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Boronphenylalanine insertion in cationic liposomes for Boron Neutron Capture Therapy

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Abstract

Cationic liposomes are widely used as carriers of biomolecules specifically targeted to the cell nucleus. *p*-Boronphenylalanine (BPA) is a powerful anti-tumor agent for Boron Neutron Capture Therapy (BNCT). In this paper, ^{1}H and ^{13}C NMR was used to study the insertion of BPA in mixed liposomes, made up by the positively charged 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and the zwitterionic 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE). The boronated drug was distributed between the water phase and the liposomes. The location site of BPA into the lipid bilayer was investigated and the boron-substituted aromatic ring was found inserted in the hydrophobic region, whereas the amino acidic group was oriented towards the aqueous environment. Further information was given by proton spin-lattice relaxation rates.

Keywords: BPA; BNCT; Liposomes; NMR of lipid structures; DOTAP/DOPE

1. Introduction

Boron Neutron Capture Therapy (BNCT) is a binary cancer treatment [1] based on the reaction:

$$^{10}_{5}\mathrm{B} + ^{1}_{0}n \rightarrow ^{4}_{2}\mathrm{He} + ^{7}_{3}\mathrm{Li} \quad (1)$$

which occurs when ^{10}B, delivered to tumor tissues, is irradiated with low energy neutrons. Both $^{4}_{2}He$ and $^{7}_{3}Li$ particles have a short path-length (<10 μm) in tissues and can damage only the cells in which they are located, before being absorbed. The efficacy of BNCT depends primarily on selective boron uptake by tumor cells with respect to the healthy ones; the location of boron atoms close to the nucleus also represents a basic requirement [2–4] and, for this purpose, specific transport peptide units (TPU) have been recently proposed as nucleus-directed bioshuttles of boron atoms [5].

In recent years, a huge number of boron–containing molecules have been designed and synthesized for BNCT purposes [6–13] and two of them are currently used in clinical applications, namely the sodium salt of mercaptoundecahydrododecaborate (BSH) and *p*-boronphenylalanine (BPA). In particular, BPA is the most commonly used. A selective uptake of BPA by tumor cells has clearly been established [14–17], due to an increased amino acid transport through the membranes of malignant cells [18]. However, BPA solubility in water and in physiological media ($\sim 7\times10^{-3}$ mol/l) [19,20] is not high enough for BNCT applications, and BPA it has often been used in the form of complex with simple monosaccharides, e.g. glucose and fructose [20–23].

It is therefore of the outmost importance to find good carriers for BPA which are able to enhance the compatibility of this molecule for aqueous media and, possibly, its affinity toward the cell nucleus, while preserving its positive therapeutic characteristics. Among the most used drug delivery agents, liposomes [24] of diameter not exceeding 100–200 nm are able to escape over the inherently leaky endothelium of the blood vessel walls and to accumulate in the under-

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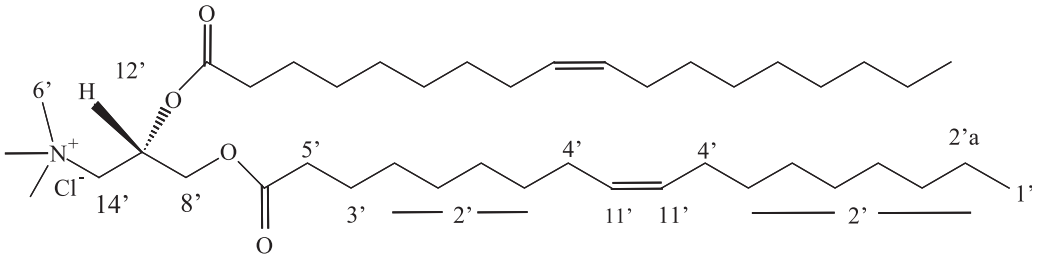
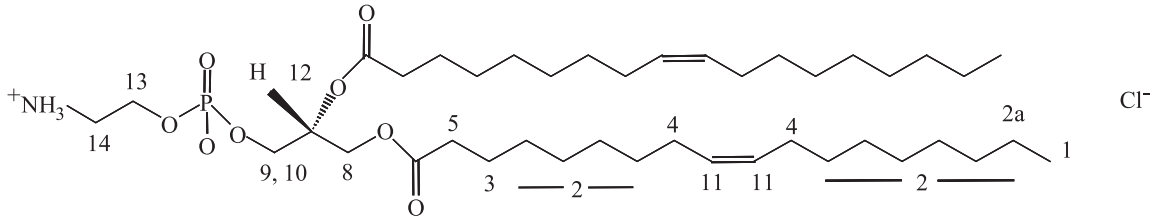
lying tumor tissue [25]. In addition, positively charged liposomes have been shown to interact with the cell nucleus and are currently employed as non-viral vectors in gene therapy [26–31].

In this work, we studied the insertion of BPA into mixed liposomes, made with the positively charged lipid 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP, **1**) and with the zwitterionic lipid 1,2-dioleoyl-*sn*-glycerol-3-phosphoethanolamine (DOPE, **2**), in order to establish whether or not these liposomes are suitable carriers for BPA. High resolution nuclear magnetic resonance was chosen as the investigation tool, due to its ability to provide important information on the interactions that take place at molecular level, and it has been successfully used to establish the insertion of biologically active molecules inside liposomes, as well as to characterize the loaded structures [32–35].

A few studies on BPA inserted in conventional and stealth liposomes have been published [36,37], but, to the best of our knowledge, none concerning cationic liposomes has been reported in the literature, yet.



DOTAP, 1



DOPE, 2

2. Experimental

2.1. Materials

DOTAP (**1**) and DOPE (**2**) were purchased from Avanti-lipids (Alabama) and used as received.

Liposomes made by DOTAP and DOPE, in the weight ratio 1:1, were prepared as following. First, multilamellar dispersions were obtained by evaporating the solvent from a stock $CHCl_3$ solution and by adding the appropriate amount of PBS in D_2O. Total lipid composition was 2×10^{-2} M. PBS (phosphate buffer 10^{-2} mol/l at pH = 7.4 and 0.15 NaCl) was used as dispersing medium. Then, liposomes were downsized by extrusion (Liposofast apparatus, Avestin) with 27 passages through polycarbonate membranes of 100 nm pore diameter.

In order to obtain BPA-liposome samples, the borocompound was added in the form of a water solution to the lipids in chloroform, while gently stirring. This allowed to obtain an emulsion, which improved mixibility. Both solvents were then evaporated under vacuum overnight.

Boron concentration in the final samples was measured by inductively coupled plasma-atomic emission spectrometry (ICP-AES, Publiacqua, Firenze). Boron concentration turned out to be dependent on the specific sample preparation and only liposome solutions with at least 90% of the starting boron content were used for NMR measurements.

2.2. Methods

^1H and ^{13}C spectra were obtained on a Bruker DRX 600 spectrometer, operating at 600.13 and 150.89 MHz for ^1H and ^{13}C, respectively. As probeheads we used a reverse triple resonance (^1H, ^{13}C, BB) probe for ^1H monodimensional experiments and a double resonance (^1H, BB) probe for ^{13}C experiments. ^1H spectra were acquired with 8192 complex points with 64 transients (15s recycle delay). ^{13}C spectra were acquired with 8192 complex points, 4096 scansions, using a 20-s recycle delay, under broad band proton decoupling (WALTZ-11006) [38].

Proton spin-lattice relaxation rates were measured using the (180°-τ-90°-t)n sequence. The τ values were: 0.01, 0.02, 0.04, 0.06, 0.08, 0.1, 0.2, 0.4, 0.8, 1, 1.5, 2, 3, 4, 5, 7, 10 and 20 s, respectively, and the delay time t was 20 s in all experiments. Since the recovery of proton longitudinal

magnetization after a 180° pulse is not generally represented by a single exponential, due to the sum of different relaxation terms, the spin-lattice relaxation rates were calculated using the initial slope approximation [39] and, subsequently, a three parameter exponential regression analysis of the longitudinal recovery curves was performed. The maximum experimental error in the relaxation rate measurements was 7%.

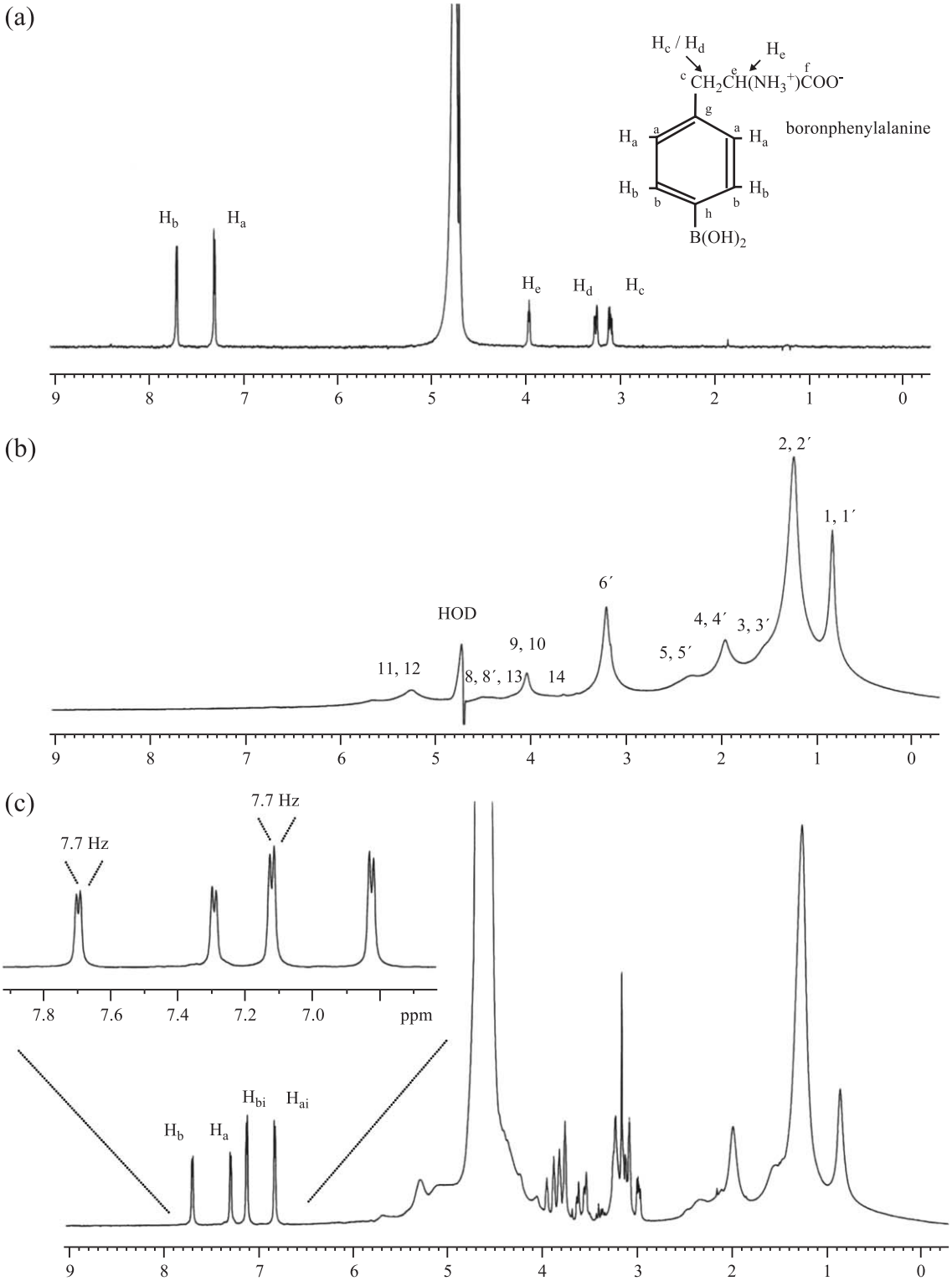


Fig. 1. Comparison of ^1H spectra of: (a) BPA in D_2O/PBS solution, (b) DOTAP/DOPE in D_2O/PBS solution, (c) BPA-DOTAP/DOPE system in D_2O/PBS solution, at 600 MHz and 298 K.

3. Results and discussion

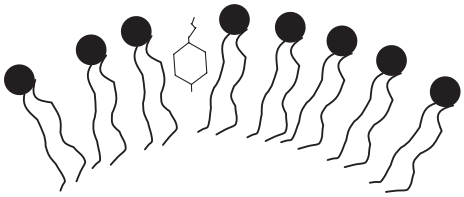
Fig. 1 shows the proton NMR spectra of boronphenylalanine in PBS/D_2O solution (in a), of pure DOTAP/DOPE (50:50 by weight) mixed monolamellar liposomes (in b), and of BPA-containing (92%) DOTAP-DOPE liposomes (in c). The molecular structure and numbering of BPA are also reported in Fig. 1. The assignment of relevant ^1H peaks is shown on the spectra and the corresponding analysis is available as complementary material. Peak attribution was carried out according to the phenylalanine proton assignment published by Wüthrich [40]. Zuo et al. [22] also report a partial spectral assignment of BPA, which is in agreement with ours.

In the ^1H NMR spectrum of mixed DOTAP/DOPE liposomes (Fig. 1b) all the observed signals were broadened, due to partially unaveraged dipolar interactions, as it is usually encountered in microscopically ordered systems. The signals do not have the resolution, which could allow to observe proton–proton scalar couplings. However, line broadening was not too severe in the present case, since liposomes were sufficiently small and monodisperse. Thus, most of the relevant signals were clearly distinct and easily detectable. Peak assignment was mainly based on values reported in the literature for similar systems [41–44] and they are shown directly in Fig. 1b and c.

Spectrum c was the superposition of three absorptions: the first two were due to free BPA and to liposome-inserted BPA; the third absorption was due to the liposome components. New proton signals in the aromatic ring region of the BPA spectrum (6.5–8 ppm), as well as in the methylene (3–3.5 ppm) and methine (~ 4 ppm) regions were indeed observed when BPA was added to DOTAP/DOPE liposomes. The two relatively strong peaks (H_{bi} and H_{ai}) around 7 ppm were identified as two doublets as it is shown in the enlargement of Fig. 1c. These signals had the same scalar coupling constant (7.7 Hz) as those belonging to the aromatic protons of BPA in PBS/D_2O. It is known from the literature that NMR signals usually shift to higher fields when molecules are located in non-polar environments [45–47]. These results suggested the assignment of the two resonances at 7.12 and 6.83 ppm to a fraction of BPA inserted in the bilayer, whose amount was larger than that of free BPA. Area calculation of all peaks allowed us to determine that 60% of the total BPA was inserted in liposomes, whereas the remaining 40% was in the aqueous phase. This ratio did not change over time and the whole system was indeed stable for at least one month. Table 1 reports the chemical shift values for protons of the two different kinds of BPA. The chemical shift variation of the ring signals was about 0.5 ppm, which is indeed quite large if compared with those commonly observed for the insertion of small molecules in micelles, liposomes and other self-assembled structures [33,47–50].

Table 1
Chemical shift values of BPA protons in the free and liposome-inserted environments

H_n	Chemical shift (δ, ppm) of BPA outside the bilayer	Chemical shift (δ, ppm) of BPA inside the bilayer	$\Delta\delta$ (ppm)
H_b	7.69	7.12	0.57
H_a	7.29	6.82	0.47
H_e	3.94	3.87	0.07
H_d	3.10	2.98	0.12
H_c	3.26	3.15	0.11



Scheme 1. Schematic representation of the localization site of BPA in DOTAP/DOPE double layer.

^1H nuclei chemical shift variation decreased when passing from the meta protons ($\Delta\delta = 0.57$ ppm) to the ortho protons ($\Delta\delta = 0.47$ ppm), and further on toward the amino acidic protons ($\Delta\delta \sim 0.1$ ppm), as it is reported in Table 1. This suggested that penetration of the BPA aromatic ring in the bilayer took place with the-$B(OH)_2$ moiety oriented toward the lipid tails, as it is sketched in Scheme 1. Okamura and Nakahara [33] drew similar conclusions studying the penetration of two benzene derivatives (i.e. *n*-propylbenzene and benzyl alcohol) into the bilayer of egg yolk phosphatidylcholine vesicles. Concerning the matrix protons, i.e. those belonging to DOTAP and DOPE, a narrowing of the methyl and methylene signals was observed, indicating faster local motions and, possibly, a slightly more disordered packing of the hydrocarbon chains due to the presence of host molecules [51]. The above findings were therefore a direct evidence of BPA penetration into the liposome membrane.

The spectral separation of inserted and free BPA showed that slow exchange conditions took place between the two environments.

Fig. 2 shows the ^{13}C-NMR spectra of the same systems as in Fig. 1. The spectral assignment of ^{13}C signals of BPA in PBS/D_2O solution (Fig. 2a) was performed on the basis of the corresponding proton spectrum (Fig. 1a), following the attribution of phenylalanine resonances given by Wüthrich [40]. The peaks at 136.4, 129.0 and 132.1 ppm were attributed to C_g, C_a and C_b nuclei, respectively. Line broadening, due to the high relaxation rate caused by interaction with the quadrupolar boron nuclei (^{11}B and ^{10}B) [52], induced the lack of resonances attributable to C_h, which is directly bound to the boron atom. The peak at 182.3 ppm was assigned to the carboxylic carbon C_f, and the signals at 41.2 and 58.0 ppm were attributed to the methylene and methine carbons, respectively. Assignment of

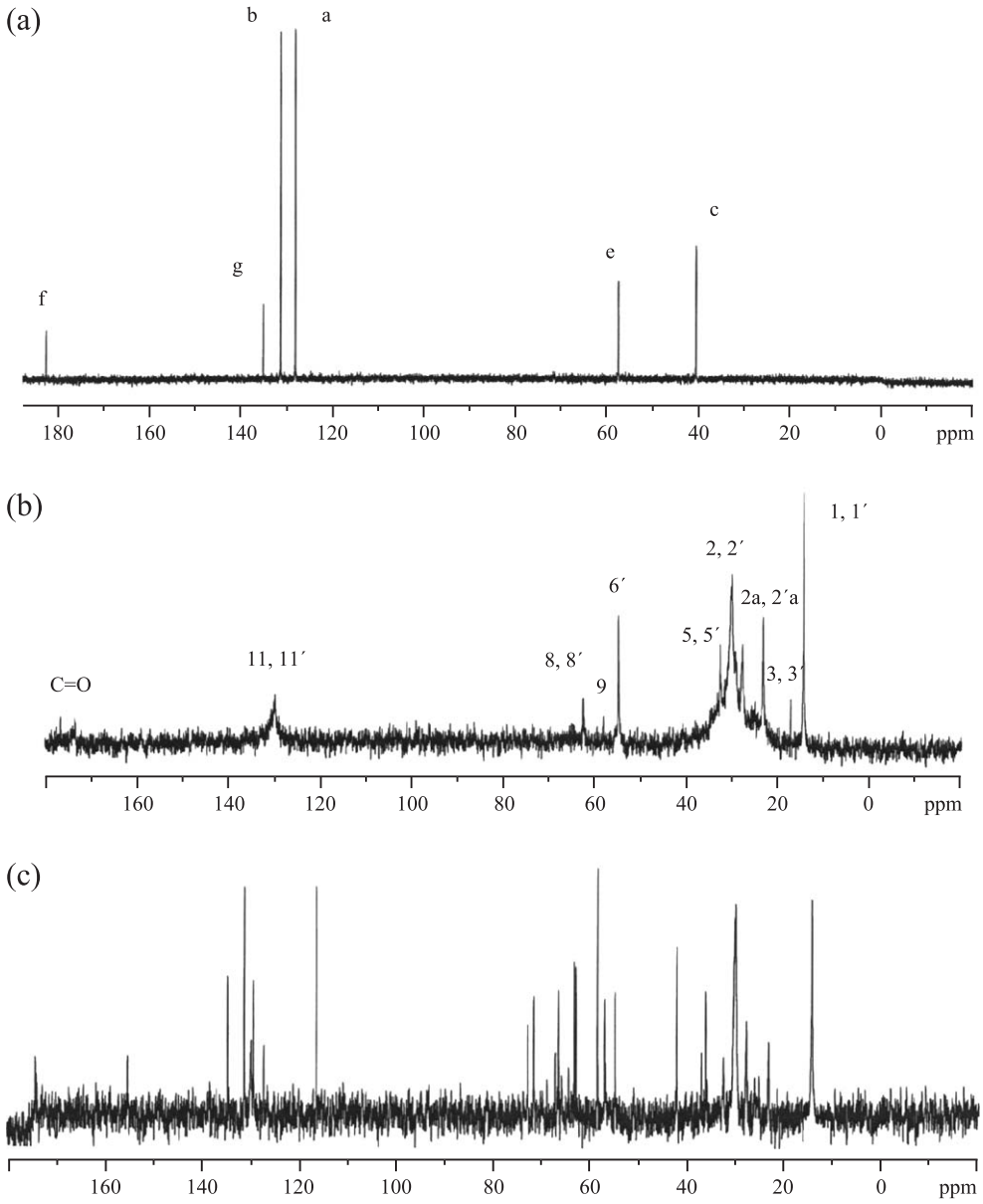


Fig. 2. Comparison of ^{13}C spectra of: (a) BPA in D_2O/PBS solution, (b) DOTAP/DOPE in D_2O/PBS solution, (c) BPA-DOTAP/DOPE system in D_2O/PBS solution at 150 MHz.

some relevant ^{13}C peaks for the DOTAP and DOPE molecules is reported in Fig. 2b.

The above discussed narrowing effect for lipid signals was much more evident in the ^{13}C spectrum of the composite system BPA-liposomes than in the corresponding proton spectrum, as it can be shown by comparing Fig. 2b and c. In this case, the line width decrease was so strong that new signals became detectable. Jezowska et al. [53] observed a similar effect upon addition of β-carotene to dipalmitoylphosphatidylcholine multilamellar liposomes.

The increase of lipid chain molecular motion in the presence of inserted boronphenylalanine suggested the investigation of the molecular dynamics of BPA in the free and liposome-inserted environments through nuclear spin relaxation experiments. ^{13}C-NMR relaxation measurements must be considered one of the most powerful approaches to study local motions in self-assembled systems [54–60]. In this case, the concentration of free and bound species prevented an accurate evaluation of ^{13}C relaxation rates. Nevertheless, the analysis of proton spin-lattice relaxation rates as a function of temperature provided additional information about the motional regime of BPA in the two environments.

Fig. 3 reports the spin-lattice relaxation rates (R_1) of the aromatic protons for free and liposome-inserted BPA versus temperature. All R_1 values decreased with increasing temperature; this indicates that the fast motion conditions in the Larmor frequency time scale ($\omega_0\tau_c \ll 1$, with τ_c being the correlation time which modulates the proton–proton dipole

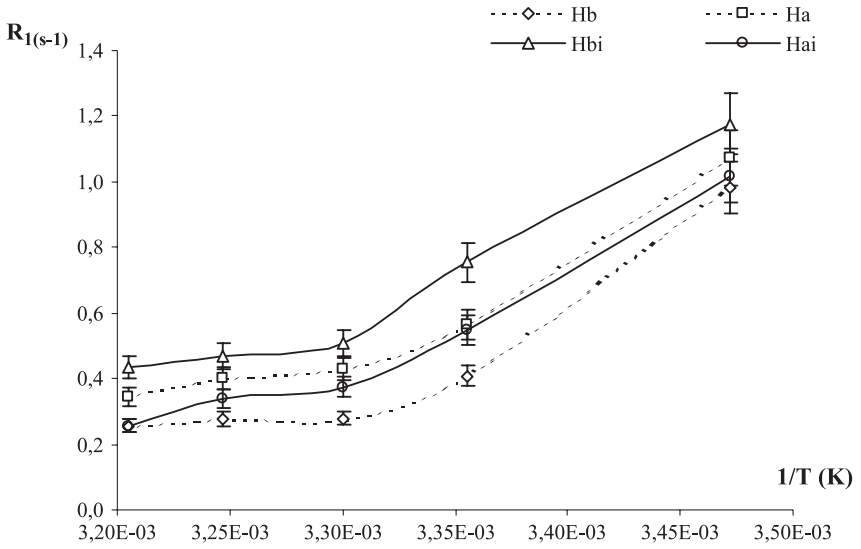


Fig. 3. Spin-lattice relaxation rate values obtained for the aromatic protons of BPA in the BPA-DOTAP/DOPE system as a function of temperature.

interactions) held for both BPA environments. This fact could seem somehow unusual for small molecules trapped in a locally ordered matrix. However, it should be considered that DOTAP and DOPE chains are in a fluid state above ~ 263 K [61] and local motions are fast. The overall R_1 behaviour suggested a complex temperature dependence of the relaxation rates with two different activation barriers for molecular motions. These should be related to local effects only, since exchange mechanisms (such as those among the monomer and aggregate state that can be found in micelles) do not take place in liposomes that are intrinsically out of equilibrium and kinetically stabilized structures. The calculation of activation energy values would require the assumption of fixed and known proton–proton distances, in order to obtain the corresponding τ_c. However, such calculation was beyond the aims of the present work. From a closer look at Fig. 3, it was possible to note that H_i nuclei relaxed faster than non-inserted protons. This indicated a slightly hindered motion, particularly with respect to the corresponding H_b protons of uninserted BPA.

4. Conclusions

In this work, we analysed the NMR spectral properties of boronphenylalanine which interacts with cationic liposomes. Valuable information concerning the insertion of BPA in the lipid bilayer was mainly obtained from the aromatic region of the proton spectrum. In fact, the presence of the benzene ring is an important peculiarity, which is not ubiquitous in cell membranes and it has also been proposed as a diagnostic tool for clinical applications [22].

^{13}C spectra and proton spin-lattice relaxation rate measurements also confirmed that BPA insertion took place and gave details on the dynamics of BPA molecules, as well as of the lipid matrix.

Moreover, as a positive result, we found that the tumor-targeting portion of BPA, the amino acidic group, was not masked by liposome insertion, since it was oriented from the lipid bilayer toward the aqueous phase.

All these findings clearly suggested that the system under study is to be considered a good boron carrier in BNCT.

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