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Structural changes induced by interactions between thyroid hormones and phospholipid membranes: a Raman Spectroscopy study

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Previous reports from our laboratory have shown that thyroid hormones induce changes in the fluidity and permeability of phospholipid bilayers and modify the transmembrane dipolar organization according to their iodine content. The interactions of T2, T3, and T4 with a model membrane of dilauroylphosphatidylcholine in the liquid-crystalline phase were analyzed by confocal Raman spectroscopy. Insights into the nature of the hormone effects on the membrane properties, as well as the structural adaptations of the hormones in response to the lipid environment were derived from the spectral changes. The series of progressive iodine substituents on the ring-β allowed us to correlate the hormone effects according to the number and the orientation of the iodine atoms. T4 was responsible for producing the highest alteration in the hydrophobic region of the membrane. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: thyroid hormones; phospholipids; vibrational analysis; Confocal Raman Microscopy

Introduction

Thyroid hormones are important for the growth and development of various tissues, in particular the brains, and for the regulation of their basal metabolic rate throughout life.[1] Best known actions of thyroid hormones are initiated by binding of 3,5,3-triiodo-L-thyronine (T3) to thyroid nuclear receptors in target cells.[2] Although these nuclear receptors mediate the biological activities of T3 via transcriptional regulation, thyroxine (T4) is the predominant iodothyronine secreted by the thyroid gland under normal conditions. As consequence, T4 has to be converted by deiodination into T3 in the cytoplasm of target tissues.[3]

Actions of T3 or T4 that are not initiated by binding of the hormone to the thyroid nuclear receptors induce very fast responses in cells and are called nongenomic or extranuclear effects. These nongenomic actions have been described at the level of the plasma membrane, cytoskeleton, cytoplasm, and organelles of mammalian cells.[4] They include alterations in solute transport (Ca^{2+}, Na^{+}, H^{+}, glucose), and changes in activities of signal transducing kinases like protein kinase A, protein kinase C, pyruvate kinase M2, phosphatidylinositol 3-kinase, and the MAPK pathway.[5–7] It has been suggested that some of these effects are mediated via alterations of the membrane fluidity by the hormone. These can be considered the first nongenomic effects of thyroid hormones.[8,9] However, the mechanisms by which thyroid hormones nongenomically affect the activity of plasma membrane ion channels and ion pumps are not well understood.[10,11]

The hydrophobic nature of the thyroid hormones allows a fraction of free hormone (not bound to cellular proteins) to be located within the lipid matrix of the various cellular membranes, normally rigidifying these membranes by affecting their lipid composition.[3,12–14] Previous reports from our laboratory have shown that thyroid hormones induce changes in the fluidity and permeability of membrane liposomes,[15–17] and they modify the transmembrane dipolar organization of phospholipid monolayers according to the iodine content in the hormone molecule.[18] In order to accomplish a more detailed understanding of these specific hormone-membrane interactions, our previous studies have been solely focused on the analysis of the induced changes in the molecular structures of T3 and T4 by the phospholipid surroundings by means of FT-Raman spectroscopy[19,20] and molecular dynamic (MD) simulations.[21] Those results have clearly shown three important aspects of the mentioned interactions: (1) T3 and T4 insert into the lipid medium in such a manner that the ring-β and part of ring-α remain anchored between the aliphatic chains of the lipid via hydrophobic interactions; (2) the progressive iodine substitution on the ring-β lowers the possibility of membrane penetration; (3) the orientation of the single iodine substitution on ring-β of T3 is also a relevant factor of the hormone behavior in the lipid medium.

In the present study, we perform a direct comparison among the behavior of T3, T4, and 3,5-diiodo-L-thyronine (T2) upon interaction with a model membrane of dilauroylphosphatidylcholine (DLPC) in the liquid-crystalline (L_α) phase, together with the spectral analysis of specific lipid bands that may supply clues concerning the membrane's response to the hormone insertion.

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The inclusion of T2 in this study allows completing the series of progressive substitution on the ring-β and performing a better correlation according to the number and the orientation of the iodine substituents in the molecule (Fig. 1). It has been proven that T2 is also able to induce similar nongenomic actions on plasma membrane,[4] and evidence of its actions at metabolic levels has been published.[22–24]

Raman spectroscopy is a well-suited technique to study phospholipids. When integrated with a confocal microscope, its efficiency can be greatly improved since the Raman scattering is collected from a small volume with relatively high local concentration of lipid. Therefore, the contributions from the surroundings are minimized.[25,26] We demonstrate here the usefulness of confocal Raman microscopy for the analysis of iodothyronine–membrane systems.

Materials and methods

Sample preparation

T2, T3, T4, and DLPC were purchased from Sigma and used without further purification. Methanol solutions of each thyroid hormone (100 mg/ml) and the phospholipid (40 mg/ml) were prepared. Appropriate amounts of phospholipids or hormone/phospholipid mixtures (molar ratio of 1:5) were dried under a nitrogen stream and suspended by vortexing in 50 nM acetate-acetic acid buffer (pH 5.0) at ambient temperature to give a final concentration of 1 mM phospholipid. A minimum ionization degree of the phenolic hydroxyl substituent occurs at pH 5.0 (pK = 9.29, 8.45, and 6.73 for T2, T3, and T4, respectively)[27] yielding the maximum lipophilicity of thyroid hormones and a particularly high partition coefficient between the lipid and the aqueous phase. The suspensions were centrifuged at 2000 rpm and at 298 K for 15 min. The pellets were used for spectroscopic measurements.

Raman spectra

Raman spectra between 3500 and 50 cm^{-1} were collected using a DXR Raman Microscope (Thermo Fisher Scientific). Data were collected using a diode-pump, solid state laser of 532 nm (5 cm^{-1} spectral resolution). A confocal aperture of 25 μm pinhole was used. A 10X objective was used when collecting Raman data. The samples were placed on gold-coated sample slides. In order to achieve a sufficient signal-to-noise ratio, 200 expositions with exposure time of 2 s were accumulated for all samples. To avoid photodegradation, the laser power was maintained at 4 mW when collecting data from the iodothyronines in pure state and the T4/DLPC complex. 9 mW was the laser power used for DLPC in pure state and the T2/DLPC and T3/DLPC mixtures. All spectroscopic experiments were carried out at ambient temperature.

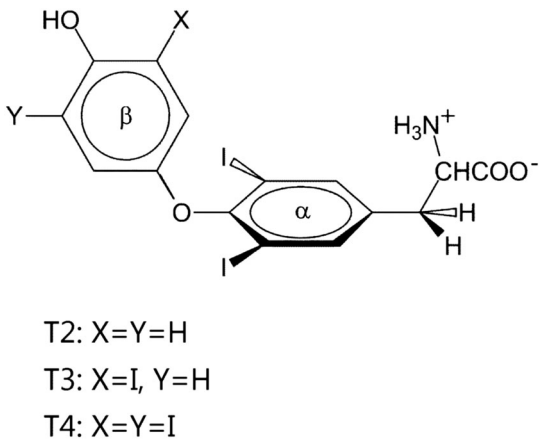

Figure 1. Schematic representation of thyroid hormones. According to the substitution in ortho-positions of the ring-β, the iodothyronine is T2, T3, or T4. Substituents in X and Y are oriented 'proximal' and 'distal' to ring-α, respectively.

Data analysis includes the spectral subtraction of the Raman spectrum of the pure DLPC in the entire frequency range from the iodothyronine/DLPC mixture spectrum, yielding the difference spectrum corresponding to the hormone bound to the membrane. Although the DLPC bands were not completely eliminated by the subtraction process due to molecular interactions between the hormone and the lipid, most of them were minimized when a subtraction factor of 0.9 was used. Additionally, the overlapping components in a specific spectral region of DLPC were mathematically decomposed by using an iterative curve-fitting process. This process has been widely applied to decompose the complex bands in proteins and is described elsewhere.[28,29] Briefly, the number and position of component bands were obtained through deconvolutions and derivations. These, together with the band shape (a combination of Lorentzian and Gaussian functions), were fixed during the first 500 iterations. The fitting was further refined by allowing the band position to vary for 50 additional iterations. Finally, the fitting result was visually evaluated by overlapping the reconstituted overall curve on the original spectrum.

Results

Raman spectra of T2, T3, T4, and DLPC in pure states and the T2/DLPC, T3/DLPC, and T4/DLPC complexes were recorded in the range between 3500 and 50 cm^{-1}. At ambient temperature (291 K), the DLPC bilayer is in the L_α phase (Tm ~278 K). The spectral differences shown by the Raman spectrum of each complex sample in respect to the spectrum of the corresponding hormone or lipid, both in pure states, were analyzed separately. The evaluation of these spectral differences was restricted to a few bands that supply structural information concerning specific parts of the iodothyronine molecules as of the DLPC bilayer.

DLPC effects on thyroid hormone molecules

Many DLPC bands partly overlap with some of the thyroid hormones bands leading to uncertainty in the determination of possible membrane-induced spectral changes. Then, the analysis was restricted to the following spectral regions, characterized by the absence of lipid bands: (1) 1650–1500 cm^{-1}, corresponding to aromatic-ring vibrations, mainly consisting in C = C stretching modes, (2) 1250–1170 cm^{-1}, representing the C–O stretching vibrations of the ether bridge and phenol group, (3) around 850 cm^{-1}, where a Fermi doublet typical of tyrosine and symmetric *para*-substituted aromatic rings appears, and (4) 250–150 cm^{-1}, where the C–I stretching bands are observed as the strongest Raman bands of iodothyronines. All of these vibrations are expected to be sensitive to the surroundings.[19,20] The corresponding bands together with their spectral shifts are listed in Table 1.

Figure 2 shows the Raman spectra of each iodothyronine in pure state and in interaction with DLPC in the region between 1650 and 1500 cm^{-1}. In general, *para*-substituted aromatic rings

Table 1. Wavenumbers (cm^{-1}) of selected Raman bands of thyroid hormones. Tentative assignment of the features observed in the spectra of the hormones in pure state and the iodothyronine/DLPC mixtures

Assignment[a,b]	T2	T2/DLPC	T3	T3/DLPC	T4	T4/DLPC
β1	1613	1610	~1603	1597	1581[c]	1583
β2	1604	1600	~1593		1569	1565
α1	1580	1576	1576	1575	1581	1572
α2	1539	1536	1536	1535	1539	-
α5			1282	1285[d]	1293	-
CH_2 twisting	1267	1265	1257	1255[d]	1273	1273
						1256
$\nu(C_\alpha–O/C–OH)_{o.o.ph.}$	1258	-	1247	1247[d]	1240	~1246
$\nu(C_\alpha–O/C–OH)_{i.ph.}$	1238	1235	1235	1240	~1230	1239
$\nu(C_\beta–O)$	1195	1191	1187	1182[d]	1179	-
δ(OH)	1160	1157				
ν(C–N)	1054	1050	1052	1052	1055	1050
α9					1040	1042
β12	849	846	~851	847	858	847
2τβ	860	856			848	
δ(α/β)	815	812	830	822	830	821
$\nu_a(C_\alpha–I)$	245	241	245	243	~245	~245
$\nu_a(C_\beta–I)$					~245	~245
$\nu(C_\beta–I)$			-	226		
$\nu_s(C_\beta–I)$					221	213
$\nu_s(C_\alpha–I)$	186	182	187	187	192	187

[a] αi and βi (i = 1,2,5,9,12) correspond to modes of the ring-α and ring-β, respectively, described in Reference 27.
[b] ν: stretching; τ: torsion; δ: deformation; o.o.ph: out-of-phase; i.ph.: in-phase; s: symmetric; a: antisymmetric.
[c] The coupling of the vibrations gives rise to the modes (α1 + β1) and (α1 - β1) that may contribute to the same band.
[d] In the difference Raman spectrum of T3.

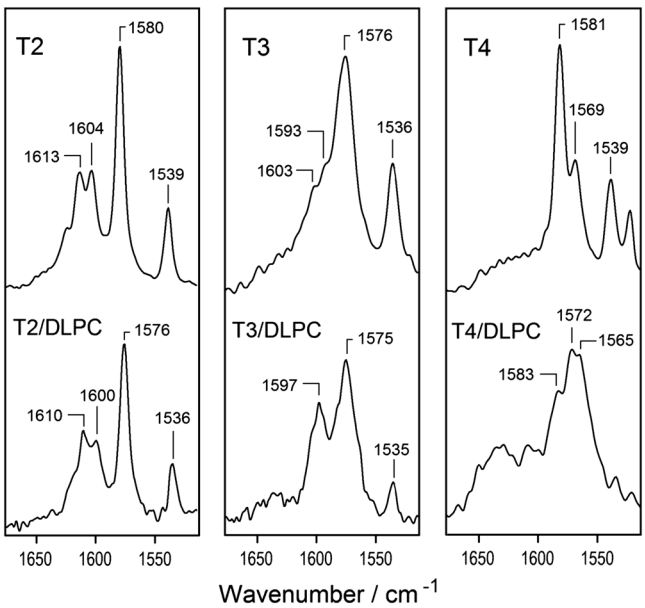

Figure 2. Raman spectra of iodothyronines in pure state (above) and iodothyronine/DLPC complexes (bellow) in the region corresponding to the symmetric and antisymmetric C=C stretchings characteristic of para-substituted aromatic rings (the α1, α2, β1, and β2 modes are observed).

show two strong ring vibrations around 1605 and 1580 cm^{-1}. These vibrations are analogous to the tyrosine modes ν8a and ν8b, respectively,[30] and appear duplicated in the iodothyronines studied: the vibrations located in the ring-α of the hormone (here named α1 and α2 modes, where the numbers 1 and 2 correspond to labels a and b in tyrosine) and those located in the phenolic ring-β, (here, the β1 and β2 modes). Due to the different substitution patterns, differences are observed for the ring-β modes in the spectra of T2, T3, and T4, while the modes originating from ring-α show similar characteristics.[31] In the Raman spectrum of T2, the β1, β2, and α2 appear like medium intensity bands at 1613, 1604, and 1539 cm^{-1}, respectively, and the strongest band of this region (1580 cm^{-1}) is assigned to the α1 mode. The effect of lipid surroundings on T2 is manifested by downshifts of all these bands by ~4 cm^{-1}. The Raman spectrum of T3 in pure state shows the α1 and α2 modes at 1576 and 1536 cm^{-1}, respectively, while the vibrations of the single iodine-substituted aromatic ring are observed like shoulders of the strong α1 band, at approximately 1603 (β1) and 1593 (β2) cm^{-1}. In the Raman spectrum of T3/DLPC complex, a new band at 1597 cm^{-1} is defined which, together with the α1 band (1575 cm^{-1}), show irregular contours suggesting the presence of several components. The α2 band of T3 is not affected upon lipid interaction. The identical iodine substitution at the *ortho*-position on both aromatic rings of T4 leads to the coupling of the symmetric α1 and β1 modes.[31] Then, the (α1 + β1) and (α1 - β1) complex vibrations are assigned to the strong band at 1581 cm^{-1} in the Raman spectrum of the pure hormone. The α2 and β2 modes of T4 appear at 1539 and 1569 cm^{-1}, respectively. With exception of the α2 mode that does not exceed the noise level, the Raman spectrum of T4/DLPC complex shows drastic changes in this spectral region, manifested by the decoupling of the symmetric vibrations, now appearing at 1583 and 1572 cm^{-1} and the shift to 1565 cm^{-1} of the β2 mode.

The Raman spectra of the three iodothyronines show several bands between 1100 and 1300 cm^{-1} corresponding to the CH_2 twisting, C–O stretchings, and phenolic OH deformation.[31] These bands are depicted in Fig. 3. We focus our study on the C–O bands since they may provide valuable skeletal information. According to quantum-chemical calculations, the coupling between the C_α–O and C–OH bonds yields the in-phase $\nu(C_\alpha\text{–O/C–OH})_{i.ph.}$ and the out-of-phase $\nu(C_\alpha\text{–O/C–OH})_{o.o.ph.}$ vibrations, which appear between 1230 and 1260 cm^{-1}.[31] The $\nu(C_\beta\text{–O})$ mode is observed at 1195–1180 cm^{-1}. Each hormone shows a distinct behavior when interacting with phospholipid, which is manifested by specific spectral changes in the Raman spectrum of the corresponding complex sample. The lipid medium induces downshifts of all the T2 bands of this region and intensity loss of the C–O bands, which makes difficult to detect the signal corresponding to the $\nu(C_\alpha\text{–O/C–OH})_{o.o.ph.}$ mode. The effect produced by the lipid environment on T3 is better observed in the difference spectrum that results from the subtraction of the DLPC spectrum from the T3/DLPC complex spectrum (included in Fig. 3). In the difference spectrum, the band corresponding to the $\nu(C_\alpha\text{–O/C–OH})_{i.ph.}$ is up shifted from 1235 cm^{-1} to 1240 cm^{-1} and shows a shoulder at ~1230 cm^{-1}, the $\nu(C_\alpha\text{–O/C–OH})_{o.o.ph.}$ vibration remains apparently unaffected by the lipid medium, and the $\nu(C_\beta\text{–O})$ is downshifted by 5 cm^{-1} appearing as a broad and irregular band. The C–O bands of T4 are also significantly affected by the lipid medium; however, an unambiguous assignment of the features in the T4/DLPC spectrum is difficult: the $\nu(C_\alpha\text{–O/C–OH})_{o.o.ph.}$ and $\nu(C_\alpha\text{–O/C–OH})_{i.ph.}$ modes of T4 are observed as a medium intensity band at 1240 cm^{-1} and a shoulder at ~1230 cm^{-1}, respectively; the spectrum of the complex sample shows a band a 1239 cm^{-1}, a shoulder at higher wavenumbers (~1246 cm^{-1}), and a new band at 1256 cm^{-1}. These features indicate that, at least one of the coupled $\nu(C_\alpha\text{–O/C–OH})$ vibrations resulted significantly up shifted upon lipid insertion. In addition, the strong band assigned to the νC–N of T4 (1055 cm^{-1}) is downshifted 5 cm^{-1}. The level of noise of this spectral region in the T4/DLPC spectrum does not allow a confident identification of the $\nu(C_\beta\text{–O})$ band.

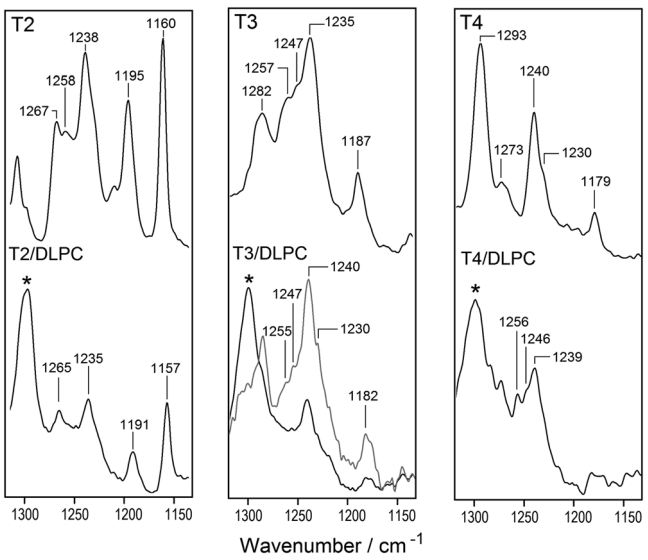

Figure 3. Raman spectra of iodothyronines in pure state (above) and iodothyronine/DLPC complexes (bellow) in the region corresponding to the C–O stretching vibrations. The trace in gray corresponds to the difference Raman spectrum, obtained by subtracting the Raman spectrum of pure DLPC from the T3/DLPC mixture.

Tyrosine, and in general, *para*-substituted benzenes with *C2v* local symmetry display a doublet at ca 850–830 cm^{-1} in the Raman spectrum, which is due to a Fermi resonance between the ring-breathing mode and the overtone of an out-of-plane ring-bending vibration.[32,33] The intensity ratio I_{850}/I_{830} depends on the hydrogen-bonding state of the phenol OH-group.[32] Figure 4 shows the spectral region comprised between 800 and 900 cm^{-1} of the three iodothyronines in pure state and in interaction with DLPC. As expected, this doublet is observed in the Raman spectra of T2 and T4 at 860/849 cm^{-1} and 858/848 cm^{-1}, respectively, originated by the fundamental β12 symmetric mode and the overtone 2τβ.[19,31] In the spectrum of T2, the ratio $I_{860}/I_{849} \cong 0.8$ indicates that the hormone in the solid state acts as a hydrogen donor.[34] On the contrary, T4 acts as a strong hydrogen-bonding acceptor according to the $I_{858}/I_{848} \cong 1.8$. Differences in the doublet ratio of T2 and T4 should represent the variation in the local environment of the hormones due to their incorporation to the membrane. However, due to the proximity of these T2 bands to the $\tau(CH_2)$ band of DLPC at 1298 cm^{-1}, a confident evaluation of the Fermi doublet of T2 upon lipid interaction is not accomplished from the analysis of the T2/DLPC complex spectrum, where the intensity ratio appears slightly affected. In the spectrum of T4/DLPC complex, a single band at 847 cm^{-1} is observed, indicative of the disappearance of the Fermi resonance due to the spectral shift of the β12 vibration upon membrane insertion. In the Raman spectrum of T3, the β12 mode appears as a shoulder at ~851 cm^{-1}, but is notably enhanced appearing as a well-defined band at 847 cm^{-1} in the spectrum of T3/DLPC complex. The skeletal $\delta(\alpha/\beta)$ deformation involving both aromatic rings is also observed in this spectral region of the iodothyronines (815 cm^{-1} in T2 and 830 cm^{-1} in T3 and T4). In T3/DLPC, the $\delta(\alpha/\beta)$ band loses intensity and downshifts to 822 cm^{-1}, while in T4/DLPC spectrum, a new feature 821 cm^{-1} is also observed.

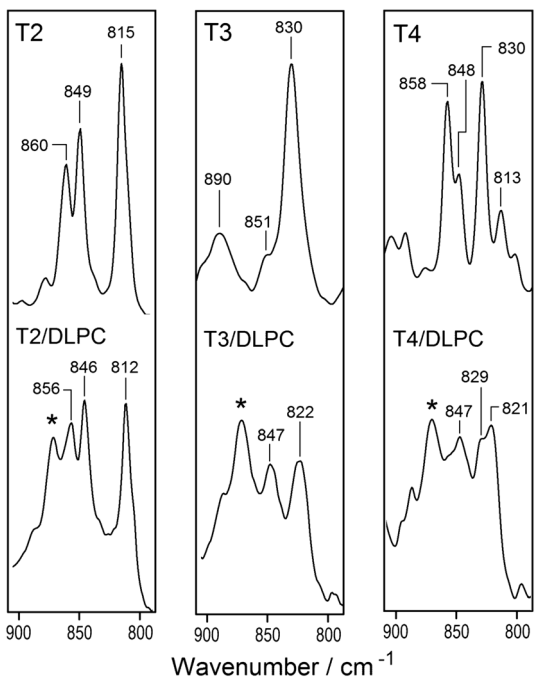

Figure 4. Raman spectra of iodothyronines in pure state (above) and iodothyronine/DLPC complexes (bellow) in the range between 900 and 800 cm^{-1}. The Fermi doublet characteristic of tyrosine and symmetric derivatives is shown by T2 and T4.

The spectral changes observed in the region corresponding to the C–I stretching modes (Fig. 5) are consistent with the interaction degree shown by each iodothyronine with phospholipid. In the case of T2, both the symmetric and antisymmetric $\nu(C_\alpha–I)$ modes (186 and 245 cm^{-1}, respectively, in the spectrum of pure T2)[31] downshift 4 cm^{-1} upon lipid interaction. No further spectral changes are evidenced. The T3/DLPC spectrum shows the C–I bands with irregular contours suggesting the presence of an additional signal contributing to each vibration: the symmetric $\nu(C_\alpha–I)$ (245 cm^{-1} in T3 spectrum) shows at least three component signals centered at 243, ~232, and 226 cm^{-1} (better solved in the difference spectrum of T3, Fig. 5). The feature at 226 cm^{-1} is assigned to the $\nu(C_\beta–I)$ mode which, in the spectrum of the pure hormone, is not observed due to overlapping of the stronger $\nu(C_\alpha–I)$ bands.[20,31] The band corresponding to the antisymmetric $\nu(C_\alpha–I)$ (187 cm^{-1} in T3) is not shifted but shows a shoulder at ~175 cm^{-1} in T3/DLPC. The shoulders of the main symmetric and antisymmetric $\nu(C_\alpha–I)$ bands are interpreted as evidence of the presence of two conformers of T3, in agreement with published reports.[20,35] The Raman spectrum of pure T4 shows two very strong bands at 221 and 192 cm^{-1} which are associated to the symmetric $\nu(C_\beta–I)$ and $\nu(C_\alpha–I)$ modes, respectively. Upon lipid interaction, both bands downshift, but the vibration in the ring-β is the most affected ($\Delta\nu=-8\ cm^{-1}$). A subtle shape change is observed concerning the antisymmetric $\nu(C_\beta–I)$ and $\nu(C_\alpha–I)$ modes. They appear as a weak shoulder at ~245 cm^{-1} in T4 and T4/DLPC spectra.

Thyroid hormones effects on DLPC

The regions comprised between 3200–2600 and 1800–250 cm^{-1} of the Raman spectrum of DLPC in pure state and the Raman spectra of the hormone-lipid complexes, are shown in Fig. 6. In order to determine the effects of the three iodothyronines on the membrane properties, we pay special attention to the bands belongings to the acyl chain ν(C–H) and ν(C–C) stretching vibrations. Other signals appearing in these spectral regions, like those associated to $\delta(CH_2)$, $\delta(CH_3)$, and $\tau(CH_2)$ are known to contain a great deal of information about the conformational order;[36–44] however, iodothyronines present strong vibrations overlapping with several of these phospholipid bands (Fig. 6) and make it difficult to arrive to conclusions that rule out or confirm induced structural changes in the lipids from their evaluation. Table 2 lists the wavenumbers of the C–H and C–C stretching bands of DLPC in pure state and in interaction with the hormones.

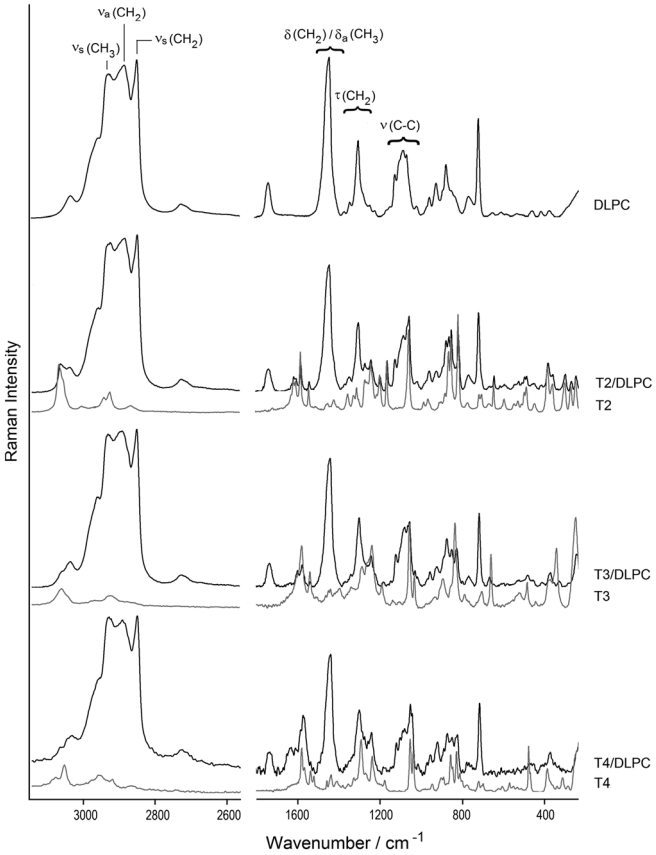

Figure 6. Raman spectra of DLPC in pure state and iodothyronine–DLPC complexes between 3200–2600 and 1800–250 cm^{-1}. Band assignment of the main lipid vibrations is included in the spectrum of DLPC. The Raman bands of the iodothyronines are easily identified in the complex spectra by overlaying the respective Raman spectrum (gray traces).

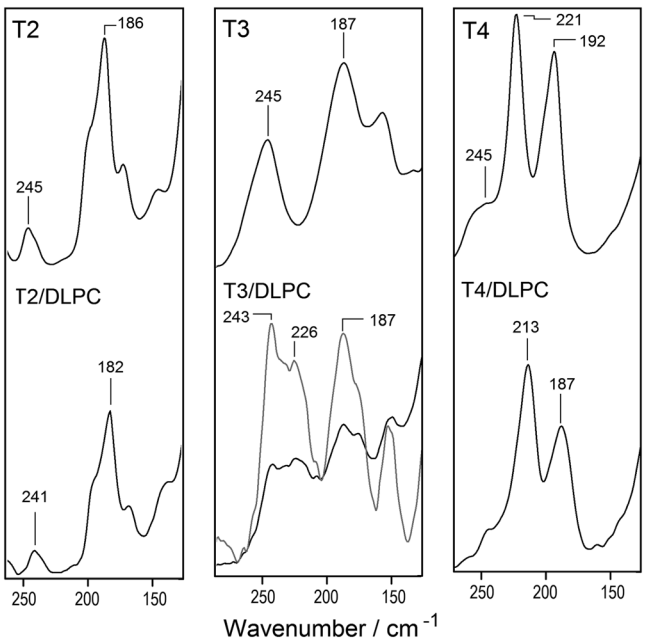

Figure 5. Raman spectra of iodothyronines in pure state (above) and iodothyronine/DLPC complexes (bellow) in the region corresponding to the C–I stretching vibrations. The trace in gray corresponds to the difference Raman spectrum, obtained by subtracting the Raman spectrum of pure DLPC from the T3/DLPC mixture.

The C–H stretching region is well characterized by three principal bands:[38,44] the symmetric stretching $\nu_s(CH_2)$ at 2849 cm^{-1}, the asymmetric stretching $\nu_a(CH_2)$ at 2885 cm^{-1}, and the symmetric stretching $\nu_s(CH_3)$ at 2930 cm^{-1}. The peak intensity ratios among these vibrations are indicative of the acyl chain rotational disorder and intermolecular chain coupling. In addition, the wavenumbers of the $\nu_s(CH_2)$ and $\nu_a(CH_2)$ bands also reflect conformational order and interchain coupling.[38,44] In the spectrum of pure DLPC, both the wavenumbers and the band shapes are characteristic of lipids in the liquid-crystalline phase (Fig. 6). Upon T2 incorporation, negligible changes are revealed by these features in wavenumbers and in relative intensities, pointing out a poor effect of this hormone on the membrane. On the contrary, the Raman spectra of T3/DLPC and T4/DLPC show that the $I[\nu_s(CH_3)]/I[\nu_s(CH_2)]$ peak intensity ratio increases of ca. 10% in relation to the ratio in the DLPC spectrum, indicating that T3

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Table 2. Experimental Raman bands (cm^{-1}) corresponding to the C–H stretchings of DLPC. Calculated band positions (cm^{-1}) and intensities (arbitrary units, in parenthesis) corresponding to the components of the C–C stretching bands, obtained after the curve-fitting procedure. Tentative assignments of the features are presented

Assignment[a]	DLPC	T2/DLPC	T3/DLPC	T4/DLPC
ν_s CH_3	2930	2929	2929	2927
ν_a CH_2	2885	2883	2892	2890
ν_s CH_2	2849	2849	2850	2849
$\nu(C–C)_{T(i.ph.)}$	1120 (130)	1120 (176)	1120 (111)	1119 (598)
$\nu_s(PO_2^-)$	1100 (160)	1099 (199)	1102 (85)	1109 (268)
				1101 (626)
$\nu(C–C)_G$	1087 (144)	1087 (50)	1087 (180)	1089 (891)
$\nu(C–C)_G$	1076 (182)	1076 (310)	1075 (167)	1079 (455)
				1071 (786)
$\nu(C–C)_{T(o.o.ph.)}$	1062 (162)	1061 (223)	1062 (182)	1060 (920)

[a]ν: stretching; s: symmetric; a: antisymmetric; o.o.ph: out-of-phase; i.ph.: in-phase; T: trans; G: gauche.

and T4 induce increase in the rotational and vibrational freedom of the terminal methyl groups. In addition, the $\nu_a(CH_2)$ band upshifts 7 and 5 cm^{-1}, according to the incorporation of T3 and T4, respectively, which evidences increasing chain decoupling.

The spectral region comprised between 1150 and 1000 cm^{-1} in the spectra of lipids membranes is characteristic of methylene C–C stretching modes (Fig. 7). Their relative intensities give information about the *trans/gauche* conformers ratio in the acyl chains of lipids.[38,44] In the spectrum of pure DLPC, the peaks at 1063 cm^{-1} and 1120 cm^{-1} are assigned to the out-of-phase $\nu(C–C)_{T(o.o.ph.)}$ and in-phase $\nu(C–C)_{T(i.ph.)}$ *trans* stretchings, respectively, while the broad and complex signal centered at 1080 cm^{-1} represents the C–C stretching of *gauche* conformers ($\nu(C–C)_G$). The comparative strong intensity of this last peak is in agreement with the relatively high population of *gauche* conformers, typical of L_α phase. The shoulder at ~1100 cm^{-1} is attributed to the symmetric $\nu_s(PO_2^-)$stretching. The Raman spectra of the iodothyronine/DLPC complexes show a strong band at ~1050 cm^{-1}, corresponding to the ν(C–N) stretching of the hormones that partially overlaps the

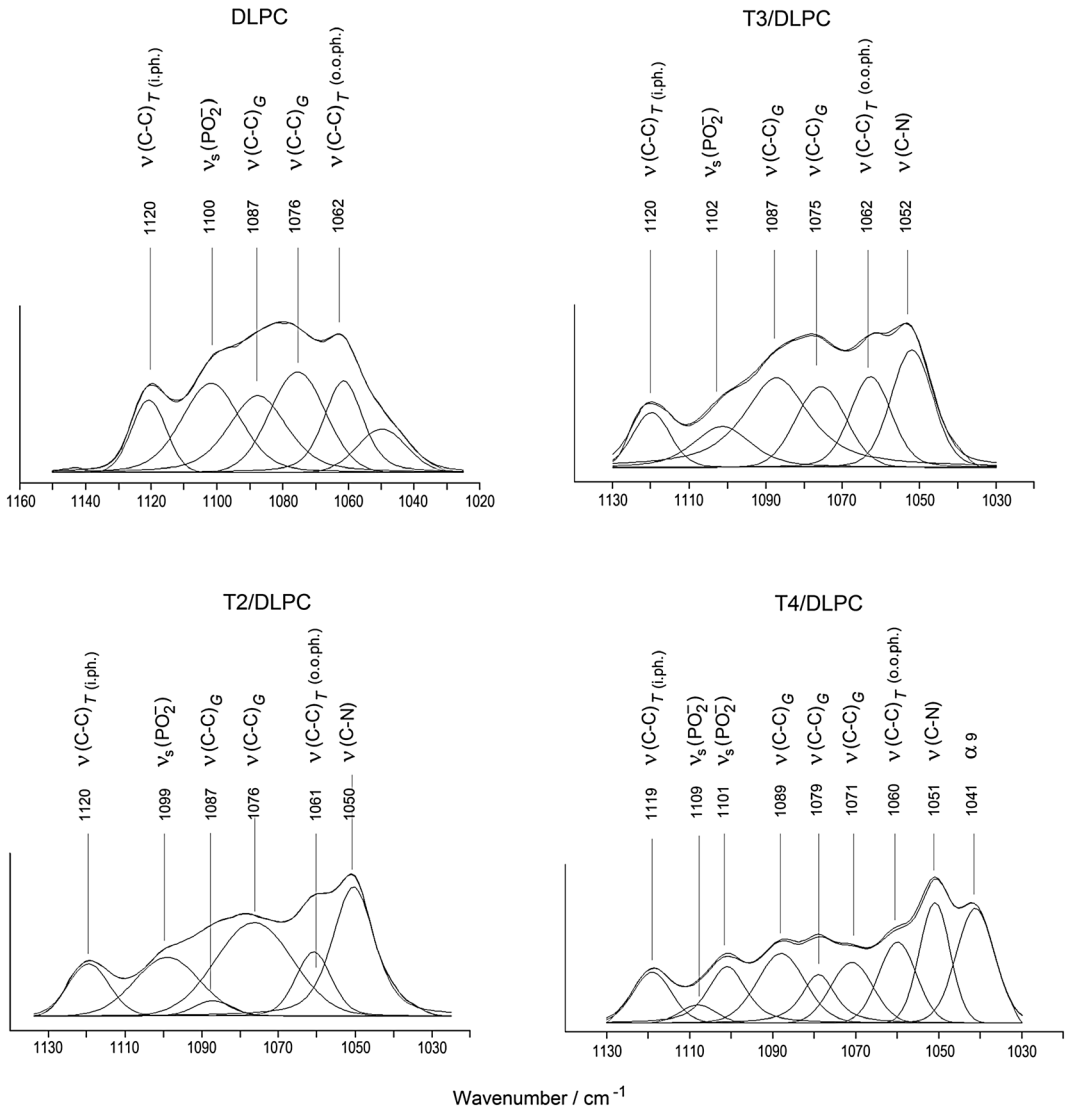

Figure 7. Spectral region corresponding to the C–C stretchings, characteristic of the acyl chains. Decomposed bands after the curve-fitting procedure are shown. The reconstructed band is superimposed on the original spectrum in order to show the level of fit attained. The spectral assignment of the bands is extended to the iodothyronine vibrations appearing in this region.

lipid ν(C–C)$_{T(o.o.ph.)}$. The Raman spectra of the complex samples show the *trans/gauche* population altered upon hormone incorporation. Based on the Iν(C–C)$_G$/Iν(C–C)$_{T(i.ph.)}$ peak intensity ratio, it is evidenced that T2 and T3 incorporation increases the *gauche* conformer content by approximately 6 and 19%, respectively. The evaluation of this intensity ratio in the T4/DLPC spectrum is not straightforward due to the splitting of the ν(C–C)$_G$ band into two components, at 1087 and 1079 cm^{-1}. This fact leads to a reevaluation of this spectral region based on derivation and deconvolution and a subsequent curve-fitting procedure.[28,29] This procedure allows a better visualization of possible band components present in this region. Then, a qualitative analysis is performed. The results of the decomposition of the C–C bands by the curve-fitting procedure together with a tentative assignment are shown in Fig. 7. DLPC spectrum shows the ν(C–C)$_G$ band resolved into two signals (1087 and 1076 cm^{-1}). The band at 1100 cm^{-1} is assigned to the $\nu_s(PO_2^-)$ mode. Similar C–C band patterns are obtained from the analysis of the T2/DLPC and T3/DLPC spectra. The main effect of T2 on the membrane is evidenced by the increased area of the band at 1076 cm^{-1} at the expense of the band at 1087 cm^{-1}, while T3 tends to induce the opposite situation. On the contrary, more drastic changes are observed in T4/DLPC, mainly due to the splitting of the 1076 cm^{-1} signal into the 1079 and 1071 cm^{-1} bands and the enhancement of the 1089 cm^{-1} component. These spectral alterations of the ν(C–C)$_G$ band correlate with the changes observed in the C–H stretching region (see above). The $\nu_s(PO_2^-)$ band appears also split into two bands. This fact is supported by MD simulations that predict strong hydrogen-bond interactions between the $-NH_3^+$ group of T4 with the PO_2^- group of the lipid.[21] The strongest bands at ~1050 cm^{-1} in the iodothyronine/DLPC spectra correspond to the ν(C–N) stretching of the hormones, and the band at 1041 cm^{-1} in T4/DLPC, to the symmetric ring mode α9.[31]

The spectral region around 1450–1300 cm^{-1} contains a number of overlapping deformation modes of lipids predominantly dominated by the methylene scissor mode $\delta(CH_2)$ which is observed in the Raman spectrum of DLPC as an asymmetric and broad peak with maximum at 1440 cm^{-1} (Fig. 6). The shoulder at higher frequencies is attributed to the asymmetric methyl bend $\delta_a(CH_3)$.[38,44] Both, the frequencies and the band shapes show subtle changes upon hormone incorporation. However, considering the complexity of the bands, a confident analysis of these spectral changes is not possible.

Discussion

The results previously obtained by MD simulations[21] and differential calorimetry[18] have indicated that T2 is able to penetrate the lipid bilayer in the liquid-crystalline phase and get both aromatic rings embedded into the acyl chains of the lipids, with the ring planes oriented perpendicularly to the membrane surface. This orientation clearly results in a minimum structural alteration of the hormone.[21] The spectral analysis presented here confirms these statements since most of the Raman bands of T2 in interaction with lipids experience similar downshifts, indicative of similar surroundings to both rings. However, no changes in relative intensities or appearance/disappearance of bands that could indicate any structural alteration of T2 upon lipid interaction are observed.

Several Raman bands of T3 in lipid interaction show differences (e.g. shifts and intensity changes) regarding those in the spectrum of the pure hormone. In addition, many of these affected bands show irregular contours that appear as better defined shoulders upon spectral subtraction of the DLPC spectrum. NMR experiments of T3 in methanol solution have shown the presence of two conformers of the hormone, coexisting in equilibrium.[35] They are called T3p and T3d when the iodine atom in the ring-β is in proximal or distal orientation to the ring-α, respectively (see Fig. 1). In addition, a previous spectral study of the behavior of T3 in PC membranes has evidenced the existence of two conformers of this hormone in the lipid medium.[20] Now, the concept of both conformers coexisting in interaction with DLPC is also adopted in order to explain the presence of shoulders exhibited by several fundamental bands in the spectrum of T3/DLPC. According to MD simulations, the T3d conformer adopts a similar position and orientation into the lipid medium as T2, while T3p experiences a considerable limitation to get inserted into the hydrophobic region.[21] This differential behavior is supported by the C–I stretching modes since $\Delta\nu$ up to 11 and 13 cm^{-1} are observed for the $\nu(C_\alpha–I)$ bands, indicative of different surroundings to the ring-α.

The Raman spectrum of T4/DLPC shows several drastic differences with respect to that of the pure hormone. The split of the band at 1581 cm^{-1} is interpreted as the uncoupling of the symmetric ring-stretching vibrations induced by the lipid medium. One plausible explanation of this behavior consists in considering that each aromatic ring of T4 feels a distinct effect from its surroundings. This is in agreement with the MD simulations that show that the system T4-membrane is in stable conformation when the hormone has its ring-β immersed in the hydrophobic region and part of the ring-α is localized in the polar region of the membrane.[21] Other spectral differences like the 5 cm^{-1} downshift of the ν(C–N) band and the appearance of a medium intensity band at 821 cm^{-1}, which can be associated to the NH_3^+ wagging (814 cm^{-1} in the spectrum of T4), point out that T4 also strongly interacts with the polar region of the membrane. In addition, significant band shifts corresponding to the β2 ring-stretching, the $\nu(C_\alpha–O/C–OH)_{o.o.ph.}$ and/or $\nu(C_\alpha–O/C–OH)_{i.ph.}$, and the symmetric $\nu(C_\beta–I)$, together with the disappearance of the Fermi doublet are strong indicators of structural distortions on T4 molecule, mainly affecting the orientation of the ring-β.

In concomitance with the structural effects exerted on the iodothyronine molecules by the lipid environment, the membrane also shows sensitivity to their incorporation. The utility of Raman spectroscopy for determining acyl chain conformational order in lipid and lipid–drug complexes has been extensively demonstrated.[25,26,38,43,44] We focus our analysis mainly in the perturbation of the hydrophobic region of the membrane by evaluating the spectral markers concerning the C–C and C–H stretchings in the acyl chains. Upon T2 incorporation, these features show negligible changes with respect to their characteristics in the spectrum of pure DLPC, indicating that T2 produces minor effects on the membrane, although MD simulations show that this hormone introduces both aromatic rings inside the hydrophobic region and this iodothyronine reaches the deepest in the lipid.[21]

On the contrary, it is demonstrated that T3 and T4 induce changes in the interchain coupling in the lipid as well as in the C–C *gauche* conformation content. The incorporations of T3 and T4 to DLPC induce similar increases in the freedom of motion of the CH_3 methyl terminal groups and in the rotational disorder of the acyl chains. In addition, the molecular interactions between acyl chains are also decreased by both hormones since upshifts in the $\nu_a(CH_2)$ bands are observed. Correlatively, alterations in the C–C vibrations are also observed. A better understanding of the effect exerted by these hormones on the C–C

gauche content is attained by considering the relative intensities of the decomposed C–C bands. The components at 1087 and 1076 cm^{-1} (in pure DLPC) are associated with the two predominant kinked-chain structures of *n*-alkanes and acyl chains that were determined by Raman spectroscopy: the 'end-*gauche*' conformation at the chain end and the *gauche-trans-gauche* (*g-t-g*) conformation in the body of the chain.[45–49] Spectroscopic observations have pointed out that chain-end defects are more important at lower temperature while the *g-t-g* conformation rises as the temperature increases and the membrane becomes more fluid.[46,50] Then, we assign the feature at 1087 cm^{-1} to the end-*gauche* conformation and that at 1076 cm^{-1} to the *g-t-g* conformation. It is expected that this last band reaches the maximum intensity in the liquid-crystalline state. The incorporation of T3 to DLPC membrane enhances both bands associated to *gauche* conformation. Upon T4 interaction, two significant changes are observed in the $\nu(C–C)_G$ region: the enhancement of the 1089 cm^{-1} band, compared to the $\nu(C–C)_{T(i.ph.)}$ band, which suggests an increased population of end-*gauche* conformations, and the split and shift of the *g-t-g* band. A preliminary interpretation of this last fact consists in considering that T4 induces the appearance of a third type of kinked-chain structure, the 'double-*gauche*' (g-g) conformation,[46] but further studies are necessary to confirm this acyl chain behavior. In addition, T4 evidences the strongest interactions with the polar region of the membrane which is manifested by the appearance of a new band attributable to the PO_2^- group. As was predicted by MD simulations, this fact suggests that T4 adopts a different position (e.g. penetration level) in the membrane than T3, in concordance with the iodine content, and hence with the molecular size.[21]

Conclusions

Confocal Raman microscopy has shown to be an effective technique to elucidate thyroid hormone–DLPC interactions at a molecular level, since the Raman scattering is collected from a small sample volume where the concentration of lipid and the partitioned hormone results sufficiently high. This minimizes the contributions from the surroundings. Conformational information concerning the interacting lipid and hormone are extracted from the Raman spectra presented here. A clear correlation between the effects produced by the iodothyronines on the membrane properties and the structural conformation of the hormones inside the lipid environment is derived. The alteration in the membrane properties is associated to the iodine content in the ring-β instead of the lipid penetration attained. Thus, T2 produces minimum lipid perturbation due to the lack of iodine atoms in the ring-β, regardless of its deep penetration predicted by MD simulations. On the contrary, the number of iodine atoms in the ring-β of T4 is responsible for producing the highest alteration in the hydrophobic region of the membrane. In addition, the interactions between the polar groups of both, T4 and phospholipid, are expected to play important roles in the process of membrane penetration by the hormones.

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