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^{31}P-NMR and Differential Scanning Calorimetry Studies for Determining Vesicle's Drug Physical State and Fraction in Alendronate Liposomes

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Abstract

Background: A liposomal delivery system requires a complete understanding of the physicochemical characteristics of the drug–liposome system in order to predict their behavior and stability *in-vitro* and *in-vivo*.

Objectives: Develop a rapid and simple experimental method to determine the fractions of the drug, alendronate (ALN), encapsulated and as a free form distributed in the liposomal suspension, and the physical state of the encapsulated drug.

Methods: ^{31}P-NMR measurements utilizing Ga^{+3} as a shifting reagent in comparison to HPLC determinations, theoretical calculations and differential scanning calorimetry (DSC) studies of various liposomal ALN formulations.

Results: The ^{31}P-NMR demonstrated that titrating liposomal ALN with increasing amounts of Ga^{+3} induced a significant shift in the exterior fraction without changing the interior fraction. Quantitative determination of the encapsulated and non-encapsulated fractions of ALN has been achieved at Ga^{+3} concentrations of 3.2-25mM. The DSC study revealed that none of the formulation ingredients is in a solid phase.

Conclusions: ^{31}P-NMR was found to be sensitive enough to allow accurate differentiation of the distributed fractions of ALN, encapsulated and the non-encapsulated free form. Based on theoretical calculations and DSC analysis it can be concluded that ALN is dissolved in the aqueous core of the liposome.

Keywords: ^{31}P-NMR; Liposomes; Encapsulation; Differential scanning calorimetry (DSC); Alendronate; Gallium nitrate

Introduction

It has been demonstrated that partial and transient systemic inactivation of circulating monocytes by liposomal bisphosphonates (BPs) reduces neointimal hyperplasia and restenosis in animal models (Danenberg et al., 2002; Danenberg et al., 2003a; Danenberg et al., 2003b; Epstein et al., 2007). Restenosis, re-obstruction of the coronary arteries following percutaneuos coronary interventions such as stent angioplasty, can be prevented by diminishing the inflammatory trigger of smooth muscle cells (SMC) proliferation and migration (Libby et al., 1992; Danenberg et al., 2002; Danenberg et al., 2003a; Danenberg et al., 2003b; Colombo and Sangiorgi, 2004; Cohen-Sela et al., 2006). One single IV injection of liposomal formulation containing a BP such as clodronate or alendronate (ALN), at the time of injury, can treat several vessels with a long-term effect and minimal side effects (Danenberg et al., 2002; Danenberg et al., 2003a; Danenberg et al., 2003b; Epstein et al., 2007). The anti-restenotic effect (now in Phase II clinical trials (Web, 2009)) is due to the reduced number of macrophages at the injury site mediated by the transient (5 to 7 days) systemic inactivation of circulating monocytes. BPs do not cross the cell membrane (Lin et al., 1999) and the intracellular effects of BPs on osteoclasts in bone-related disorders is enabled only after ingestion of bone-adsorbed drug (Rodan, 1998). Similarly, only when the BP is in a particulated dosage form, such as liposomes, endocytosis by monocytes/macrophages occurs, and following intracellular release of the BP the cell is inactivated (Danenberg et al., 2002; Danenberg et al., 2003a; Danenberg et al., 2003b; Epstein et al., 2008).

A liposomal delivery system requires a complete understanding of the physicochemical characteristics of the drug–liposome system in order to predict their behavior and stability in vivo (Thomas and Verkleij, 1990; Seydel, 1991; Mayhew et al., 1997). Key parameters include zeta potential, stability, release kinetics, and vesicle size (Torchilin and Weissig, 1990; Moghimi et al., 2001; Panyam and Labhasetwar, 2003; Epstein et al., 2008). An important parameter in liposomal characterization is the drug partitioning inside vs. outside the vesicle. The existence of un-encapsulated (free) drug in the vehicle (typically buffer), whether a BP or other drug, could affect bioactivity as well as elicit untoward effects since the target of treatment with liposomal ALN is the phagocytic monocyte. There are few quantitative methods available for such specific physicochemical characterization of liposomal BPs. However, these methods require sacrificing part of the formulation, and these techniques suffer from certain disadvantages including laborious work with organic solvents. The limitations of these techniques are amplified when multiple and repeated tests are required in stability analyses over time. Thus, a facile experimental non-destructive method for differentiating between the interior and exterior BP fractions is advantageous. ^{31}P NMR with a shift reagent, ($Nd(NO_3)_3$), has been used to determine the outside/inside distribution of phosphatidylcholine molecular species over the vesicle membrane (de Kruijff et al., 1975). Recently a method for determining encapsulation efficiency by proton NMR spectroscopy and a chemical shifting agent has been reported (Zhang et al., 2004). Herein we envisioned the use of lanthanide (Ga^{+3}) to induced shift and relaxation in the ^{31}P-NMR of ALN phosphorus. Since Ga^{+3} forms stable coordinative complexes with various anions and oxygenated ligands such as hydroxylates, carboxylates, phosphates

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and phosphonates (Hoffer, 1980; Adding et al., 2001; Nelson et al., 2002), we hypothesized that it could be utilized for differentiating ALN fractions in the liposomal formulation. In addition, differential scanning calorimetry (DSC) studies were performed in order to verify crystallization of the drug and the bilayer lipids physical state.

We report here on a facile experimental approach exploiting ^{31}P-NMR to determine entrapment and partitioning of ALN in liposomal formulations. In addition, DSC studies and theoretical calculations elucidated that the drug is in dissolved in the vesicle's aqueous core.

Materials and Methods

Materials

Monosodium alendronate trihydrate [(4-Amino-1-hydroxybutylidene) bisphosphonic acid monosodium salt trihydrate] was obtained from Unipharm, Tel-Aviv, Israel. Gallium nitrate hydrate [$Ga(NO_3)_3.7H_2O$] of at least 99% purity and cholesterol of at least 95% purity were obtained from Sigma-Aldrich, Israel and used with no further purification. Gadoliniumdiethylenetriamine pentaacetic acid [Gd-DTPA] (Magnetol, 0.5 M) was obtained from Soreq Radiopharmaceuticals, Israel. The phospholipids 2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and distearoyl-phosphatidylglycerol (DSPG) were obtained from Lipoid, Ludwigshafen, Germany.

Methods

Preparation of liposomes: Small unilamellar vesicles were prepared by a modified thin lipid film hydration method (Epstein et al., 2008). DSPC, negatively charged DSPG, and cholesterol, at a molar ratio of 3:1:2 were dissolved in *t*-butanol and lyophilized overnight at -50°C. The lyophilized cake was hydrated with 8 ml aqueous solution containing 200 mM monosodium ALN trihydrate at 55- 60°C, and was left to stand for 1hr. The suspension was then extruded three times through double polycarbonate membranes of 0.8, 0.4 and 0.2 μm pore sizes (Nucleopore, CA, USA) by means of a thermobarrel extruder (Northern Lipids Inc, Vancouver, Canada). The obtained liposomes were purified on a Sephadex G-50 column, and eluted in PBS isotonic buffer (Biological Industries, Israel) to remove free ALN. The liposomes were sterilized by microfiltration (Minisart 0.22 μm, Sartorius) and stored at 4°C.

Liposomes characterization: Liposomes size and morphology were determined by photon correlation spectroscopy (ALV-GmBH, Langen Germany). The zeta potential was measured by means of a NanoZ (Malvern instruments, Malvern UK). The phospholipid content was determined colorimetrically with the Bartlett assay, and cholesterol and ALN content were measured by means of HPLC (Cohen-Sela et al., 2006).

NMR studies: All ^{31}P-NMR spectra were recorded on Varian Inova in the resonance frequency of 500 MHz by a spectrometer equipped with a 5mm computer switchable probe. Chemical shifts were processed using the standard Varian NMR software. The spectra were collected with broadband decoupling of the protons, acquisition time (AT) = 1.6 and relaxation delay (d1) = 2 seconds each. Usually, 32 pulses were acquired and line broadening (lb = 3) was applied prior to processing and integration. The samples were measured without spinning at 25°C. Chemical shifts were assigned relative to the external reference signal of H_3PO_4 set at δ = 0 ppm. All samples contained about 10% D_2O (v/v) for solvent locking.

To determine the effect of Ga^{+3} cation on free ALN and liposomal ALN, a solution of free ALN (700μl, 100 mM) or a liposomal formulation (400μl) were transferred to the NMR tube that was inserted into the NMR machine, and the system was locked and shimmed accordingly. Following initial measurements of these samples, further measurements were performed on titrated samples by using 50μl fractions of gallium nitrate (100mM stock solution), shaked for 1-2 minutes prior measurements. In a third arm of the experiments, in order to distinguish between the two forms of ALN (encapsulated *vs.* free), ALN solution (200μl) was added to liposomal ALN (400μl). The solution mixture was titrated with gallium nitrate (20μl) in the same manner. All measurements were performed with and without shifting reagent. Integrating and comparing the areas under the relevant NMR peaks enabled quantification.

DSC studies: Thermograms of selected specimens were recorded with a DSC (Malvern Toledo, USA), under purged nitrogen. After equilibration of the analyzer at 25°C, specimens were hermetically sealed in a 40 μl pan; an empty specimen pan was used as the reference. The melting temperatures were determined using a heating rate of 10 °C/min from 25 to 290°C. The resulting thermograms were analyzed with the Star^e software (Mettler Toledo, USA). The temperature range at half peak height was used to determine the range of the transition.

Results

Characterization of the liposomal formulations

The encapsulation yield was 30±5%. The concentration of encapsulated ALN and lipids in the liposomes was 5.20±0.05 mg/ml and 23.4±1.6 mg/ml, respectively, with a drug to lipids ratio of 1:4.5. The non-encapsulated free drug concentration in the suspension was less than 0.05 mg/ml. The liposomes obtained had a mean diameter of 175±21nm, with a polydispersity index below 0.1, and a negative zeta potential of –26.3±1.9 mV.

Characterization of ALN by ^{31}P-NMR

In order to affirm purity, the ^{31}P-NMR of ALN solution was measured and the chemical shift of the drug was assigned to δ =18.44 ppm (in reference to phosphoric acid).The spectrum was collected over a wide spectral width (from +60 ppm to -45 ppm), and no other phosphorus containing species were detected all over the range indicating that the compound was pure (Figure 1a).

Effect of a lanthanide (gallium nitrate) on free ALN

Titrating free ALN (100mM) with gallium nitrate at an equimolar concentration (100mM) resulted in additive weakening of the peak assigned for free ALN, as shown in the continuous reduction of the signal to noise (s/n) ratio, and the appearance of a widened new peak shifted to δ =16.59 ppm (Figure 1). In addition, a white precipitate was formed with the cumulative addition of the titrant (probably Ga-ALN salt/complex). As more portions of Ga^{+3} were added, a total disappearance of the original peak at δ =18.44 ppm (Figure 1g) was observed, and the precipitation was intensified. However, when excess Ga^{+3} was added (>500 μl) the precipitate started to dissolve and two additional peaks, one to the right (δ =15.25 ppm) and one to the left (δ =16.51 ppm), appeared (Figure 1h). This continued even after addition of a larger excess of Ga^{+3}. Not only the signal at δ =18.44 ppm was weakened with successive addition of Ga^{+3} but the peak was also widened. It should be noted that the formation of the precipitate weakened the intensity of both, the original peak of ALN and the shifted fraction of ALN-Ga^{+3}. In addition, the formation and the dissolution of the precipitate induced marked changes in the viscosity of the sample, which in turn affected the chemical shifts of both free ALN as well as the shifted peaks.

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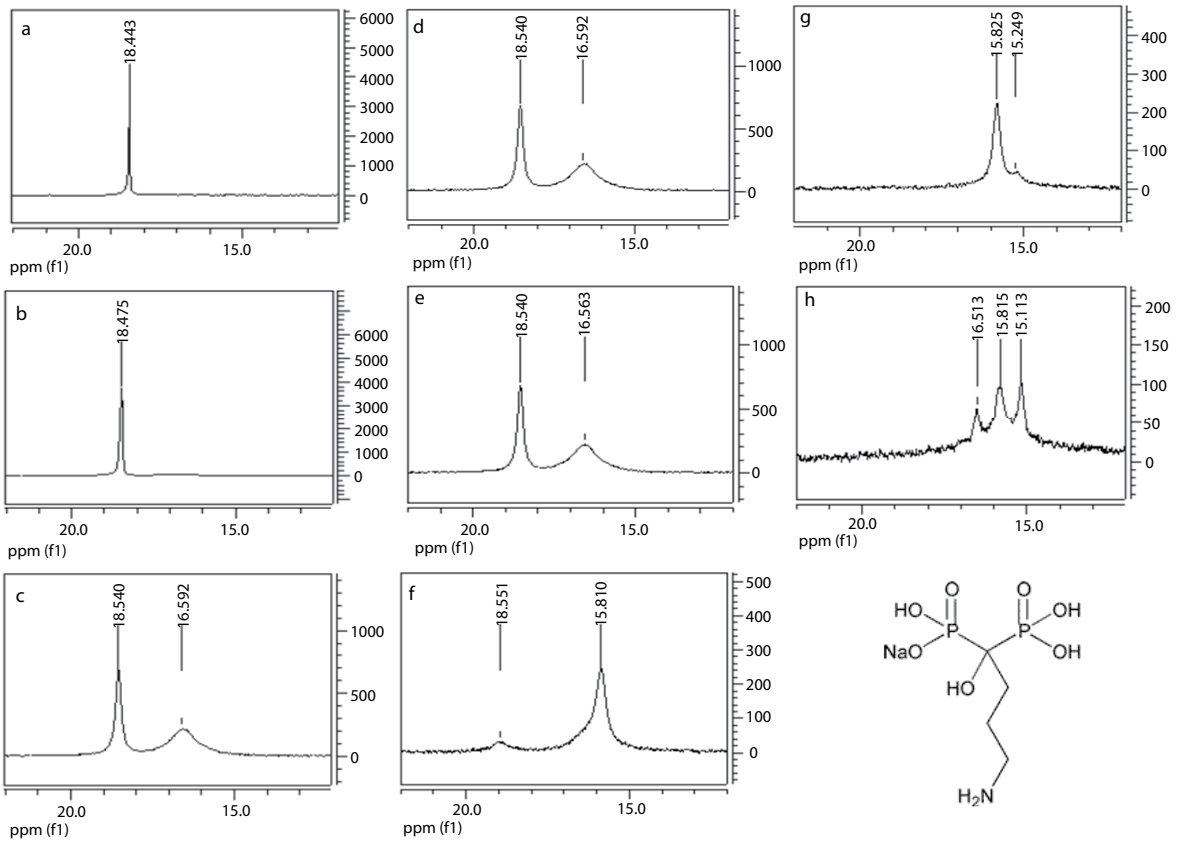

Figure 1: ^{31}P-NMR spectra of free ALN solution (700 µl, 100 mM) following titration with gallium nitrate (100 mM). **a,** free ALN; free ALN in the presence of gallium nitrate solution (100 mM), 50 µl **(b)**, 100 µl **(c),** 150 µl **(d)**, 250 µl **(d)**, 350 µl **(e)**, 450 µl **(f)**, and 650 µl **(g).** Inset shows alendronic acid.

Effects of the lanthanide, gallium nitrate, on liposomal ALN

The ^{31}P-NMR spectrum of liposomal ALN (without the addition of Ga^{+3}) exhibited two separated signals. In addition to the characteristic chemical shift of encapsulated ALN at δ =18.75 ppm, a 2nd chemical shift at δ =2.31 ppm is attributed to the buffer phosphates (Figure 2a). Thus, the chemical shift of encapsulated ALN (δ =18.75 ppm) was downfield by $\Delta\delta$ =0.31 ppm, in comparison to free ALN resonance (δ=18.44 ppm). This shift is most probably due to a certain physicochemical environment induced by ALN on the surrounding membrane of anionic phospholipids. Nevertheless, in order for this difference of ~0.31 ppm to differentiate between encapsulated and non-encapsulated ALN, particularly when existing simultaneously in the same sample, a proof of the peaks assignment should be provided. This was achieved by utilizing Ga^{+3} as a shifting reagent (see below). It is worth noting that ^{31}P NMR measurements performed for longer time (weeks) on the formulation revealed no new peaks.

We wished to assess the effect of Ga^{+3} on ALN residing in the liposomal core. To this end, liposomal ALN (400 µl) was titrated with increasing amounts of gallium nitrate (100mM), and ^{31}P-NMR was measured following each titration. It was noticed that by adding Ga^{+3} to the formulation, the phosphates of the buffer were affected first (the peak at δ =2.31 wasshifted up field toward δ =0.62 ppm). This effect was strengthened as more portions of Ga^{+3} were added. In contrast, encapsulated ALN was not affected significantly by titration with the Ga^{+3} cation (Figures 2b-d). Under the experimental conditions the buffer acts as a shield, and Ga^{+3} reacts with the buffer's phosphates prior to interacting with the phosphates of the outer liposomal phospholipids.

Encapsulated *vs.* free ALN

In order to distinguish between the two forms of ALN (encapsulated *vs.* free), ALN solution was added to a liposomal ALN preparation. ^{31}P-NMR has shown three chemical shifts, two peaks in the relevant region of ALN resonance, liposomal and free ALN (δ =18-19ppm), one was assigned for the encapsulated (δ =18.7 ppm) and the other for the nonencapsulated form (free, δ =18.4 ppm), and a third one shifted up field (δ =0.09 ppm), which was attributed to the phosphate buffer. Integrating the area under the peaks of the two ALN forms suggested that the concentration of free ALN (exterior) is 3.4 times higher than that of the encapsulated form (interior). This is in accord with the concentrations of ALN determined by HPLC (33 mM and 10.66 mM, free and encapsulated ALN, respectively, Figure 3).

In the next step we aimed at affirming whether it would be possible to distinguish between the two ALN forms by selectively titrating the exterior ALN using Ga^{+3} as a chemical shifting agent. The mixture of formulated (interior/encapsulated) and added (exterior/free) ALN was titrated with gallium nitrate solution (100 mM). At first, gradual addition of 20µl portions of Ga^{+3} to the mixture caused a continuous decrease in the intensity of the peak representing the exterior ALN (δ =18.46 ppm), while interior ALN (δ =18.77ppm) was not affected. This was affirmed by the relative integration of the area under the

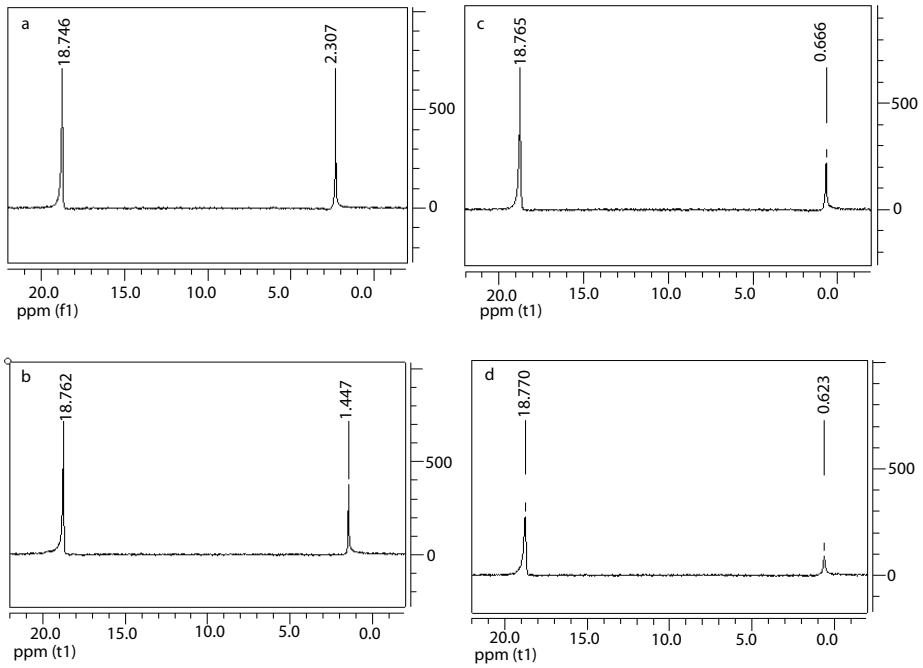

Figure 2: ^{31}P-NMR spectra of liposomal ALN following titration with $Ga(NO_3)_3$. **a**, ALN liposomes, and liposomal ALN in the presence of gallium nitrate solution (100 mM), 20 µl **(b)**, 40 µl **(c)** and 60 µl **(d)**. Note the peaks at δ = 18.75 and δ = 2.31 ppm attributed to liposomal ALN and phosphate groups of the buffer, respectively.

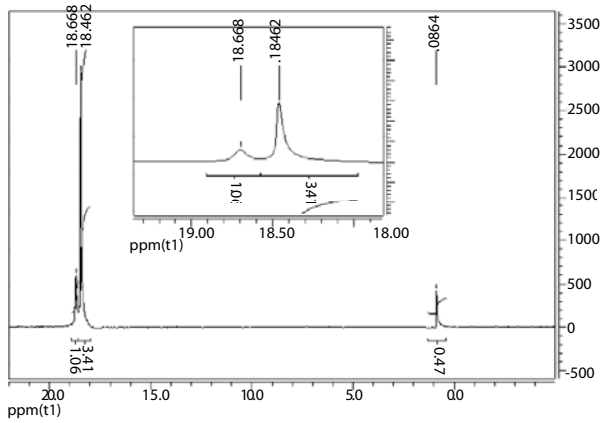

Figure 3: ^{31}P-NMR spectra of ALN liposomal suspension (400 µl) with added exterior ALN solution (200 µl). Note the peaks at δ = 18.67, δ = 18.46 and δ = 0.86 ppm attributed to interior ALN, exterior ALN, and buffer-phosphate groups, respectively.

peaks corresponding to the exterior *vs.* interior drug. As more Ga^{+3} was added (~100 µl) the broad shifted-peak at δ= 16.50 ppm (Figure 4c), a characteristic of gallium-ALN association, was intensified and clearly detected. The exterior ALN fraction (δ =18.46 ppm) was completely shifted after the addition of 200 µl gallium nitrate in total (Figure 4). Additional Ga^{+3} portions resulted in the total breakdown of the liposomal formulation and the instant release of the ALN. Most of the ALN was gallium-associated (δ= 16.38 ppm), and one-third was seemingly protected with phospholipid aggregates that still resonate at δ~18.77 ppm. Additional portions of excess Ga^{+3} did not change the ratio (Figure 5), which is probably because Ga^{+3} is unable to access the portion of insoluble aggregated alendronate-lipids.

DSC studies

The results obtained by the DSC analyses are summarized in Table 1. As expected the lipids exhibited endothermic peaks of transition and melting, and disodium alendronate trihydrate exhibited 2 endothermal peaks (loss of crystalline water and melting). In contrast, no endothermal peaks were exhibited by the ingredients of the liposomal formulation when in water, except for the evaporated water (high enthalpy of ca. 1500 j/g). Small differences in the onset temperatures of water evaporation were detected for empty liposome, ALN solution, and empty liposomes spiked with alendronate solution, and liposomal alendronate, 88.58, 85.44, 76.98 and 90.54ºC, respectively, but with a similar relatively high heat of water evaporation (ca. 1500 j/g).

Discussion

In the present study, we report a facile experimental approach using ^{31}P-NMR to differentiate between interior and exterior BP in a liposomal formulation. The developed method enables to determine the concentration of encapsulated BP, and of possibly other phosphorus containing drugs, in the aqueous liposomal core. Furthermore, this method can differentiate between encapsulated and non-encapsulated BP. The developed method is facile with no need for other cumbersome techniques.

It should be noted that ^{31}P is privileged with 100% naturally abundant isotope that resonates at higher frequency (νP = 121 MHz) and 320×more sensitive in comparison with ^{13}C making ^{31}P-NMR easy to perform. ^{31}P-NMR was used extensively in lipid bilayer characterization and to probe vesicular dynamics, conformational restrictions and other properties (Hinton and Johnson, 1994). The use of ^{31}P-NMR in combination with chemical shifting reagents has been extensively described in the context of MRI (Zhang et al., 1996; Noseworthy and Bray, 1998; Cea et al., 2002). After trying other

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lanthanides, gallium(III) proved to be the reagent of choice due to its moderate shift and relaxation effects on phosphorus nuclei in comparison to gadolinium(III). The relaxative effect of Gd^{+3} was drastic as evidenced by instant and broad widening of the ^{31}P-NMR chemical shift of ALN (data not shown). Ga^{+3} is capable of forming stable coordinative complexes with various anions and oxygenated ligands such as hydroxylates, carboxylates, phosphates and phosphonates (Hoffer, 1980; Adding et al., 2001; Nelson et al., 2002). This enabled the development of a facile experimental approach for determining ALN encapsulated fraction in comparison to free ALN in the vehicle, utilizing ^{31}P-NMR spectroscopy and Ga^{+3} as a chemical shift reagent. The process of coordinative complexation of the metal cation, Ga^{+3}, with the anionic BP ligand, ALN, was found feasible. This was shown by the binding of gallium(III) to the BP that induced a shift of $\Delta\delta$ = 2 ppm up field, δ= 18.44 and 16.59 ppm, free and Ga^{+3}-bound ALN, respectively, and widening of the peak corresponding to the bound ligand (Figure 1). Such differences in the ^{31}P-NMR chemical shift could arise from close and direct coordination of the negatively charged oxygens comprising the BP with the metal cationic center. These findings made ^{31}P-NMR suitable for the analysis of free ALN using Ga^{+3}. When free ALN solution was titrated with Ga^{+3} the intensity of the peak characteristic for ALN decreased constantly (δ =18.44 ppm), whereas the new shifted and broadened peak appeared and its intensity increased as more portions of the titrant were added (Figure 1).

Encapsulated ALN has a characteristic peak that was slightly shifted downfield (δ~18.75 ppm, Figure 2a). This shift is caused by the physicochemical environment induced by the interior lipid bilayer membrane, which in turn resulted in de-shielding of the phosphorus nuclei shifting its resonance downfield by $\Delta\delta$ = 0.31 ppm. Adding Ga^{+3} to the liposomal suspension was not accompanied with the up field shifting that is observed when Ga^{+3} is added to free ALN solution. However, there was a minor detectable effect on ALN inside the liposome ($\Delta\delta$ = 0.016 ppm). Such a minor shift at the corresponding peak of liposomal ALN is most probably not due to direct coordinative binding with the anionic moieties of the BP ligands since the membrane is impermeable to charged ions, but rather to indirect effect of the affected phosphates of the membrane. The liposomal formulation proved to be stable under these conditions unless large quantities of Ga^{+3} were added (>300 µl). Nevertheless, both the liposomal viscosity and the tendency to precipitate increased significantly when gallium nitrate was added. This could be due to the neutralizing affect of the cationic metal, Ga^{+3}, when complexing with the anionic phosphate groups of the liposomal shell.

Adding Ga^{+3} to a mixture of free ALN and encapsulated ALN markedly affected the exterior fraction (at δ =18.46 ppm), but not the interior ALN fraction (at δ = 18.67 ppm), indicating that the two peaks belong to ALN partitioning between the two sides of the liposome membrane (Figure 3). Under the experimental conditions the buffer acts as a shield, and Ga^{+3} reacts with the buffer's phosphates prior to interacting with the phosphates of the outer liposomal phospholipids. The shifting and the decrease in the intensity of the corresponding chemical shift support this notion. It is suggested that

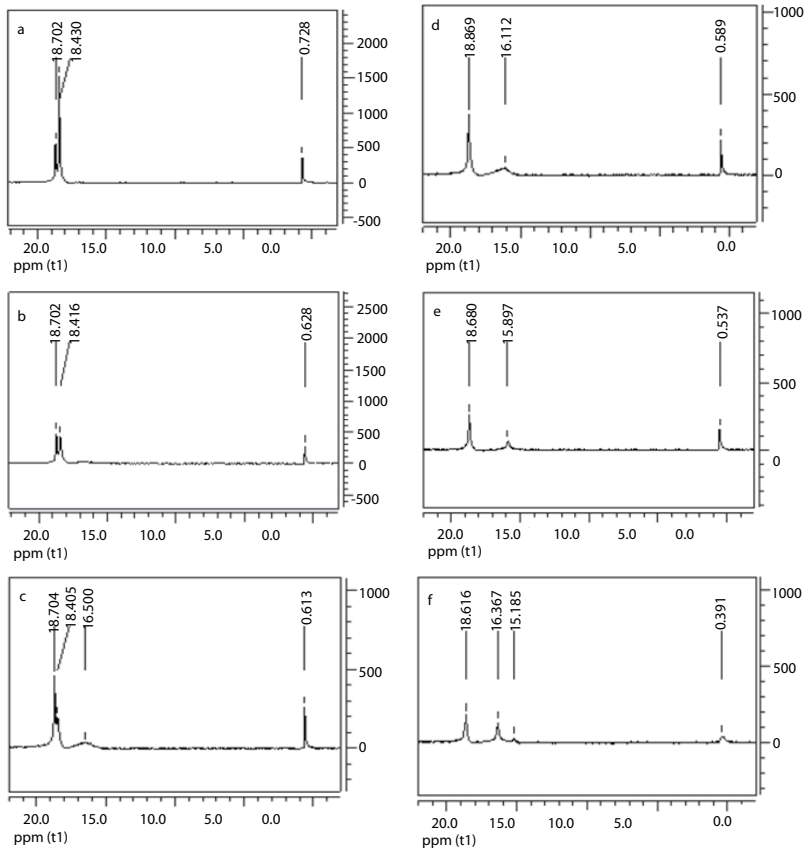

Figure 4: ^{31}P-NMR spectra of liposomal ALN suspension (400 µl) with added exterior ALN solution (200µl) following titration with $Ga(NO_3)_3$ (100 mM). **a,** 20µl; **b**, 60µl; **c,** 100µl; **d**, 140µl; **e**, 200µl; **f**, 400 µl.

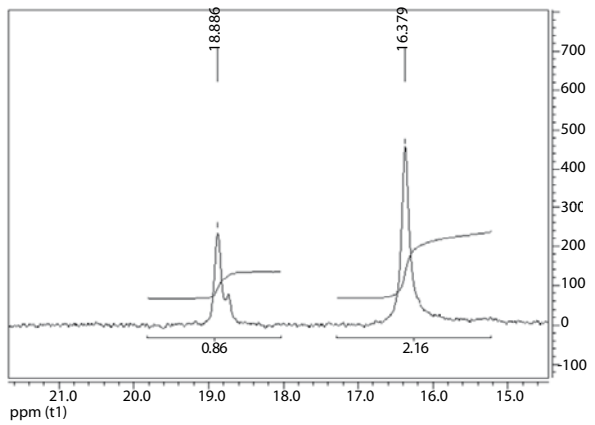

Figure 5: ^{31}P-NMR spectra of ALN liposomes following breakdown of the liposome membrane with 820 µl Gallium nitrate. Note that more than two thirds of ALN is released and is associated with gallium (δ =16.379), and that less than one third is lipids-associated (δ = 18.89 ppm).

Sample	Endothermal Peak (Onset temp.,°c)	Energy (- j/g)
DSPC powder	52.44	0.13
DSPG powder	57.45	0.72
Cholesterol powder	143.09	56.26
Empty liposomes suspension	88.58	1523.19
Alendronate powder	118.49, 259.37	415.08, 24.41
Alendronate solution	85.44	1542.88
Empty liposomes spiked with alendronate solution	76.98	1504.90
Liposomal alendronate suspension	90.54	1556.31

Table 1: Differential scanning calorimetry analyses of liposomal alendronate and of the formulation ingredients.

the mere entrapment of ALN in liposomal formulation is sufficient to provoke a change in the physicochemical environment that induces a slight change in the magnetization of ALN dwelling inside the aqueous core of the liposome, making it resonates at a new downfield shifted signal. The two distinct ^{31}P-NMR chemical shifts detected indicate the existence of two forms of ALN, inside and outside the vesicles. ^{31}P-NMR measurements performed for longer time (weeks) on the formulation revealed no new peaks. This indicates that no significant amount of ALN leaked out of the liposome core and that ALN is non-leachable from the aqueous core across the lipids bilayer. Thus, the liposome bilayer comprises a physical barrier that prohibits transport of these two forms, and prevents the metal from penetrating into the vesicle complexing with the inside drug molecules, on the other hand. This finding is corroborated by the long-term stability of alendronate liposomes (Epstein et al., 2008).

When certain amounts of ALN were titrated with a liposomal ALN preparation, ^{31}PNMR revealed two peaks, differentiated by $\Delta\delta$ = 0.24 ppm (Figure 4). Using 500 MHz NMR, these two forms (exterior *vs.* interior) were distinguishable. In addition, when more ALN was added, only the peak characteristic of the exterior ALN (δ = 18.46 ppm) was intensified (data not shown). Integrating the area under the peaks of the two forms of ALN revealed that free ALN concentration is 3.4-fold of the encapsulated form. This difference correlates well with ALN concentrations determined by HPLC, 33 mM and 10.66 mM, free and encapsulated ALN, respectively. These results confirm that ALN concentration ratio, encapsulated vs. free, can be determined by ^{31}P-NMR. After it was verified that the 2 peaks are indeed of encapsulated and free ALN, it is suggested that further studies for analyzing liposomal ALN formulations could utilize this technique, with no need for adding Ga^{+3}.

The DSC studies (Table 1) confirmed that ALN is not crystallized in the liposomal formulation, and encapsulated ALN is dissolved in the inner aqueous liposome core. It should be noted that in certain liposomal formulations, such as doxorubicin liposomes, some of the drug is in a solid state in the aqueous core (Li et al., 1998). Differences in solubility and aqueous core content can determine the physical state of drug in the vesicle's core. Since the theoretically calculated total inner volume of the vesicles is 213µl/ml (Epstein et al., 2006), the calculated concentration of ALN in the vesicles is 24.4 mg/ml. This concentration, which is much lower than the drug solubility and the concentration of the initial solution (65mg/ml), suggests that ALN is dissolved in the aqueous core.

Conclusions

^{31}P-NMR was found to be a useful tool for characterizing liposomal ALN. The formulation is pure, with one form of drug encapsulated inside, as shown by a single narrow signal in the BP region. The formulation is stable and no leakage of the hydrophilic ALN was detected under the experimental conditions over weeks. Encapsulated ALN in liposomes formulation interacts with membrane phosphates as evidenced by a slight change in the magnetization of ALN resonating at a downfield shifted signal. Differentiation between interior and exterior in the liposomal formulation was achieved by utilizing Ga^{+3} as a shifting reagent. Rigidification and phase separation was noticed when titrating the formulation with Ga^{+3}. It is suggested that ^{31}P-NMR could be used to determine the concentration of the encapsulated BP without any special treatments, and to differentiate between the interior and exterior fractions. However, the robustness of the should be validated on liposomal formulations of other BP. The theoretical calculations and DSC analyses support the conclusion that ALN is dissolved in the aqueous core of the liposome.

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