lipid bilayer samples display overlapping conventional ESR spectra. In fact, in addition to the immobilized protein component, a second, sharper lipid component appears that is attributed to stearic acid spin labels transferred from the protein to the lipid bilayers (see below in the text). Due to the different rotational mobility of the label in the two environments, the two spectral components are resolved and quantified by means of spectral subtraction and double integration of the spectra [19,20]. Briefly, computer-aided subtraction of the digitized spectra are performed as follows. The first step is to take the composite spectrum and the immobilized (fluid) component to be subtracted. Then, the two spectra are adjusted for slanting baseline and offset and shifted horizontally relative to each other so that they are in the register. Finally, incremental subtractions are carried out until an obvious endpoint is reached. In this way, the fluid (immobilized) component difference spectrum is obtained. Once the spectral components are resolved, by normalising to the same double integral the original spectra (i.e., the composite one and the single component used for the subtraction) the fraction of the spectrum that was subtracted from the total spectrum is obtained.

3. Results and discussion

3.1. Spin-labelled HSA

The ESR spectra at 20 °C of 5- and 16-SASL non-covalently bound to HSA dispersed in PBS at pH 7.2 are given in Fig. 1A and B, respectively. They are single-component spectra without the presence of any free doxyl stearic acid spin label. This indicates the ability of the protein to bind spin-labelled fatty acids. The spectrum of 5-SASL in HSA in buffered solutions (Fig. 1A) shows a high degree of anisotropy with an outermost peak separation, $2A_{max}$, of ca. 65 G. It corresponds to a powder pattern of spin labels immobilized by the protein that undergo slow anisotropic motion on the conventional ESR timescale. The spectrum of 16-SASL in HSA in buffered solutions (Fig. 1B) also corresponds to the slow-motion regime, although $2A_{max}$ is appreciably lower (61.5 G) with respect to the hyperfine splitting of 5-SASL in dispersions of HSA. These results are in agreement with ESR literature data on bovine serum albumin (BSA) spin-labelled with nitroxide stearic acids [10–12]. The increased rotational mobility of 16-SALS relative to 5-SALS in HSA dispersions suggests that the terminal part of the fatty acid molecules is located in protein environments that allow more freedom of motion.

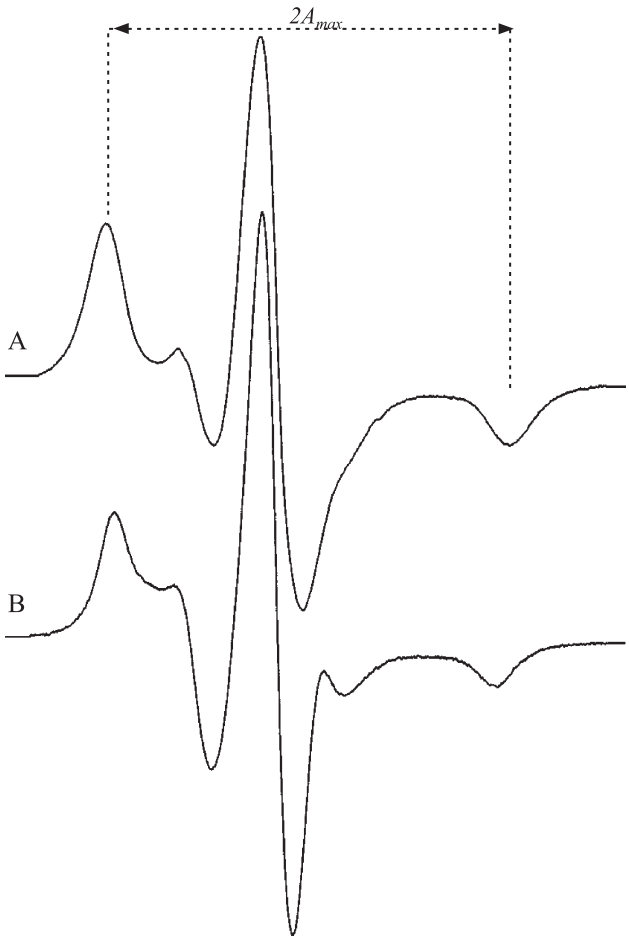

Fig. 1. Conventional ESR spectra at 20 °C of (A) 5- and (B) 16-SASL non-covalently bound to HSA dispersed in phosphate buffer solutions at pH 7.2. Total spectral width=100 G. $2A_{max}$ is the outer hyperfine splitting.

This conclusion is also supported by other structural and dynamic information. In fact, ^{13}C-NMR studies on the interactions of myristic acid with BSA have also indicated that the motion of the C-3 methylene carbons near the polar head is more restricted than that of the terminal methyl carbon of the fatty acid molecule [21]. Moreover, a static picture of the myristate complex with HSA by crystal structure at 2.5 Å resolution [18] has revealed that the fatty acids binding sites in the protein are long and narrow hydrophobic channels located in different domains of the protein. In each case, the hydrocarbon tail of the fatty acid is accommodated inside a deep cavity, from which the terminal methyl end protrudes somewhat in a more polar environment on the protein surface. Additionally, ion-induced spin-lattice relaxation enhancement in non-linear ESR spectra has evidenced that the nitroxide group of 16-SASL is more accessible to water-soluble $K_3Fe(CN)_6$ than are those of 5- and 12-SASL [12].

The spectral anisotropy of both 5- and 16-SASL non-covalently bound to HSA shows a slow decrease when the temperature is increased up to 50 °C (data not shown). At this temperature, the $2A_{max}$-values are reduced to ca. 61 and

57 G for 5- and 16-SASL, respectively. The trend of $2A_{max}$ vs. temperature is in agreement with differential scanning calorimetric data on aqueous dispersions of HSA in the same temperature range that have shown a high thermal stability of the protein [22].

3.2. Spin-labelled HSA in the presence of DPPC dispersions

The ESR spectrum at 32 °C of 16-SASL incorporated in the protein HSA, in the presence of DPPC multilayers in the dispersion buffer, is given in the upper part of Fig. 2A. It consists of distinct spectral components. The outer, more immobilized, anisotropic component corresponds to the spin-labelled protein in interaction with DPPC liposomes. The inner, more mobile, isotropic component is rather similar to the spectrum of 16-SASL in DPPC multilayers in the ripple phase at 32 °C shown in the lower part of Fig. 2A. This latter spectrum is the superposition of ordered- and disordered-type spectra, that are typical features of the nonhomogeneous structure in the ripple phase of DPPC liposomes. This has been established in the ESR studies on the ripple phase of DPPC liposomes using 16-SASL by Tsuchida and Hatta [15]. Therefore, the composite ESR spectrum of 16-SASL bound to HSA in the presence of DPPC bilayers in the dispersion medium in Fig. 2A is due to two label populations with different rotational mobility sampling different environments, namely the protein and the bilayer phases. Since only the protein has been spin-labelled, the bilayer-component represents the nitroxide stearic acid spin labels transferred from the protein to DPPC multilayers. In a similar way, Abreu et al. [23] have shown the binding of a fluorescent lipid amphiphile to bovine serum albumin and its transfer to unilamellar lipid bilayer membrane model systems. Moreover, the movement of long-chain fatty acids from multilamellar liposomes to soluble proteins capable of binding fatty acids was also studied [24]. More generally, theoretical models were proposed to describe the transport of fatty acids between intracellular membranes and between binding proteins and model membrane vesicles [23,25,26].

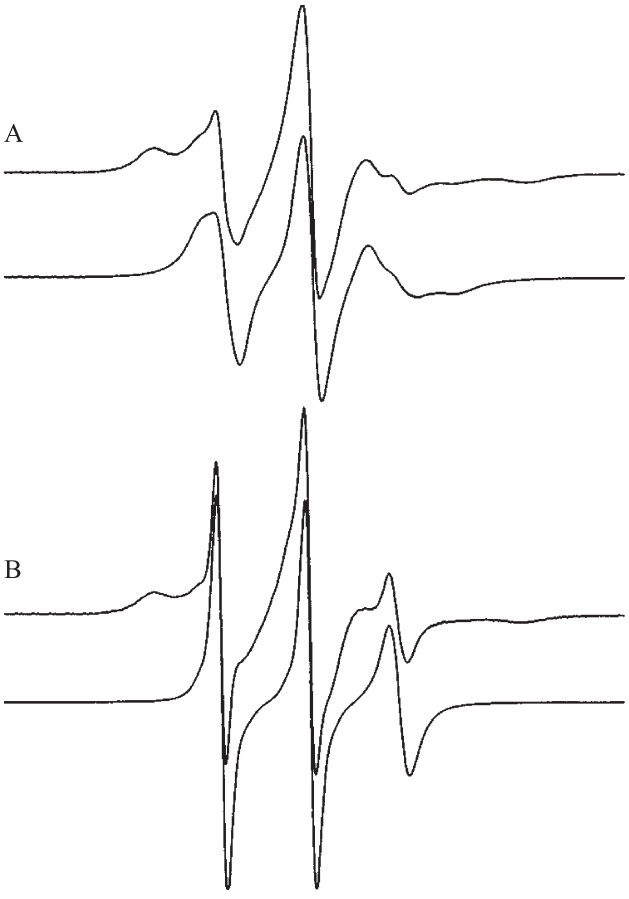

Fig. 2. (A) Conventional ESR spectra at 32 °C of HSA spin-labelled with 16-SASL in presence of DPPC bilayers in the dispersion medium (upper spectrum) and of 16-SASL in DPPC bilayers (lower spectrum). (B) as in (A) but at 43 °C. Total spectral width=100 G.

A composite ESR spectrum of a protein-like signal overlapping a bilayer-like signal is also obtained for 16-SASL incorporated in HSA in presence of DPPC multilayers in the dispersion medium at 43 °C (upper spectrum of Fig. 2B). Indeed, an anisotropic protein signal is still evident in the outer wings of the spectrum, whereas a well defined isotropic bilayer triplet is present in the inner region of the spectrum. For comparison, the spectrum of 16-SASL in DPPC multilayers in the liquid crystalline phase at 43 °C is reported in the bottom of Fig. 2B.

Comparing the composite ESR spectra given in the upper part of Fig. 2A and B, it is interesting to note that the relative intensity of the two overlapping signals changes with the temperature. The signal of 16-SASL in DPPC bilayers becomes more intense at 43 °C, accounting for an augmented transfer of stearic acids from albumin to DPPC bilayers in the fluid phase.

3.3. Spin-labelled HSA in the presence of DPPC/PEG:2000-DPPE dispersions

Two spectral components with different rotational mobility are also obtained in a wide temperature range for HSA spin-labelled with 16-SASL in presence of DPPC/PEG:2000-DPPE mixed dispersions. The spectrum at 32 °C of 16-SASL non-covalently bound to HSA in presence of mixtures of DPPC and 1 mol% PEG:2000-DPPE is reported in Fig. 3A. The coexistence of protein- and bilayer-labelled ESR signals is clearly seen.

Taking advantage of the good resolution of the lipid and protein components in the composite ESR spectra, it is possible to separate their contribution by performing spectral subtraction as described in Material and Methods. Subtracting from the composite spectrum in Fig. 3A the spectrum of Fig. 3B, i.e., the spectrum of 16-SASL in mixtures of DPPC and 1 mol% of PEG:2000-DPPE, the spectrum of HSA labelled with 16-SASL is obtained. This latter is reported in Fig. 3C and it is, indeed, very similar to the spectrum of 16-SASL incorporated in the protein reported in Fig. 3D.

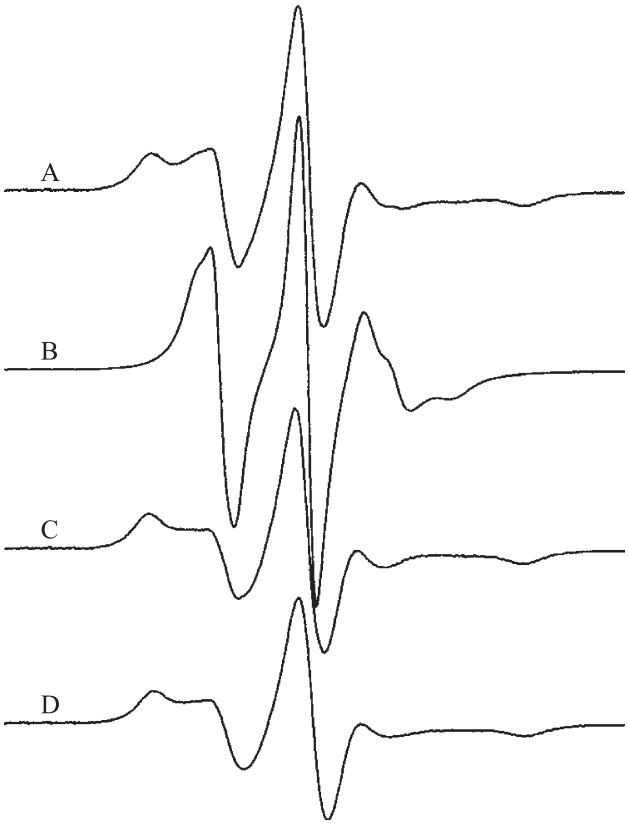

Fig. 3. (A) Conventional ESR spectrum of 16-SASL in buffered dispersion of HSA in presence of mixtures of DPPC and 1 mol% of PEG:2000-DPPE. (B) ESR spectrum of 16-SASL in mixtures of DPPC and 1 mol% of PEG:2000-DPPE. (C) difference spectrum obtained subtracting from the composite spectrum in (A) a percentage of the spectrum in (B). (D) Conventional ESR spectrum of 16-SASL in buffered dispersion of HSA. Total spectral width=100 G and temperature T=32 °C.

It is worthy to note that two spectral components with different degree of anisotropy are also evident in the spectra of 5-SASL non-covalently bound to HSA when SSL of DPPC/PEG:2000-DPPE mixtures are present in the dispersion medium (data not shown). However, the difference in chain motional anisotropy between the protein and the bilayer signals of 5-SASL is insufficient for resolution of the two overlapping components in the composite ESR spectra. This is because 5-SASL in the lamellar polymer-grafted lipid containing membranes probes the region close to the polar/apolar interface and its motion is restricted to an extent comparable to that of 5-SASL non-covalently bound to HSA. In contrast, when the nitroxide moiety is stepped down the chain toward the centre of the membranes (i.e., 16-SASL), more isotropic spectra are obtained. They can be easily separated from those more anisotropic which are obtained when 16-SASL is in the protein.

Comparing the upper spectrum in Fig. 2A with that in Fig. 3A, it is interesting to note that the intensity of the signal of the label in the bilayer is markedly reduced by the presence of polymer-lipids at 1 mol%.

Quantitatively, using the spectral subtraction methods, it is possible to obtain the fraction, f_L(16-SASL), of stearic acids transferred from HSA to mixed lipid/polymer-lipid dispersions.

The influence of both polymer-lipid concentration and temperature on f_L(16-SASL) is reported in Fig. 4. At 20 °C, the percentage of fatty acid transferred from protein to polymer-grafted membranes first rapidly decreases at low concentration of the polymer-lipid, going from 0.37 in the presence of DPPC bilayers to ~0.13 at 0.5 mol% of PEG:2000-DPPE, then it decreases more slowly to ~0.07 at 1.5 mol%, and finally levels off at 0.04 for high content of the polymer-lipid. A concentration dependent decrease of f_L(16-SASL) vs. PEG:2000-DPPE concentration is also obtained at 32 and at 45 °C, although the values are progressively higher than those corresponding to 20 °C. However, the difference of f_L(16-SASL) with different temperatures is more important for PEG:2000-DPPE between 0.5 and 1.5 mol% in the mushroom regime of PEG:2000 and it decreases on increasing the content of the polymer-lipid mixed with DPPC in the brush regime, until it is almost suppressed from 3 mol% onwards.

Among several physicochemical parameters (such as the incubation time of the protein/lipid bilayers mixtures, the concentration of the lipids and of the protein, the pH-value of the dispersion medium, the (multi)lamellarity of the bilayer structures), the transfer of fatty acids between fatty acid binding proteins and membranes is also influenced by the temperature and, hence, by the physical state of the phospholipid bilayers. The present result, i.e., the increase of the fraction of nitroxide stearic acids that leaves the protein and incorporates in the polymer-grafted membranes on a temperature increase, is consistent with the data reported in the literature. In particular, the transfer of palmitic acid from DPPC or dimyristoylphosphatidylcholine (DMPC) multilamellar liposomes to albumin or fatty acid binding proteins is favoured in the fluid state of the lipid bilayers, although decreased binding was observed at temperatures corresponding to the main transition temperatures for those phospholipids [24].

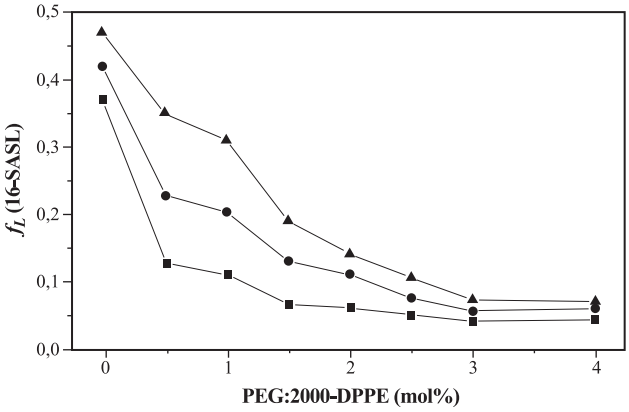

Fig. 4. Fraction, f_L(16-SASL), of the stearic acid spin labels transferred from HSA to the polymer-grafted membranes vs. PEG:2000-DPPE concentration as obtained from spectral subtraction at 20 (squares), 32 (circles) and 45 °C (triangles).

Moreover, the transfer of fluorescent lipid amphiphiles from BSA to unilamellar lipid vesicles of different composition shows highest levels in the case of liquid-disordered phases of pure DMPC and pure palmitoyl-oleoyl-phosphatidylcholine (POPC) at 30 °C, well above their phase transition temperatures [23]. For better interpreting our result, it is worthy to note that the characteristics of the main lamellar phase transition in mixtures of DPPC and low content of PEG:2000-DPPE dispersions are very similar to those of the parent DPPC liposomes. Indeed, only a modest down shift in the main transition temperature (≤1 °C) is induced by polymer-grafted lipid additives in DPPC bilayers [27].

The observed decreases of the fraction of fatty acids transferred from HSA to DPPC/PEG:2000-DPPE dispersions by polymer-lipid could be due to the steric barrier created by the polymer chains at the surface of the liposomes. The polymeric coating prevents the adsorption, i.e., the close approach, of HSA to SSL [5,8,9]. Therefore, it is likely that it prevents, in a concentration dependent manner, the passage of the stearic acids from the protein to the lamellar polymer-grafted membranes as well. Previously, we have shown that the adsorption of HSA to DPPC/PEG:2000-DPPE is first reduced at low grafting density mushroom regime of the polymer-lipid (when the large protein HSA is partially excluded from the liposomes surface) and then totally inhibited on entering the high density brush regime of PEG:2000-DPPE (when it is totally excluded) [8]. The trend of f_L(16-SASL) as function of the content of the polymer-lipid in Fig. 4 mirrors that of adsorption of the protein to SSL of DPPC/PEG:2000-DPPE [8].

4. Conclusions

Hydrating dried non-labelled lipid/polymer-lipid films of DPPC/PEG:2000-DPPE with buffered solution of HSA spin-labelled with nitroxide steric acids, we have shown the transfer of fatty acids from the protein to sterically stabilized liposomes by conventional spin label ESR spectroscopy. It is found that this process is dependent both on the concentration of the PEG-lipid in the mixed lipid lamellar dispersions and on the physical state of the sterically stabilized membranes. Indeed, it is first strongly reduced and then almost abolished on increasing the content of PEG:2000-DPPE mixed with DPPC from the mushroom to the brush regime of the polymer-lipid. Moreover, the fraction of stearic acids transferred in the sterically stabilized liposomes increases when the temperature is increased from the gel to the fluid lamellar phases. This is much more evident at low concentration in the mushroom regime of PEG-lipids.

The results are consistent with the steric stabilization exerted by the PEG-lipids and are relevant to the use of binding- and transporting-proteins in pharmacology.

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