



# Thyroid hormones-membrane interaction: Reversible association of hormones with organized phospholipids with changes in fluidity and dipole potential



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## ABSTRACT

Differential scanning calorimetry (DSC), mixed monomolecular layers and fluorescence spectroscopy techniques were applied to investigate the effect of thyroid hormones (THs) on the biophysical properties of model membranes. We found that both 3,3',5-triiodo-L-thyronine (T3) and 3,5,3',5'-tetraiodo-L-thyronine (T4) induce a broadening of the calorimetric main phase transition profile and reduce the transition enthalpy in liquid-crystalline state of dipalmitoylphosphatylcholine (DPPC) multilamellar vesicles.  $T_m$  changes from 41 °C to 40 °C compared to pure DPPC. When the experiments were done by adding THs to preformed multilamellar vesicles a second broader component in the DSC scan also appears at 20 min of incubation and becomes gradually more prominent with time, indicating a progressive alteration of lipid phase induced by THs. Analysis of surface pressure-molecular area isotherms in mixed monolayers of THs with either DPPC or 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) at air-water interface indicated a reduction in molecular area for THs/lipid mixtures at all surface pressures. A substantial decrease in surface potential in mixed lipid/THs monolayers at all surface pressures were observed for both phospholipids without affecting the mixed monolayer integrity. The data of mixed lipid/THs behavior support the establishment of lateral miscibility. Alterations of bidimensional liquid expanded → liquid condensed phase transition observed for DPPC/THs mixed monolayers are compatible with the changes observed in DSC. The transverse movement of THs and the decrease of dipole potential were also observed in single unilamellar vesicles by using appropriate fluorescent probes.

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## 1. Introduction

There is evidence that thyroid hormones (THs), mainly the pro-hormone T4, are normal constituents of biological membranes in vertebrates, as previously reviewed (Hulbert, 2000). The genomic actions of THs begin with T3 binding to nuclear thyroid hormone receptor. The effects of THs are mediated by a direct modulation of nuclear gene activity and by nongenomic mechanisms

(Barreiro Arcos et al., 2011; Davis and Davis, 2002) that can be membrane mediated (Zamoner et al., 2011). THs were able to interact with plasma membranes modifying the membrane fluidity and the cooperative behavior of associated membrane enzymes, used as a probe of the capability of the hormone molecule to modify the physicochemical membrane microenvironment (Farias, 1980). The interaction of THs with membrane lipids and the consequent modulation of membrane fluidity can be considered as the first nongenomic effect of THs found in our group (de Mendoza et al., 1978; Farias, 1980; Farias et al., 2006). The studies on membrane fluidity and the possible role of hormones in the modulation of membrane fluidity were reported by using liposome as membrane model systems (Chehin et al., 1999, 1995; Farias et al., 1995). THs also can penetrate phospholipid monolayers and modify the transmembrane dipolar organization; where a larger effect on the phospholipid interface is correlated with a larger number of iodine atoms in the hormone molecule (Isse et al., 2003). It was postulated

**Abbreviations:** T3, 3,3',5-triiodo-L-thyronine; T4, 3,5,3',5'-tetraiodo-L-thyronine; DPPC, dipalmitoylphosphatylcholine; DOPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; THs, thyroid hormones; DSC, differential scanning calorimetry.

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that this effect could explain, in part, the non-genomic action of THs at the cellular level (Isse et al., 2003). We now extend our previous biophysical studies by investigating the thermodynamic features on the phase transition of multilamellar DPPC as a sensitive system to prove the transmembrane movement of THs by using differential scanning calorimetry. We also explore the miscibility properties of mixed THs/lipid systems by visualizing the effects of these hormones brought about by intermolecular packing, surface pressure and dipolar changes of phospholipids in mixed monolayer studies.

The ability of THs to translocate inside unilamellar phospholipid lipid vesicles and how the interaction affects the transmembrane potential were also studied by using appropriate fluorescent probes. These findings correlate well with those found either in DSC for multilamellar DPPC or in THs/phospholipid mixed monolayers.

## 2. Materials and methods

### 2.1. Chemicals

Dipalmitoylphosphatidylcholine (DPPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA); 3,5,3',5'-tetraiodo-L-thyronine (T4), 3,3',5-triiodo-L-thyronine (T3) and Valinomycin were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and were used without further purification. Oxonol VI (bis(3-propyl-5-oxoisoxazol-4-il)pentamethin oxonol) and octadecil rhodamine B chloride were purchased from Molecular Probes (Eugene, OR, USA). Phospholipid stock solutions were prepared in chloroform-methanol (2:1, v/v) under nitrogen and stored at -20 °C.

### 2.2. DSC measurement

DPPC multilamellar vesicles were prepared from lipid stock solution by drying under N<sub>2</sub> stream. Organic solvent residuals were removed exposing the lipid film in high vacuum overnight. Lipid films was suspended in 50 mM sodium phosphate buffer (pH 7.4) and the temperature was maintained 10 °C above the main phase transition temperature (41 °C) and the dispersion was extensively vortexed to obtain multilamellar DPPC vesicles or after the addition of THs to obtained DPPC/T4 or DPPC/T3 vesicles before loading to the calorimetric sample cell. To mimic in vivo condition the THs were added to preformed DPPC multilamellar vesicles maintained at 60 °C (above the gel → liquid-crystalline phase transition temperature) and incubated at different time (0, 30, 60 and 120 min) before scanning. A 15:1 mole ratio phospholipid:hormone was used in the mixtures in order to maximize the observable effects of THs on phospholipid thermotropic phase behavior. Calorimetric measurements were taken in a Microcal MC-2D differential scanning calorimeter. A heating rate of 45 °C h<sup>-1</sup> within a temperature range from 25 °C to 60 °C was used. After loading the sample and reference calorimeter cells, both were equilibrated for at least 30 min at 25 °C before the first heating thermogram was recorded. A pure buffer-buffer scan was used as reference. Crude scans were normalized to lipid concentration after subtracting the reference trace. Data were analyzed with the Origin® program provided by Micro-Cal.

### 2.3. Mixed monolayers

We used the Wilhelmy film balance to determine the interactions on mixed monolayers, a validated technique to explore lipid interaction with soluble and membrane proteins, amphiphatic peptides and amphiphiles in general (Ambroggio et al., 2004; Fidelio et al., 1986; Seelig, 1999). Compression isotherms were carried out with either in homemade Langmuir balance equipped with Wilhelmy plate method via platinized-wire for surface pressure

measurements and air-ionizing <sup>241</sup>Am plate and calomel electrode pair (via millivoltmeter) for surface potential (Fidelio et al., 1986); or a KSV-Minitrough (Biolin Scientific) equipped with Teflon barriers and a vibrating plate for surface potential measurement (trough width: 7.5 cm, compression rate: 7.5 cm<sup>2</sup>/min, 5.1 Å<sup>2</sup>/mol/min, subphase: 185 mL NaH<sub>2</sub>PO<sub>4</sub>/Na<sub>2</sub>HPO<sub>4</sub> 50 mM pH = 7.4). Lipids were dissolved in chloroform:methanol 2:1 (v/v) using HPLC grade solvents (Merck) at 1 mM and stored at -20 °C until use in pyrex glass tubes (Corning) sealed with Teflon coated caps. THs were dissolved firstly in DMSO:MeOH 1:1 (v/v) up to 10 mM. 1 mM Aliquots were prepared by dilution in C:M 2:1 (v/v). Just before spreading, the THs/lipid mixtures were prepared by taking aliquots from the corresponding lipid and hormone solutions with Hamilton syringes into in a small 0.5 ml V-vial with Teflon cap glass immersed in an ice bath.

As pure molecules, THs failed to form stable monolayers by themselves, the molecular area corresponding to thyroid hormone in the mixed film were calculated according to the additive rule (cf. ref. Fidelio et al., 1991) according to the expression:

$$[A_{12}]\pi = [A_L]_\pi X_L + [A_{TH}]\pi X_{TH} \quad (1)$$

where  $A_{12}$  is the mean molecular area at the surface pressure  $\pi$  of the experimental mixed monolayer,  $A_L$  is the molecular area of pure lipid at the same surface pressure  $\pi$ ,  $X_L$  and  $X_{TH}$  are the mole fraction of lipid and thyroid hormone in the mixed film, respectively. The mean molecular area is calculated from the number of molecules (lipid plus hormone) spread at the interface divided by the area available for each point of the surface pressure upon compression (cf. ref. Fidelio et al., 1991). Eq. (1) gives an estimation of the expected individual THs molecular areas at the mixed interface assuming that the individual lipid molecular area remains unchanged (would not be the case if some lipid-hormone interactions occur), and that hormone desorption is negligible. For penetration studies of THs into lipid monolayers, a special compartmentalized homemade Teflon trough was used with a reaction surface area of 16 cm<sup>2</sup> equipped with an automatic parallel double barrier system for transference (Bianco et al., 1992).

### 2.4. Fluorescence assays

#### 2.4.1. Membrane potential measurements

Membrane potential measurements in small unilamellar vesicles (SUVs) were done using the gradient sensitive fluorescence probe Oxonol VI (Apell and Bersch, 1987).

SUVs of POPC were prepared in low K<sup>+</sup> content buffer (Tris-HCl 50 mM pH 7.4 plus 130 mM NaCl and 10 mM KCl). For measurements, SUVs suspension were dilute 100 times with high K<sup>+</sup> content buffer (Tris-HCl 50 mM pH 7.4 plus NaCl 10 mM and KCl 130 mM), final lipid concentration of 100 μM. For membrane potential measurement Oxonol VI was added (final concentration 20 nM) into a thermostated fluorescent cuvette a 25 °C with gently stirring into high K<sup>+</sup> content buffer. After constant emission fluorescence output the liposome suspension was added. The K<sup>+</sup> current induced by the ionophore valinomycin (10 nM) was recorded in presence or absence of THs. The Oxonol VI working diluted solution dissolved in ethanol:water mixture (1:5, v/v) was freshly prepared just before use from a 3.16 mM stock solution in pure ethanol. The final concentration working solutions of Oxonol VI were prepared in order to add no more than 5 μL to the reaction medium. The excitation and emission wavelength was 590 nm and 634 nm, respectively. Fluorescence was corrected by the dilution factor (final volume/initial volume). In separated experiments were checked that neither THs nor valinomycin had any direct effect on Oxonol VI fluorescence properties. To further verify if Oxonol VI were sensitive only to membrane potential generated by K<sup>+</sup> gradient, control experiments

were performed in which the concentration of K<sup>+</sup> in SUVs preparation buffer was equal to the valinomycin solution.

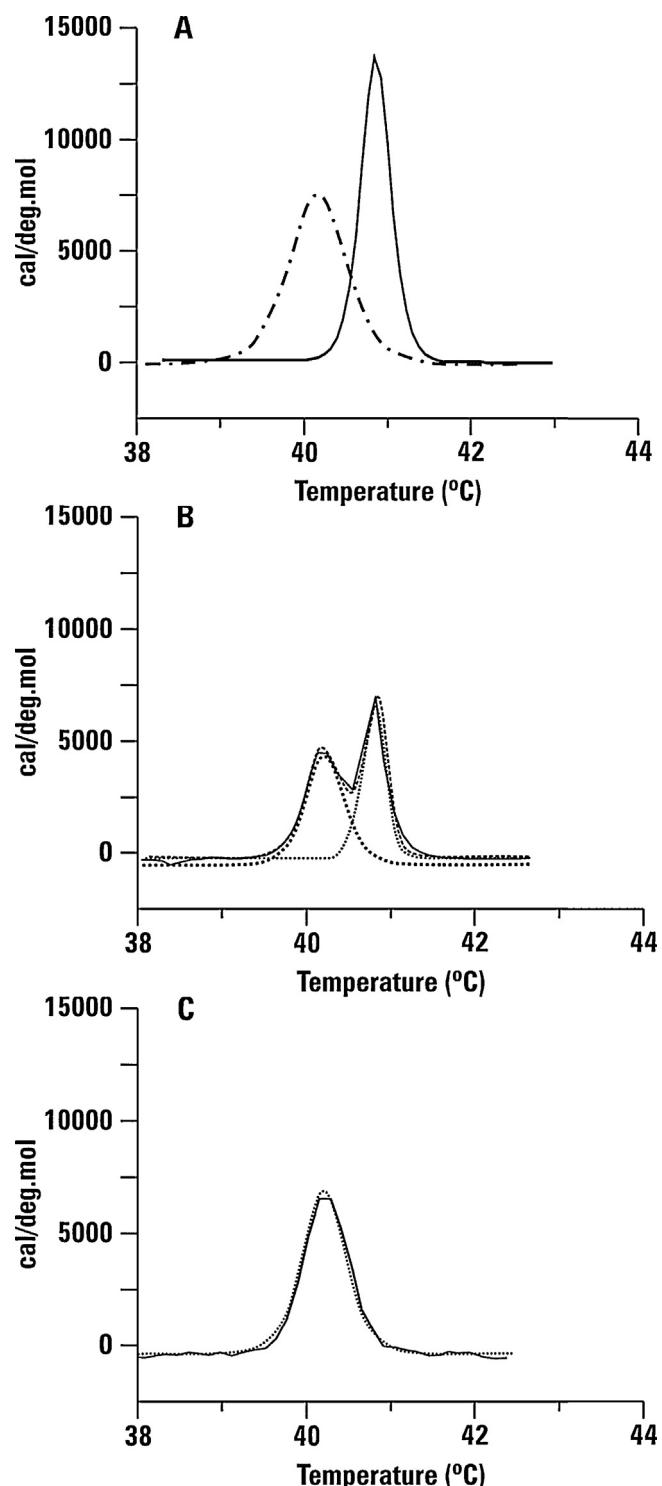
#### 2.4.2. Transference assays

For transference assays based on the property of THs to quench the fluorescence of R18 probe, two types of SUVs (prepared according to Voglino et al., 1998) were used: (i) vesicles loaded with THs (donor vesicles) were prepared by incubating a 500 μM POPC SUVs with 50 μM of THs during 60 min at 25 °C sodium phosphate 50 mM, pH 7.4 buffer. The unbound hormones were immediately separated before the experiments were done by using ion exchange chromatography (Dowex 1 × 8 CL 200–400 mesh) as described before (Farias et al., 1995); (ii) R18 loaded SUVs (acceptor vesicles) were prepared by adding R18 probe into phospholipid solution dissolved in chloroform:methanol (2:1, v/v) to give a final lipid concentration of 50 μM in the hydrated buffer (sodium phosphate 50 mM, pH 7.4) with a 3 mol% of probe. Mixed lipid/probe samples in solvent were dried off under N<sub>2</sub> stream. After hydration the vesicles solution was sonicated and left for 30 min at 25 °C for a complete resealing. Non loaded R18 was separated with gel filtration chromatography with Sephadex G-75 column (1 cm × 15 cm) equilibrated with the same hydration buffer. To measure the extent of THs translocation into R18 loaded liposomes, acceptor and donor vesicles were mixed in a total volume of 1.5 ml and the decrease in fluorescence intensity was immediately followed as function of time. Appropriate control experiments were done to discard either non-desired R18 transfer or dilution or fusion process of both vesicles pools non attributable to the effect of THs quenching (Hoekstra et al., 1984). The excitation and emission fluorescence wavelength were 575 nm and 660 nm, respectively (slit 5 nm). All fluorescence experiments were done in a SLM spectrofluorometer (SLM Aminco, Urbana, IL) equipped with thermostated cuvette holder and magnetic stirrer.

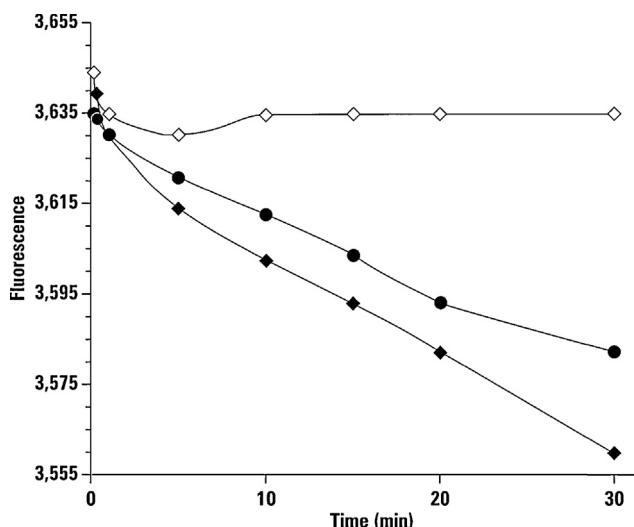
### 3. Results

#### 3.1. Differential scanning calorimetry and hormone transference between lipid lamellas

Differential scanning calorimetry (DSC) is a sensitive and non-perturbing thermodynamic technique which has been extensively used to study lipid thermotropic phase transitions in model and biological membranes (Lin et al., 1991; McMullen and McElhaney, 1995). As illustrated in Fig. 1(A), the steepness and the enthalpy of the main transition of DPPC/T4 multilayer liposomes are significantly affected. The ΔC<sub>pmax</sub> of the main transition decreases to more than half of the original value, and the T<sub>m</sub> changes from 41 °C to 40 °C compared to pure DPPC peak. The effects on the thermotropic behavior of the lipid multilayer matrix, was changed considerably when THs are added to preformed multilamellar vesicles at 60 °C (above the main lipid phase transition). A second broader component of the DSC also appears at 20 min of incubation (Fig. 1B) and becomes progressively more prominent at 30 min (not shown). The overall transition peak can be deconvoluted into two well differentiated components. One, corresponding to lower temperature, can be attributed to membrane-associated hormones into hormones-rich mixed component in the more external layer affecting the thermotropic behavior of DPPC and the other to the hormone-poor inner layers (Bradrick et al., 1989; Carrer and Maggio, 1999), suggesting that hormone molecules is gradually diffusing from one lamella to another of the multilamellar DPPC vesicles. At 60 min of incubation time, or higher, at an incubation temperate above that of the pure lipid phase transition, only the second component was found (Fig. 1C). This is indicative of a more complete homogenization of the mixed sample due to diffusion of



**Fig. 1.** Influence of T4 on the thermotropic behavior of DPPC multilayer liposomes. DSC first thermogram of: (A) Pure DPPC (—, right scan) and DPPC/T4 (----, left scan); (B) DPPC/T4 incubated for 20 min (— experimental trace and ---- deconvolution into two populations); (C) DPPC/T4 incubated for 60 min. Samples were incubated at 60 °C for the mentioned time and further equilibrated by 30 min at 25 °C before the first scan is run. Samples were incubated at 60 °C for the indicated times and further equilibrated at 25 °C for an additional 30 min before the first scan is run. A 15:1 mole ratio phospholipid:hormone was used in each mixture. Crude scans were normalized by total lipid concentration and the reference scan was subtracted. Heating rate: 45 °C h<sup>-1</sup>.



**Fig. 2.** Transference of T4 between two SUVs populations. R18 loaded SUVs emission fluorescence of: (◆) plus T4 loaded SUVs; (○) plus T4 loaded SUVs with 1  $\mu\text{M}$  of serum albumin; (●) plus T4 loaded SUVs with 1  $\mu\text{M}$  gamma-globulin. See the text for more details.

THs throughout the entire multilamellar vesicles. We have obtained similar tendency for DPPC/T3 mixtures but the magnitude of the effect is less (see Fig. S1 in the Supplementary data).

To further corroborate the capability of THs to flux throughout the lipid bilayer via membrane translocation, we designed experiments using the ability of iodine in T3 and T4 molecules to quench the fluorescence of Rhodamine B (R18) previously loaded into unilamellar POPC SUVs. The translocation was studied by mixing donor SUVs loaded with T4 with the acceptor SUVs loaded with R18. Diminution of the intensity R18 fluorescence as function of time was observed when R18 loaded POPC and POPC/T4 liposomes were mixed (Fig. 2). To probe that transfer process occur via the aqueous media, serum bovine albumin that bound THs and the gamma-globulin that does not have this capacity were added to assay media. Only the trapping hormone bovine albumin interferes significantly in the process of transference (Fig. 2). Neither of these proteins directly affects the fluorescence of the R18-PC per se (not shown). When R18 containing vesicles (R18-POPC) were mixed with pure POPC vesicles, no significant fluorescence variation was observed (not shown). The kinetics of T4 movement has a linear adjustment with time ( $t_{1/2} = 12\text{--}14 \text{ min}$ ). Similar results were found using POPC/T3 systems.

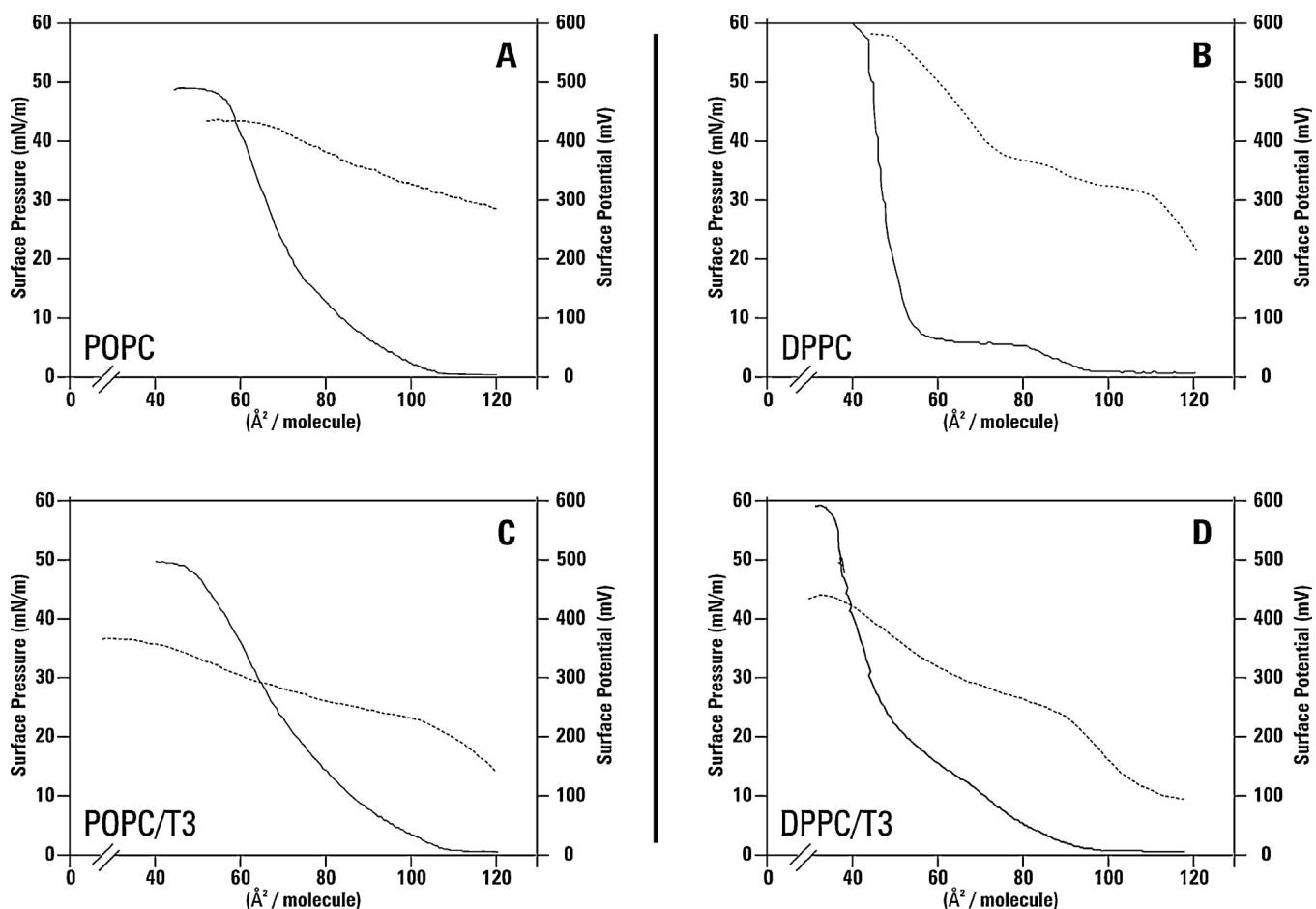
### 3.2. Mixed thyroid hormone-phospholipids monolayer and dipole potential changes

The THs behavior in mixed films has been studied with two zwitterionic phospholipids, POPC and DPPC. THs are not able to form stable Langmuir monolayers when they are directly spread at a lipid free air-water interface as pure components (Issé et al., 2003). However, T4 or T3 in mixed lipid-hormone systems acquire lateral stability at the mixed surface and become a film-forming component that support compression-decompression cycles. Representative isotherms of POPC and DPPC and POPC/T3 and DPPC/T3 monolayers are shown in Fig. 3 (Also, more data are shown in the Supplementary data). The liquid-expanded state of POPC monolayer (Dynarowicz-Latka et al., 2001; Maget-Dana, 1999) was not altered by the presence of T3 (Fig. 3A and B). It is known that upon compression a DPPC monolayer goes through three states: a liquid expanded phase at low surface pressures; a mixture of liquid expanded and liquid condensed phases in the plateau region of the isotherm (at around  $5 \text{ mN m}^{-1}$ , see Fig. 3B), and pure liquid

condensed phase at high surface pressures values (Chapman and Benga, 1984; Hollars and Dunn, 1998). The effect of T3 on pressure-area of DPPC/T3 mixed isotherms was characterized by gradually altering the bidimensional phase transition from a liquid-expanded to liquid-condensed phase (Fig. 3C and D; see also Fig. S2 and S3 in the Supplementary data). Similar behavior was also obtained for T4, with a more marked effect on bidimensional phase transition profile (see also Fig. S2 and S3 in the Supplementary data) in agreement with the DSC data. At all surface pressures, the surface potential of mixed films decrease by the presence of THs compared with pure phospholipids (Fig. 3 and Figs. S2 to S4 in the Supplementary data). At high lateral pressures, the mean molecular area and surface potential values decrease significantly compare with those values obtained for pure films of either DOPC or DPPC; whereas the maximum film stability measured by the collapse pressure values are practically not affected by the presence of THs.

During compression, no abrupt discontinuities were noted either in POPC/T3 or DPPC/T4 monolayer isotherms and a marked decrease in surface potential is still observed at high lateral pressure of mixed THs/lipid films, indicating that even when some desorption is probably occurring upon compression, a significant fraction of THs remains at the mixed surface. These evidences are indicating that: (a) THs were not substantially squeezed out into the sub-phase during the compression time ( $\sim 5\text{--}7 \text{ min}$ ), (b) that THs remaining in close association with the lipids whatever the lipid phase is: either liquid-condensed or liquid-expanded for the case of DPPC or completely liquid-expanded for POPC monolayers, (c) the change on bidimensional transition observed for both THs (see Figs. S2 and S3 in the Supplementary data) are compatible with the findings. Even when the molecular area of THs cannot be accurately calculated, since they do not form stable monolayer by themselves and the proper individual surface area cannot be measured. However, the interfacial stability of both THs is acquired in presence of lipids so that an estimation of their contribution to the mean molecular area may be estimated by using equation 1 (as indicated in Section 2.3). At medium lateral pressures, the corresponding contribution of THs molecules to the mean molecular area is estimated to be  $30\text{--}35 \text{ \AA}^2/\text{molecule}$ , similar to cholesterol, (Kwong et al., 1971). However, the equivalent molecular area of THs at the surface may be underestimated if lipid–hormone interaction is taking place or if some amount of spread hormone is gradually squeezed out into the subphase upon compression.

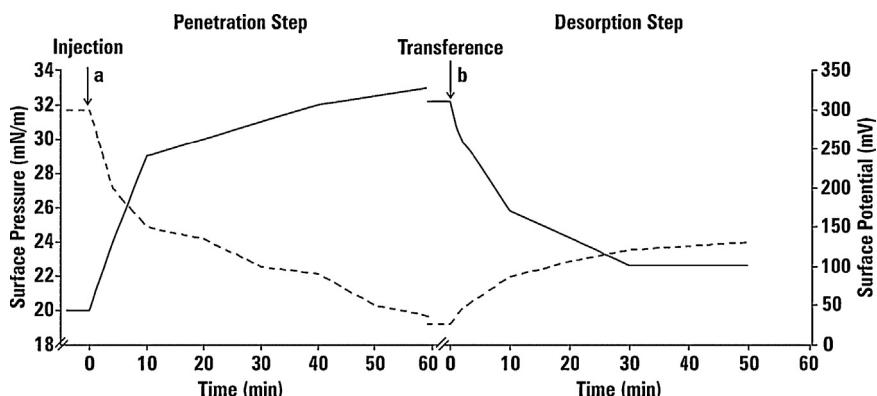
Nevertheless, the directly spread monolayer should be considered as a non-equilibrium system with respect to the subphase and allows reproducible isotherms in the time scale of compression-decompression cycle. As it was mentioned before THs fail to form insoluble monolayer films by themselves, but become incorporate at the interface if a lipid phase covers the air/water surface. As THs are amphiphiles with limited solubility in water (usual final aqueous experimental concentration in bulk is about  $1 \mu\text{M}$ ; Zamoner et al., 2008), compared with rather insoluble natural phospholipids forming biomembranes, it is to be expected that THs can be transferred reversibly into the membrane lipids from the aqueous phase and vice versa. This behavior was proved by the spontaneous adsorption-desorption of T3 into POPC monolayers. If T3 is injected beneath of a POPC monolayer set at  $20 \text{ mN m}^{-1}$ , a spontaneous incorporation of the hormone takes place with an increase in surface pressure parallel to a decrease in surface potential (see Fig. 4, first part “penetration” step), in agreement with the effect of the hormones on lipid phase behavior observed in DSC and with mixed films experiments. If the T3-penetrated monolayer is now transferred at constant lateral pressure to a hormone free subphase, a gradual reduction of the pressure and a recovery of the surface potential are observed with time (see Fig. 4, second part after transference “desorption” step) due to hormone desorption from the interface to the subphase. These facts are clearly



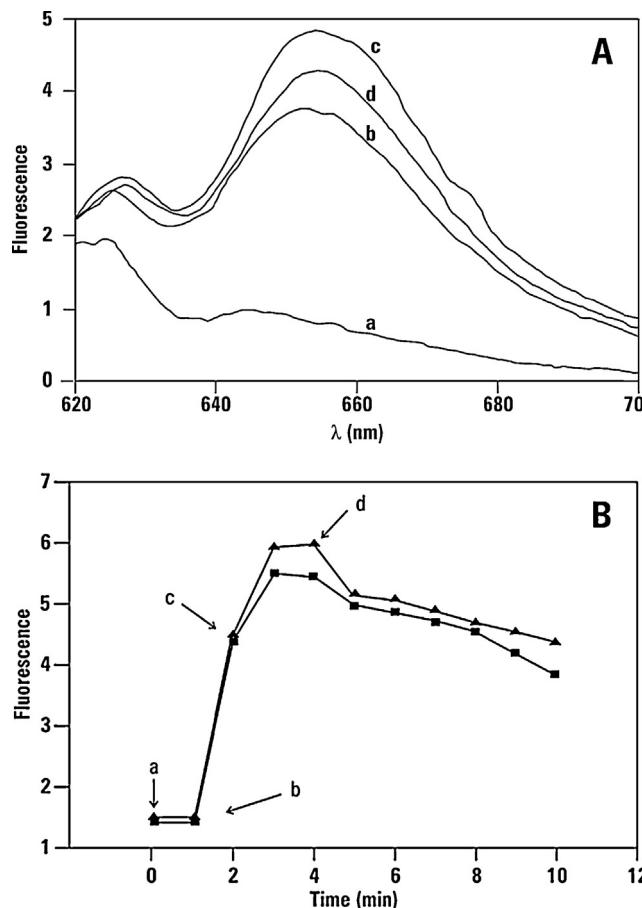
**Fig. 3.** Influence of T3 in the surface properties of phospholipids. Surface pressure ( $\text{mN m}^{-1}$ ) [—] and surface potential (mV) [---] as a function of molecular area for pure POPC (A) and DPPC (B) or mixed spread monolayer POPC/T3 (C, hormone mole fraction 0.26) and DPPC/T3 (D, hormone mole fraction 0.23). See the text for more details.

indicating the reversibility of the THs-phospholipid interaction and that the hormone is not irreversible absorbed. Nevertheless the time-course of T3 incorporation into the lipid phase is similar to desorption, the kinetics should not be necessarily completely equivalent between adsorption and desorption and some hysteresis seems to occur (Fig. 4). In this connection, it should be mentioned that the initial values are not completely recovered indicating that some lipid–hormone mixed interaction is reaching a stable equilibrium interface→subphase after transfer compatible with the overall data reported in this work.

To further verify if, the effect of THs on dipole potential changes detected with Langmuir monolayer technique is also present in vesicles (SUVs), we use Oxonol VI fluorescent probe known to be sensitive to transmembrane potential changes (Apell and Bersch, 1987). Oxonol VI shows different emission spectra in the presence of POPC liposomes, POPC liposomes plus valinomycin and POPC liposomes plus valinomycin in presence of T3 (Fig. 5A). The addition of either T3 or T4 produces a diminution of the emission fluorescence at 660 nm indicating a decrease in the membrane potential generated by valinomycin ionophore (Fig. 5B).



**Fig. 4.** Reversible adsorption–desorption of T3 in preformed lipid monolayers. Surface pressure (—) and surface potential (---) changes as function of time. The first arrow indicates the injection of 30  $\mu\text{M}$  of T3 in the subphase beneath of a POPC monolayer set at an initial surface pressure of 20  $\text{mN m}^{-1}$  (penetration step) with continuous stirring. The second arrow indicates the transference of POPC/T3 penetrated monolayer at constant lateral pressure to a T3 free subphase (desorption step).



**Fig. 5.** Influence of thyroid hormones on SUVs transmembrane potential. (A) The emission of fluorescence spectra of 20 nM Oxonol VI in: (a) high K<sup>+</sup> content buffer alone; (b) plus 100  $\mu$ M POPC-SUVs; (c) 100  $\mu$ M POPC-SUVs plus 10 nM valinomycin; (d) 100  $\mu$ M POPC-SUVs + 10 nM valinomycin plus 10  $\mu$ M of T3. (B) Effect of THs on  $\psi\Delta$ . Arrows indicate consecutive additions to the reaction medium of: (a) Oxonol VI 20 nM; (b) plus POPC-SUVs 100  $\mu$ M; (c) 100  $\mu$ M POPC-SUVs plus 10 nM valinomycin; (d) 100  $\mu$ M POPC-SUVs + 10 nM valinomycin plus 10  $\mu$ M of T3 (■) or T4 (▲). The changes in fluorescence emission output were taken at 660 nm at the indicated time. POPC-SUVs were formed in low K<sup>+</sup> buffer content and dilute in high K<sup>+</sup> buffer content (see Section 2 for more details). DPPC + T4 after 60 min of incubation. T4 interacts with lipids diffusing throughout DPPC multilamellar vesicles.

#### 4. Discussion

Although plasma membrane binding sites for iodothyronines have been described in the past for human and rat erythrocytes (Angel et al., 1989; Botta and Farias, 1985) and rat hepatocytes (Gharbi and Torresani, 1979; Hennemann et al., 2001; Pliam and Goldfine, 1977) it has not been clear that these sites are linked to intracellular events and therefore function as receptors. Details of the mechanism by which mammalian cells take up thyroid hormones remain poorly understood (Chehin et al., 1999). Furthermore, some actions of THs can be membrane-initiated events (Zamoner et al., 2011). To gain insight into the mechanisms by which T3 and T4 reach the intracellular compartment, several experiments with model phospholipid membrane systems in the different phase state were performed in our laboratory. We showed that T3 regulate membrane fluidity, similarly to cholesterol, causing an increase of the fluidity in the gel phase and a decrease in the liquid-crystalline state, while T4 decrease the fluidity in the liquid crystalline state, but did not modified the gel state (Farias et al., 1995).

The effect of T3 and T4 on the release of the liposomal content also depends on the physico-chemical state of the liposomal

membrane (Farias et al., 1995). Previous results obtained with T3 indicated that the hormone can permeate phospholipid bilayers and the diffusion time increases in the gel and liquid-ordered phase (eggPC liposomes containing up to 30 mol% of cholesterol Chehin et al., 1999). On the contrary, the transverse diffusion of T4 is more restricted in lipid gel phase or in more the liquid-ordered phase (Chehin et al., 1999, 1995). These facts correlated with a lower incorporation of T4, compared with that observed for T3 in liposomes in gel state and liquid-ordered state. The differential incorporation of THs was also observed at physiological concentration with mammalian cell membranes having more than 25 mol% of cholesterol (Chehin et al., 1995). In this regard, previous studies using Raman vibrational spectroscopy and density functional theory (DFT) calculations allowed us to detect spectral changes observed for T4 and T3 upon binding to phospholipids and to postulate that they are likely due to specific conformational changes adopted by the hormones after inserting into the lipid bilayer, according to their specific steric requirements (Alvarez et al., 2004).

In this work, we show that THs can reversible interact with organized membrane models either using lipid monolayers, small sonicated vesicles or multilayered liposomes. Both, T3 and T4 do not form stable insoluble films at an air-water interface by themselves but they can form miscible systems with phospholipids at rather low mole fraction of hormone in the mixture. So, the asymmetric lipid environment offers an adequate amphiphilic phase for THs reservoir depending on the physical state of the lipids regardless of model membrane system employed (lipid monolayers or vesicles). When DPPC lipid is used the main bulk phase transition is modified and the bidimensional liquid expanded  $\rightarrow$  liquid condensed is also altered in lipid/hormone mixed systems. The molecular area contribution of the thyroid hormone into mixed films is similar to that obtained with cholesterol. The progressive time-dependent changes observed in the thermotropic behavior of multilayer DPPC liposomes clearly indicate that both hormones T3 and T4 have the ability to interact by fluxing throughout lipid bilayer barriers by only their concentration gradient. The interaction with lipids is reversible as assessed by adsorption/desorption experiments in pre-formed phospholipid monolayers. The transference also takes place between vesicles via aqueous medium. The interaction occurs with a decrease in the surface dipole potential with a negative contribution to the hydrocarbon region of the lipid monolayers (contrary to the positive contribution of the hydrocarbon portion of phospholipids toward the air end). This singularity was also checked in vesicles by using the Oxonol VI dye, known to be sensitive to K<sup>+</sup> current, probing the influence of T3 and T4 on the dipolar organization in membrane model systems.

It should be emphasized that THs-lipid interaction (partition) takes place independently of the membrane curvature of model system assayed (lipid monolayers, multilayered DPPC-LUVs or POPC-SUVs) when they are in liquid disordered phase. Indeed, an approximate comparative estimation of  $t_{1/2}$  in any of the observed effects gives: (i) about 20–30 min in DSC experiments (THs diffusion in multilayered LUVs, Fig. 1), (ii) 12–14 min for transverse movement of THs in POPC-SUVs loaded with R 18 fluorescent probe (Fig. 2), (iii) 10–15 min for penetration of THs into POPC monolayers (Fig. 4) and, (vi) 6–8 min for halving the signal of the transmembrane potential in presence of THs previously induced by valinomycin ionophore (sensed by Oxonol VI fluorescent probe in POPC-SUVs under K<sup>+</sup> gradient, Fig. 5). Despite of the different expected rates for the several biophysical parameters measured in the distinctive experimental systems assayed, which may be influenced by membrane curvature, our results clearly demonstrate a strong interaction of THs with lipid interfaces regardless of membrane model employed (Table 1).

The possible physiological actions resulting from the property of THs to reversible transverse lipid membranes still remain elusive,

**Table 1**

Surface parameters of mixed lipids-T3 thyroid hormone.

Parameters/lipid	POPC	POPC/T3 <sup>a</sup>	DPPC	DPPC/T3 <sup>a</sup>
Collapse pressure (mN/m)	45–47	45–47	60–62	58–60
Collapse area (Å <sup>2</sup> /molecule)	55	50	39	36
Surface potential (mV)	410–430	360–370	550–660	420–440

<sup>a</sup> Mole fraction of POPC/T3 and DPPC/T3 were 0.74/0.26 and 0.77/0.23 respectively.

but this peculiar property of THs is certainly giving the biophysical basis to explain the observed non-genomic effects.

## 5. Conclusion

The present results suggest: (a) THs strongly interact with lipids in a spontaneous and reversible mode; (b) THs are freely to diffuse in the lateral plane of the lipid phase and transversally between lamellas thermodynamically governed by concentration gradient; and (c) the spontaneous partition into lipid phase affect the physical properties of the membrane. Then, the modulation of the THs on several physical parameters, such as lateral phase, fluidity and the polarity of membranes could play a role in regulation of several membrane activities (enzymes, transport, receptor, etc.) due to the requirement of such activities for a specific lipid microenvironment.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.chemphyslip.2013.08.007>.

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