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Iminothiol/thiourea tautomeric equilibrium in thiourea lipids impacts DNA compaction by inducing a cationic nucleation for complex assembly

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ABSTRACT

Our research on lipidic vectors for transfection led us to develop thiourea lipids able to interact with DNA. Hence, we developed a series of lipopolythioureas based on the strong hydrogen bond donor ability of thiourea. More recently we have reported a branched hydroxylated bis-thiourea derivative with interesting transfecting properties. The last step of the syntheses involved a strong acidic condition, leading to an unstable product upon storage. Therefore we designed a new synthesis in mild acidic conditions. Though they exhibit the same mass, the lipids obtained in the two different conditions differ by their interaction with DNA. We therefore explored the physicochemical properties of these two lipids by different means that we describe in this article. In order to insure easier and reliable ^{13}C-NMR studies of the thiourea group we have designed the synthesis of the corresponding ^{13}C-labeled thiourea lipids. We have thus shown that when the lipid was submitted to mildly acidic medium; only the thiourea group was observed; while a thiourea/charged and/or uncharged iminothiol tautomeric equilibrium formed when the last step of the synthesis was submitted to low pH. NMR experiments showed that this tautomeric equilibrium could not form in polar solvents. However, UV experiments on the liposomal form of the lipopolythiourea showed the presence of the tautomers. Lipid/DNA interaction consequently differed according to the acidic treatment applied. Eventually, these results revealed that on this particular thiourea lipid, electrostatic interactions due to cationic thioureas are likely to be responsible for DNA compaction and that this tautomeric form of the thiourea could be stabilised by hydrogen bonds in a supramolecular assembly. Nevertheless, this does not reflect a general thiourea lipid/DNA interaction as other thiourea lipids that are able to compact DNA do not undergo an acidic treatment during the final stage of their synthesis.

1. Introduction

Because of the polyanionic nature of DNA and the overall negative charge of cell membranes, cationic lipids [1,2] remain the mainly studied non viral vectors for DNA delivery. However, the positive charges of the cationic lipids induce a poor efficiency *in vivo* [3,4] as lipoplexes are captured by opsonins or other physiological components, hence resulting in a low circulation time [5]. Numerous studies have been undertaken to improve serum resistance by masking or suppressing the cationic charges [6,7]. Alternative delivery systems have been introduced, using non electrostatic interaction with DNA. For instance, Aoyama et al. succeeded in trapping DNA in virus-like saccharidic clusters [8], and Barthelemy et al. developed plasmids associated to nucleoside-based amphiphiles [9]. However, with the exception of artificial glycovirus [10], these systems require a cationic charge to show some transfection activity *in vitro* [11]. Strictly neutral systems, such as liposomes [12] or spherulites [13] have been investigated, but as far as we know, they have not shown any transfection properties.

Our laboratory has developed a new type of neutral synthetic vectors to avoid the burden of the cationic charge. This system had to be able to compact DNA thanks to numerous hydrogen bonds between a properly chosen chemical function and the DNA phosphate groups. Our choice fell on the thiourea function which is known for its strong hydrogen donor ability [14]. Moreover, bis-thiourea moieties selectively bind to dihydrogenphosphate via multitopic hydrogen bonding [15], giving stronger complexes with $H_2PO_4^-$ than any synthetic neutral receptor known so far.

In previous articles, we have described the synthesis of several lipopolythioureas. We demonstrated that different families of lipopolythioureas could compact DNA [16] and transfect cells [17]. We improved the structure of the lipids until the molecules were hydrophilic enough to be easily formulated. We developed syntheses which involved only a few steps with good yields. Two families showed the best results, one that derives from a serinol compound [18], the other from a lysine moiety [19]. Among these lipids, some showed a blood circulation time twice that of the cationic lipid

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RPR209120/DOPE [19] and *in vitro* transfection in the same magnitude. We also demonstrated that it was possible to induce gene expression *in vivo*. Hence, these lipids represent a very interesting alternative for gene transfection.

However, from these two families, lipids of the serinol family were found to be unstable upon storage of the lyophilisate. The last step of the synthesis of these compounds involves a deprotection of an acetal group using hydrochloric acid, which is not used in the lysine derivatives. Remaining traces of this acid could explain this instability. Therefore we modified the last step of this synthesis by using a much milder acid: the acetic acid. The first biological results were rather surprising, as only the serinol deprotected by HCl showed an efficient transfection on B16 cells. It seemed to indicate that we obtained a different serinol product even though it bore the same mass. We postulated that the thiourea lipids could be in a tautomeric equilibrium (thiourea and iminothiol). The aim of this article is to characterize these different molecules.

The tautomeric equilibrium of thiourea has been studied by mass spectrometry and theoretical calculations [20]. Results indicated that tautomerism could occur but they were not completely conclusive. We also conducted a literature research on the topic of thiourea protonation but the literature is rather contradictory. The available data presents a deprotonation constant pK_A for protonated thiourea in water comprised between −4.55 [21] and +2.91 [22]. The protonation constant pK_P of thiourea in water is given between −1.58 and +1.18 [23]. Literature is also unclear as whether N-protonation or S-protonation is more likely. Nevertheless the most recent articles favor S-protonation [24]. According to Schiessl et al. [25], the protonation of thiourea is impossible in the pH range 0 to 7 in aqueous solution but protonation in other solvents has not been comprehensively reported. Facing the lack of data, we therefore decided to use different analytical techniques in synergy so as to present a characterization of the thiourea function and improve our understanding of the interaction between lipopolythiourea and DNA.

2. Experimental section

2.1. Materials and methods

All solvents were purchased from Carlo Erba-SDS. All chemicals were purchased from Sigma-Aldrich-Fluka or Lancaster. Solvents and products were used without further purification. Reactions were monitored by thin-layer chromatography using Merck precoated 60F254 silica gel plates. Inverse phase SPE chromatography was performed on a Supelco Superclean C8 cartridge using an H2O/CH3CN/CH3OH mixture.

^{1}H and ^{13}C NMR spectra were recorded on a BRUKER Advance DRX400 spectrometer operating at 400 MHz for the proton and 100 MHz for the carbon. Chemical shifts are given in ppm with respect to reference frequency of TMS. The deuterated solvents used are $CDCl_3$, MeOD, and D_2O. They are described in the caption of the figures. Sample concentration was 5 g/L for ^{1}H-NMR and 25 g/L for ^{13}C-NMR. NMR spectra were processed using XwinNMR (Bruker) or MestRec 2.3a [26]. MS were carried out on a Shimadzu 2010A LC-MS on ESI mode. S-methylisopseudothiourea hemisulfate salt was neutralized by adding one equivalent of NaOH into a solution of S-methylisopseudothiourea hemisulfate salt in D_2O.

UV spectra were recorded on a Varian Cary 100 Scan UV-visible spectrophotometer and processed using Varian Cary Win UV Scan application 3.00 [27].

Zeta potentials were measured (a minimum of 3 measurements per sample) with a Malvern Zetasizer Nano-ZS (Malvern Instruments, Southborough, USA). The samples were diluted in NaCl 20 mM to obtain a DNA concentration of 1 µg/mL.

The names of the molecules were computed using the Autonom Software by MDL, according to IUPAC rules.

2.2. Synthesis of lipopolythioureas

2.2.1. N-[1,3-Bis(2,3-dihydroxypropylthiocarbamoylamino)propan-2-yl]-2-(didecylcarbamoylmethoxy)acetamide DDSTU/HCl (1a and 1a C13)

1 N hydrochloric acid (0.5 mL) was added to a solution of 2-[1,3-Bis[(2,2-dimethyl-1,3-dioxolan-4-yl)methylthiocarbamoylamino] propan-2-ylcarbamoylmethoxy]-N,N-didecylacetamide 2 or 5 (0.19 g, 0.23 mmol) in THF (2.1 mL). The reaction mixture was stirred for 3 h at room temperature, and then most of the THF was evaporated under reduced pressure in a cold water bath. The resulting liquid was lyophilized to yield 1a or 1a C13 (0.17 g, 96%) as a colorless oil. ^{1}H NMR ($CDCl_3$) δ (ppm): 0.88 (t, 6H, $J=6.0$ Hz, CH_3), 1.23 (m, 28H, CH_2), 1.54 (m, 4H, CH_2), 2.14 (s, 4H, OH), 3.09 (m, 2H, CH_2NCO), 3.30 (m, 2H, CH_2NCO), 3.63 (m, 12H, CH_2NCS, CH_2OH), 3.92 (m, 2H, CH), 4.13 (m, 2H, CH_2O), 4.31 (m, 7H, CH, CH_2O). ^{13}C NMR ($CDCl_3$) δ (ppm): 14.18 (s, CH_3), 22.76 (s, CH_2), 27.02 (s, CH_2), 27.70 (s, CH_2), 29.45 (m, CH_2), 31.98 (s, CH_2), 40.36 (m, CH2NCS), 46.83 and 47.32 (2s, CH_2NCO), 51.00 (s, CH), 63.98 (s, CH_2OH), 69.52 (s, CH_2O), 71.16 (m, CH_2O, CHOH). MS (ESI, *m/z*) 751 (M+). This product was checked by LC-MS before use to ensure that it was not degraded.

2.2.2. N-[1,3-Bis(2,3-dihydroxypropylthiocarbamoylamino)propan-2-yl]-2-(didecylcarbamoylmethoxy)acetamide DDSTU/CH_3CO_2H (1b and 1b C13)

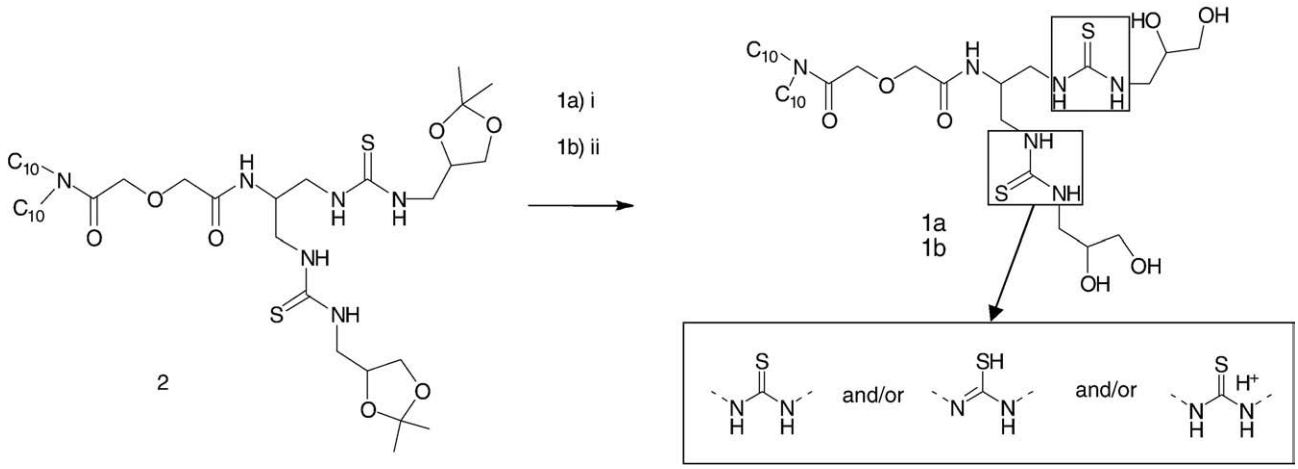
Eighty percent of acetic acid (15.2 mL) was added to a solution of 2-[1,3-Bis[(2,2-dimethyl-1,3-dioxolan-4-yl)methylthiocarbamoylamino]propan-2-ylcarbamoylmethoxy]-N,N-didecylacetamide 2 or 5 (0.30 g, 0.36 mmol) in water (3 mL). The reaction mixture was stirred for 5 min at 100 °C, and then most of the water was evaporated under reduced pressure in a cold water bath. The resulting liquid was diluted with cyclohexane and evaporated twice, then the reaction mixture was diluted with ethanol and evaporated to yield 1b or 1b C13 (0.20 g, 72%) as a colorless oil. ^{1}H NMR ($CDCl_3$) δ (ppm): 0.88 (t, 6H, $J=6.0$ Hz, CH_3), 1.23 (m, 28H, CH_2), 1.54 (m, 4H, CH_2), 3.13 (m, 2H, CH_2NCO), 3.30 (m, 2H, CH_2NCO), 3.60 (m, 8H, CH_2NCS, NCH_2CH), 3.75 (m, 4H, CH_2OH), 3.92 (m, 2H, CH), 4.13 (m, 2H, CH_2O), 4.22 (m, 1H, CH), 4.41 (m, 2H, CH_2O).^{13}C NMR ($CDCl_3$) δ (ppm): 14.18 (s, CH_3), 22.76 (s, CH_2), 27.02 (s, CH_2), 27.70 (s, CH_2), 29.45 (m, CH_2), 31.98 (s, CH_2), 46.83 and 47.32 (2 s, CH_2NCO), 60.20 (s, EtOH) 63.98 (s, CH_2OH), 69.52 (s, CH_2O), 71.16 (m, CH_2O, CHOH), 171.92 (s, CO), 183.38 (s, CS). MS (ESI, *m/z*) 751 (M+).

2.2.3. 4-Isothiocyanatomethyl-2,2-dimethyl-[1,3]dioxolane (3)

In a 10 mL round-bottom flask 2,2-dimethyl-dioxolan-4-methanamine (0.1 g, 0.76 mmol), DCC (0.16 g, 0.76 mmol), and carbon disulfide (46 µl, 0.76 mmol) were dissolved in THF (3 ml). The mixture was stirred at RT during 1 h. It was diluted with 5 mL of petroleum ether (45–65°) and washed with 0.1 N HCl, water and brine. The organic phase was dried over Na_2SO_4, filtered and concentrated to the crude product (0.1 g; 75% — caution: boiling point of the product is 30 °C) ^{1}H NMR (CDCl3) δ (ppm): 1.31 and 1.42 (s, 6H, CH_3), 3.54 and 3.64 (m, 2H, CH_2), 3.80 and 4.07 (m, 2H, CH_2), 4.26 (m, 1H, CH). ^{13}C NMR (CDCl3) δ (ppm): 25.24 (s, CH_3), 26.85 (s, CH_3), 47.47 (s, CH_2), 55.64 (s, CH_2), 66.66 (s, C), 73.39 (s, CH), 133.12 (^{13}C). MS (ESI, *m/z*) 174 (M+H)+.

2.2.4. N,N-Bis-decyl-2-({2-[3-(2,2-dimethyl-[1,3]dioxolan-4-ylmethyl)-thioureido]-1-[3-(2,2-dimethyl-[1,3]dioxolan-4-ylmethyl)-thioureidomethyl]-ethylcarbamoyl}-methoxy)-acetamide (5)

In a 5 mL round-bottom flask 2-[(2-Amino-1-aminomethyl-ethylcarbamoyl)-methoxy]-N,N-bis-decyl-acetamide 4 (16.4 mg, 0,034 mmol), 3 (11.8 mg, 0.068 mmol), and triethylamine (12 µl, 0.085 mmol) were dissolved in THF (339 µl). The reaction mixture was stirred overnight at room temperature. After evaporation, the reaction mixture was purified on silica column using cyclohexane/ethanol (85:15) to give 5 (24 mg; 85%). ^{1}H NMR (CDCl3) δ (ppm):



Scheme 1. Last step of the synthesis of DDSTU/HCl (1a) and DDSTU/CH_3CO_2H (1b). i: HCl 1N, THF, RT; ii: CH_3CO_2H 80%, H_2O, 100 °C.

0.85 (m, 6H, CH_3), 1.23 (m, 32H, CH_2), 1.30–1.49 (s, 6H, CH_3), 3.06–3.27 (m, 4H, CH_2N), 3.50–3.85 (m, 16H, CH_2N), 4.04 (m, 4H, CH_2O), 4.25 (m, 4H, CH_2O).^{13}C NMR ($CDCl_3$) δ (ppm): 14.12 (s, CH_3), 22.70 (s, CH_2); 25.39 (s, CH_2); 26.95 (s, CH_2); 27.72 (m, CH_2); 29.57 (m, CH_2), 31.91 (s, CH_2), 40.02 (s, CH2NCS), 46.64 and 47.11 (2 s, CH_2NCO), 51.30 (s, CH), 66.92–74.43 (s, CH_2O), 109.55 (s, CO2(CH3)3), 132.86 (s, C); 182.78 (^{13}C). MS (ESI, *m/z*) 831 (M + H)+, 853 (M + Na)+.

2.2.5. Plasmid preparation

Plasmid pVax2 was used for all experiments. pVax2 is a derivative of the commercial plasmid pVax1 (InVitrogen), which was digested with the restriction enzymes HincII and BamHI to excise the promoter. The plasmid was then blunted with the Klenow fragment, dephosphorylated with alkaline phosphatase. pCMVbeta (Clontech) was digested with EcoR1 and BamHI to excise the CMV promoter. The CMV promoter was blunted with Klenow enzyme and ligated into the blunted pVax1 to give pVax2. The plasmid pXL3031 was digested with EcoRI and BamHI and then treated with the Klenow fragment to produce a blunted fragment containing the luciferase cDNA. This fragment was ligated into pVax2 after EcoRV digestion and phosphatase alkaline dephosphorylation to give the pVax21- Luc. [28].

2.2.6. Liposome preparation by the ethanolic injection method

Lipopolythiourea was dissolved in ethanol and added dropwise to 10 volumes of water under vigorous agitation. The mixture was stirred overnight and then evaporated under reduced pressure at room temperature to obtain a solution of liposomes.

2.2.7. Preparation of LPT/DNA complexes

Plasmid DNA (100 µL, 0.02 g/L in H_2O) was added dropwise with constant vortexing to various amounts of liposomes (in 100 µL of H_2O) at room temperature. TU/P indicates the ratio in nmol of thiourea function (2 per lipid) versus DNA phosphates.

2.2.8. Gel retardation experiments

Samples of DNA/lipid complexes (20 µL) were mixed with 5 µL of bromophenol blue. The mixture was loaded on a 0.8% agarose gel in TBE buffer (1 M Tris, 0.9 M boric acid, 0.01 EDTA) at 80 V/cm. DNA was revealed with ethidium bromide and visualized under UV light.

3. Results

3.1. Lipids synthesis

The lipopolythiourea N,N-Bis-decyl-2-({2-[3-(2,3-dihydroxy-propyl)-thioureido]-1-[3-(2,3-dihydroxy-propyl)-thioureidomethyl]-ethylcarbamoyl}-methoxy)-acetamide 1a which we will call DDSTU/HCl (DD for didecyl, S for the serine based linker, TU for its thiourea moiety, HCl for the last step of the synthesis) synthesized as previously described [18] was found to be unstable upon storage of the lyophilisate

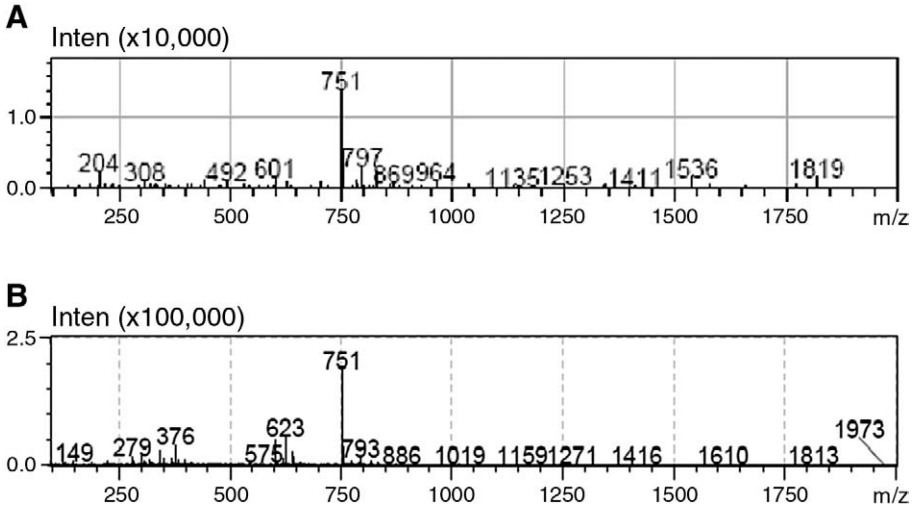


Fig. 1. Mass spectroscopy by negative ionization of DDSTU/HCl 1a (MW = 751 g mol^{-1}), and DDSTU/CH_3CO_2H 1b (MW = 751 g mol^{-1}).

at 4 °C. This seemed to be a consequence of the acidity of the last step of the synthesis. We then replaced 1 N hydrochloric acid by 80% acetic acid in water (as shown in Scheme 1). The product obtained 1b which we will call DDSTU/CH_3CO_2H (CH_3CO_2H for the last step of the synthesis) was stable over months as checked by LC-MS. The two products should have been identical, but DDSTU/CH_3CO_2H did not lead to transfection. It was therefore interesting to further investigate how the pH conditions of the last step of synthesis did affect the structure of the thiourea function and influenced its interaction with DNA.

3.2. Mass spectrometry and NMR analyses

Firstly, we have characterized the compounds 1a and 1b by classical mass spectrometry (Fig. 1) and ^1H and ^{13}C NMR analyses. The LC-MS analyses for both products were identical. They showed only one peak with a related mass of 751 on the negative ionization spectrum which corresponds to the negative ionisation of the desired product.

The next investigation was one-dimensional ^1H-NMR and ^{13}C-NMR as shown in Fig. 2. Owing to the amphiphilic nature of the

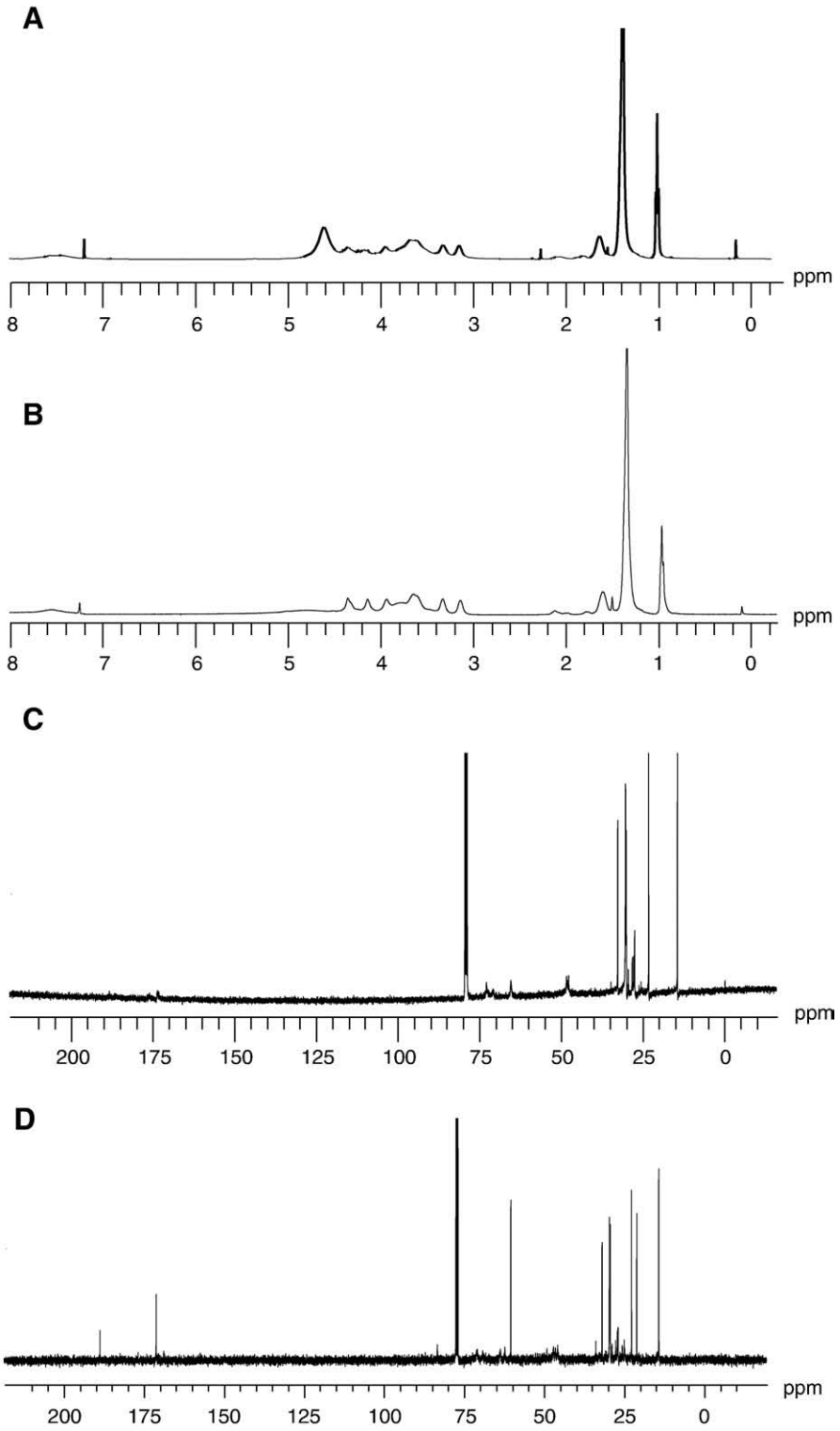


Fig. 2. ^1H-NMR spectra of DDSTU/HCl 1a (A in $CDCl_3$), and DDSTU/CH_3CO_2H 1b (B in $CDCl_3$), ^{13}C-NMR spectra of DDSTU/HCl 1a (C in $CDCl_3$), and DDSTU/CH_3CO_2H 1b (D in $CDCl_3$).

compounds the ^{1}H-NMR spectra were poorly resolved, the major differences being observable in the zone 3 to 4.5 ppm corresponding to the head part of the lipids. This chemical shift region contains the peaks of the hydrophilic head of the molecule. A precise attribution of all the hydrogen signals was possible thanks to 2D ^{1}H-NMR experiments such as COSY and heteronuclear ^{1}H-^{13}C HSQC (in Supplementary data 1–4) The ^{13}C-NMR signal of the thiourea function was clearly observed with compound 1b. The signals of compound 1a have all been attributed in a previous publication [18] however the signal corresponding to thiourea barely rose above the noise baseline in the case of compound 1a in routine NMR conditions. These preliminary observations confirmed that the differences between the two lipids are due to the thiourea group.

3.3. Effect of the acidic conditions on commercially available molecules

The acidic conditions used to remove the acetal group and obtain DDSTU/HCl were applied on two commercially available thioureas:

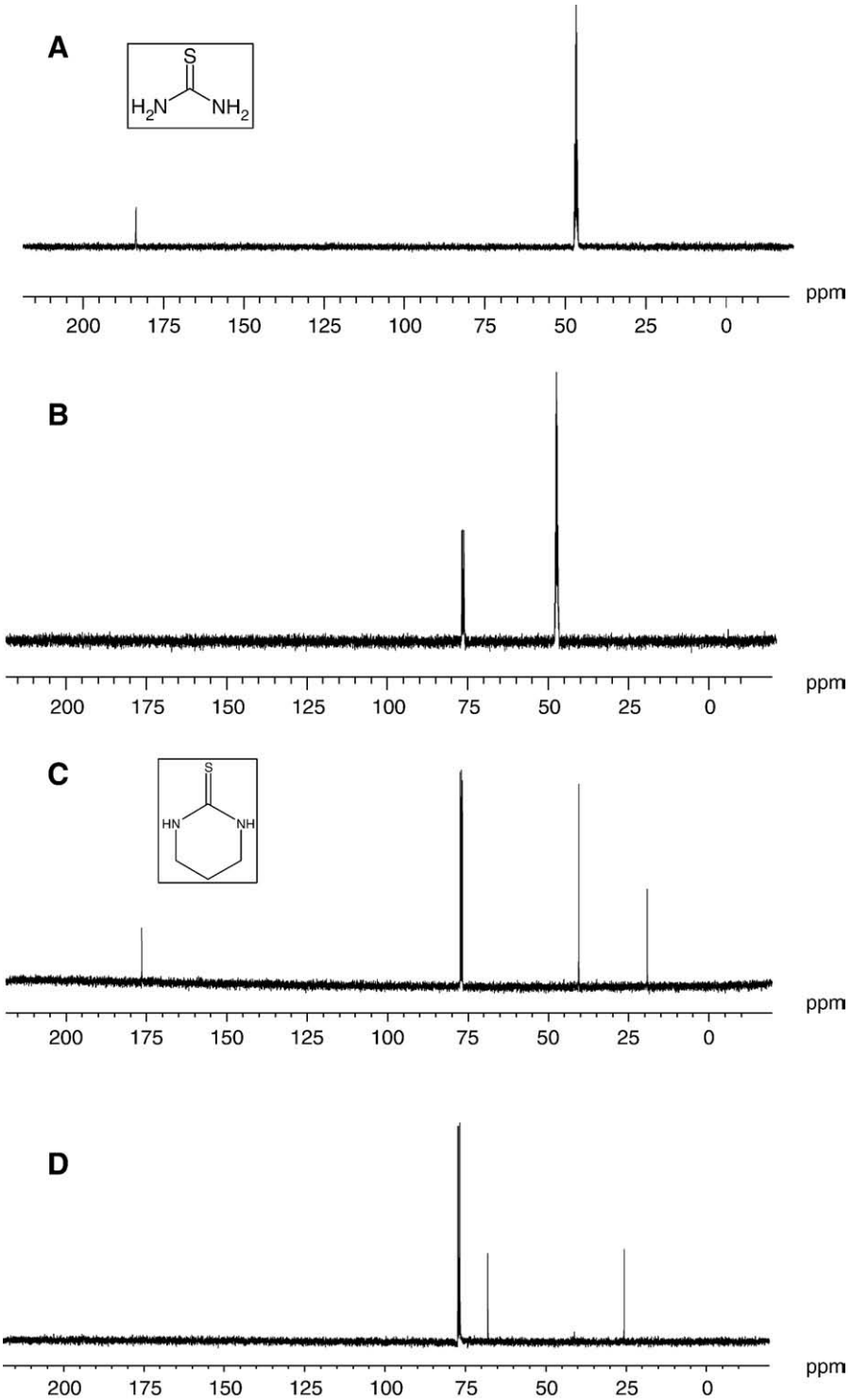
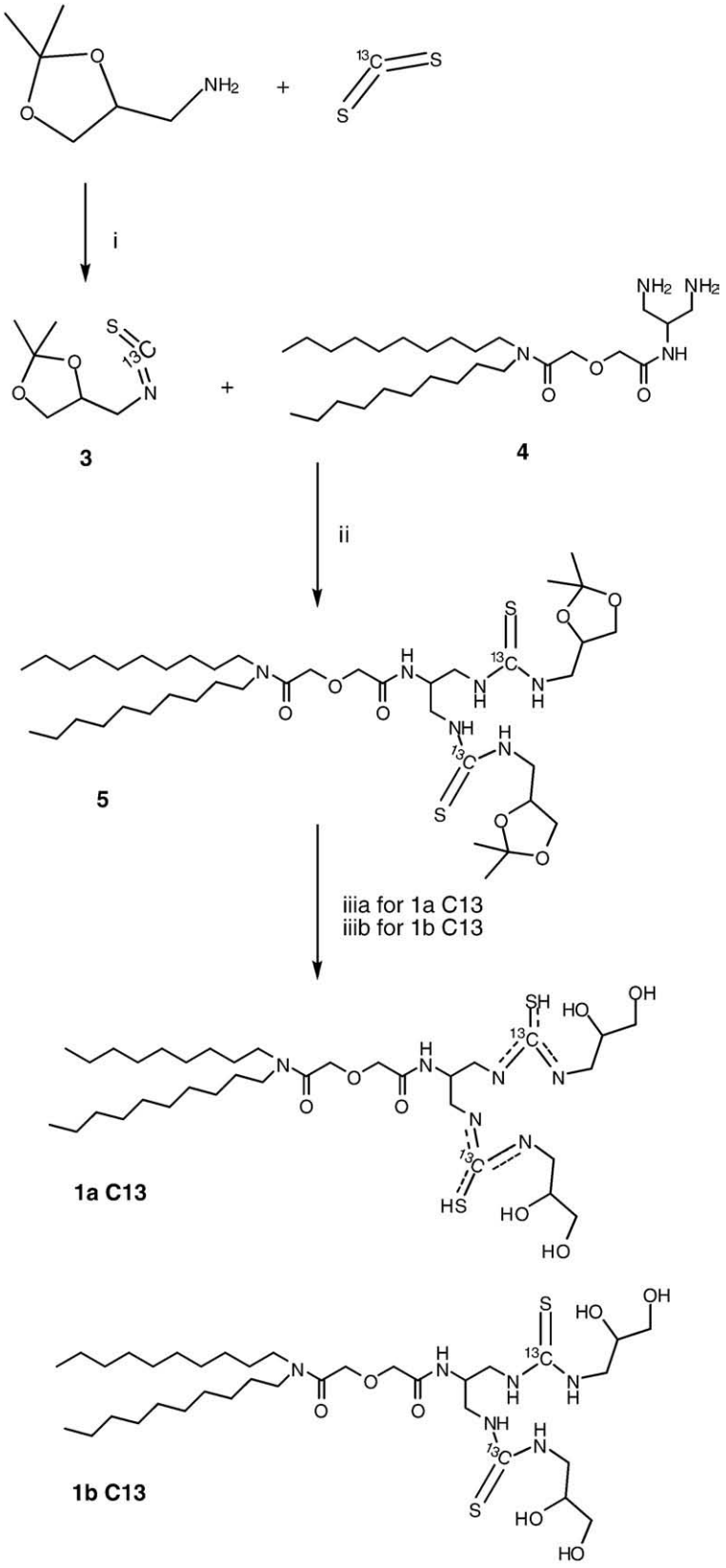


Fig. 3. ^{13}C-NMR spectra of thiourea before (A in MeOD) and after (B in MeOD/$CDCl_3$) acidic treatment and ^{13}C-NMR spectra of tetrahydropyrimidinethione (inserted figure) before (C in $CDCl_3$) and after (D in $CDCl_3$) acidic treatment.



Scheme 2. Synthesis of ^{13}C products. DDSTU/HCl-^{13}C (1a C13) and DDSTU/CH_3CO_2H-^{13}C (1b C13). i: DCC, THF, RT, 1 h; ii: NEt_3, THF, RT, 12 h; iiia: HCl 1N, THF, RT, 3h; iiib: CH_3CO_2H 80%, H_2O, 100 °C, 5 min.

thiourea and tetrahydro-pyrimidine-2-thione. These molecules have been treated with HCl 1 M in THF for 2 h keeping the same stoichiometry between the thiourea and the acid as in the synthesis of DDSTU/HCl. The ^{13}C-NMR spectra are shown in Fig. 3. Before treatment, the thiourea function peak (Fig. 3, A) was clearly visible at 185 ppm. After treatment (Fig. 3, B) the peak disappeared. The same

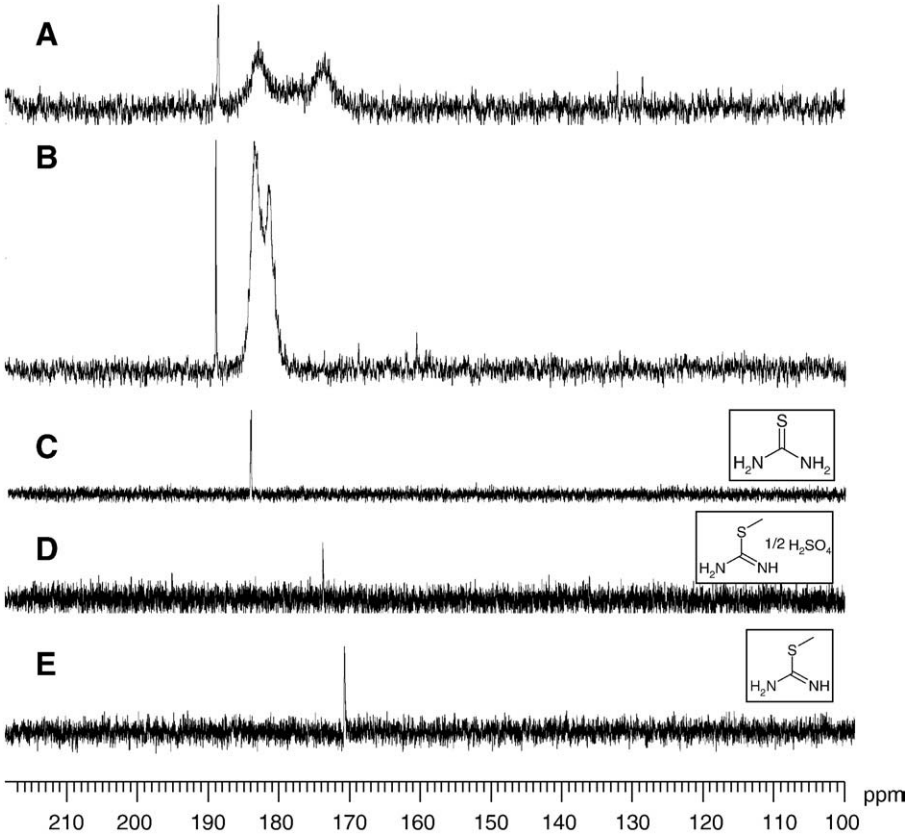


Fig. 4. ^{13}C-NMR spectra of DDSTU/HCl-^{13}C 1a C13 (A in $CDCl_3$), DDSTU/CH_3CO_2H-^{13}C 1b C13 (B in $CDCl_3$), thiourea (C in D_2O), S-methylisopseudothiourea hemisulfate salt (D in D_2O) and S-methylisopseudothiourea (E in D_2O). The signal at 189 ppm represents residual carbon disulfide.

result was obtained with the cyclic thiourea: the tetrahydropyrimidine-2-thione (Fig. 3, C). A shift of the other carbon peaks was also clearly visible (Fig. 3, D). As the mass of both products (checked by LC-MS) were unchanged after the acidic treatment, this experiment confirms that the chemical treatment applied to the lipid DDSTU/HCl is acidic enough to transform the thiourea function in another function. This function is likely to be a tautomeric form of the thiourea.

3.4. NMR using ^{13}C products

In order to increase the signal of the thiourea carbon in the spectrum of DDSTU/HCl and to better characterize these lipopolythioureas, we synthesized analogs with ^{13}C carbon disulfide as a starting reagent. This synthesis is described in Scheme 2. It is based on the synthesis described in reference [18], however, to obtain the product 3, the carbon disulfide used was 99.9% ^{13}C and it was not introduced in excess but in the same stoichiometry as the amine. The desired products were obtained as confirmed by LC-MS.

The spectrum of DDSTU/HCl-^{13}C 1a C13 (Fig. 4, A) showed two broad peaks at 183 ppm and 173 ppm. The wide shape of the peaks suggests an equilibrium between two forms. *Ab initio* and density functional theory calculations suggest that the most energetically favored prototropic form of thiourea is S-protonated thiourea (the iminothiol) over N-protonated thiourea by approximately 20 kcal/mol. [24] We therefore chose to compare the signal of our products with the signal of the commercial products thiourea, S-methylisopseudothiourea hemisulfate salt and S-methylisopseudothiourea. In the spectrum of DDSTU/HCl-^{13}C 1a C13 (Fig. 4, A) the peak at 183 ppm could then be attributed to the thiourea function (Fig. 4, C), however the peak at 173 ppm was more delicate to attribute. Indeed, positively charged S-methylisopseudothiourea showed a signal at 174 ppm while its neutralised form showed a characteristic peak at 172 ppm. Considering the broadness of the peaks for compound 1a, a difference of 2 ppm does not allow to definitely attribute the signal at 173 ppm to a charged or uncharged iminothiol, a mixture of both could also explain the broadness of the signal.

These data confirm that both tautomeric forms iminothiol and thiourea are present and that there is a balance between these two forms. Conversely, only one signal could be seen on the spectrum of DDSTU/CH_3CO_2H-^{13}C 1b C13 (Fig. 4, B) at 183 ppm corresponding to the thiourea function. This signal appeared as a pair of peaks. This could be due to two different spatial conformations of the molecule or to remaining acetic acid bound to the thiourea.

The iminothiol form could also be favored by a supramolecular structure stabilized by hydrogen bonds. We therefore set up an experiment to verify this hypothesis. Trifluoroethanol (TFE) is a solvent which has been described for its ability to create hydrogen bonds and weaken hydrophobic interactions [29,30]. In TFE, hydrogen bonds between a molecule and the TFE solvent are favored, thus reducing intramolecular hydrogen bonds between lipid molecules which would consequently reverse the equilibrium towards the thiourea form. Indeed, addition of increasing amounts of TFE to a solution of DDSTU-^{13}C/HCl in chloroform to a final ratio of 1:1 induced gradual increase of the signal at 183 ppm to the detriment of the peak at 172 ppm, which eventually disappeared. It could thus be concluded that the iminothiol form is stabilized by the presence of intramolecular hydrogen bonds between the lipid molecules. Increasing the polarity of the NMR solvent confirmed this result as the peak at 183 ppm was present in all cases, while, the peak at 173 ppm disappeared in favor of the 183 ppm resonance indicative of the thiourea form (Table 1). This influence of the polarity is consistent with a charged or uncharged iminothiol form stabilised by hydrogen bonds.

3.5. UV analysis

To confirm the hypothesis of a supramolecular effect sustained by electrostatic interactions or hydrogen bonding, we finally conducted a UV spectrophotometry experiment in aqueous medium at physiological pH. The thiourea function shows a characteristic peak at 240 nm [25] whereas the amide function of a charged or uncharged iminothiol will only show a peak at 220 nm. The ratio of the absorbance at 240 nm on the absorbance at 220 nm is rather representative of whether the molecule bears a thiourea or a charged or uncharged iminothiol function. Again, we used two commercially available molecules as standards in this experiment: thiourea and S-methylpseudoisothiourea hemisulfate salt. Our lipids 1a and 1b were not soluble in water so they were formulated as liposomal solution before the measurements. The results are displayed in Fig. 5. In the case of the thiourea, the peak at 240 nm was highly predominant, whereas the spectrum of the S-methylpseudoisothiourea which contains only the iminothiol function exhibited a higher absorption at 220 nm. Concerning the lipids 1a and 1b, DDSTU/HCl presented a major absorption peak at 220 nm confirming an iminothiol form and DDSTU/CH_3CO_2H presented a maximum absorption peak at 240 nm indicating the thiourea form.

Table 1
Chemical shifts of the thiourea peaks of DDSTU/HCl 1a in various solvents.

Solvent	$CHCl_3$	TFE/$CHCl_3$ 1/1	MeOH	Water/MeOH 1/1
Polarity	− −	+	+	+ +
Carbon chemical Shifts(ppm)	183.02 173.56	182.00	184.35	184.00

3.6. Physico-chemical properties and interaction with DNA

We then studied how the thiourea tautomeric equilibrium did affect the interaction of the lipids with DNA. As previously reported [18], the lipid 1a is able to compact DNA at a TU/P ratio of 10. A gel retardation experiment showed that the lipid 1b is also able to compact DNA but requires a TU/P ratio of 40 (Fig. 6). This result showed how the structure of the thiourea function contributed to the interaction of the particles formed with lipopolythiourea and DNA. Indeed, the lipid 1b bearing solely the thiourea form was able to fully compact DNA, however, the presence of the iminothiol function (as in product 1a) improved the interaction with DNA and its full compaction was possible at a lower TU/P ratio.

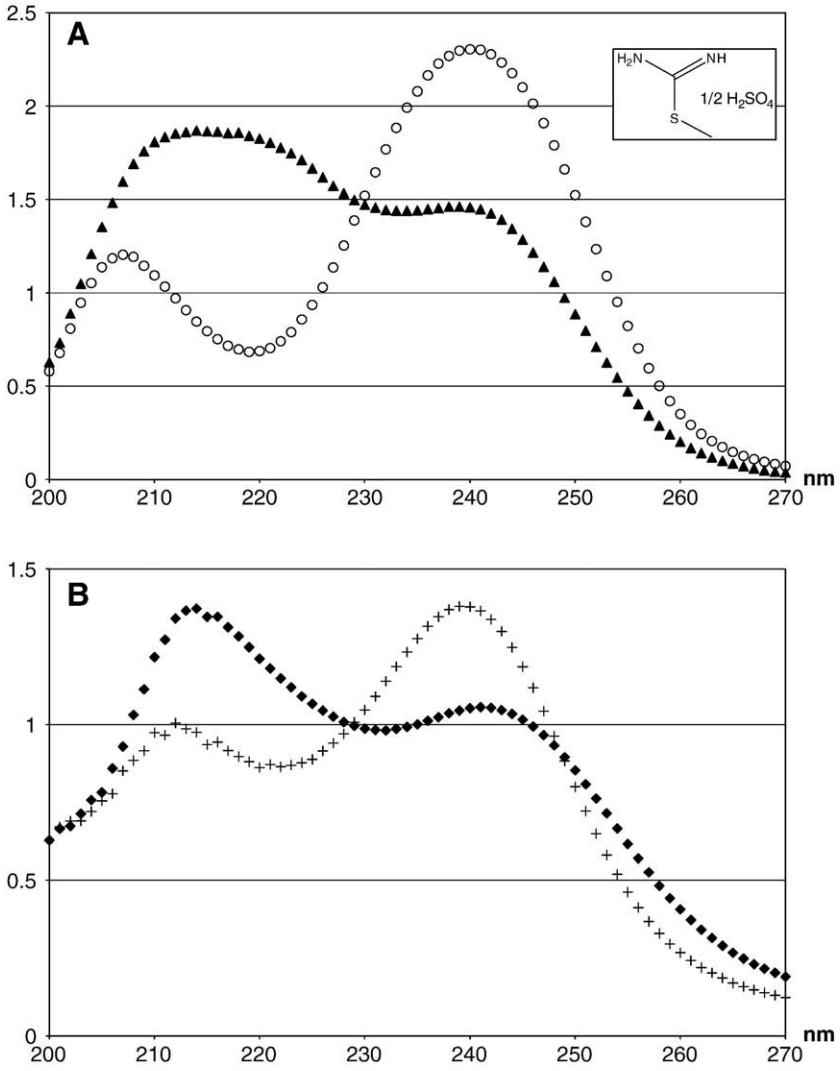


Fig. 5. UV spectra on the range 200 nm to 400 nm of (A) thiourea (OOO) and S-methylisopseudothiourea hemisulfate salt (▲▲▲ and inserted figure); (B) DDSTU/HCl 1a (♦♦♦), DDSTU/CH_3CO_2H 1b (+ + +).

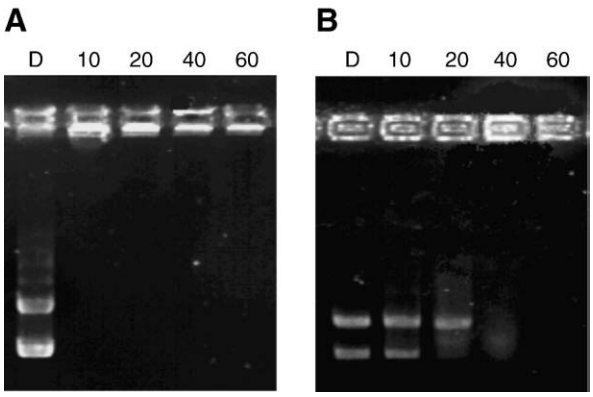


Fig. 6. Agarose gel electrophoresis of DNA complexes with DDSTU/HCl 1a (A) and DDSTU/CH_3CO_2H 1b (B) at different TU/P ratios. D: DNA alone.

4. Discussion

We have conducted different physicochemical studies to tentatively assess the differences in transfection behavior of a thiourea lipid obtained in two different synthetic pathways. ^{13}C NMR studies of the compounds 1a and 1b as well as the ^{13}C labeled analogs allowed revealing the presence of the iminothiol function in organic solvents. We showed that DDSTU/CH_3CO_2H 1b only bears the thiourea function while DDSTU/HCl 1a presents an equilibrium between the two tautomeric forms, thiourea and iminothiol. In chloroform, ^{13}C-NMR spectrum of 1a shows both thiourea and iminothiol corresponding peaks. However, ^{13}C-NMR spectrum of 1a in chloroform in presence of TFE affords only the thiourea form. As TFE shifts the hydrogen bonds, it can be concluded that the iminothiol form is stabilized in organic solvents by the presence of hydrogen bonds between lipidic molecules. The use of different solvents also showed that when the polarity of the solution increases, the iminothiol form disappears in favor of the thiourea form.

Thanks to the MarvinSketch software (v. 5.1.4, ChemAxon Ltd., Budapest, Hungary) it was possible to model the tautomer distribution of the DDSTU lipid at different pH. At pH 2, the thiourea form is present at 65% and at pH 3, it is present at 100%. Between pH 1 and 3, the charged iminothiol and the thiourea functions are in equilibrium. The ionization of the thiourea function requires very low pH and above pH 3, no iminothiol form could be expected. Nevertheless, UV spectrum of the 1a formulated as a liposome, compared to commercially available compounds respectively in the thiourea or iminothiol form, confirmed the presence of both tautomeric forms in water at a neutral pH. This discrepancy between UV, NMR experiments and the theoretical calculation of tautomer ratios can only be explained by a supramolecular effect of the liposomal formulation that stabilizes the iminothiol form. As only the thiourea forms is present within the 1b liposomes, it sustains the hypothesis that when the lipid is synthesized in very acidic conditions, it favors an iminothiol form which is then stabilized and preserved by hydrogen bonds in the liposomal structure.

The structure of the thiourea function influences its ability to form hydrogen bonds. The CS function is a good proton acceptor when enhanced by resonance effects as it is the case in thioureido species [31,32]. The iminothiol function which is a tautomeric resonance form of thiourea is then likely to be a good proton donor. According to the calculations of the MarvinSketch software at neutral pH, the lipid 1a bearing two thiourea functions has 18 acceptor or donor sites for H bonds while the lipid 1a bearing two iminothiol functions has 23 acceptor or donor sites for H bonds. It could be thus expected that the presence of the iminothiol function could improve the interaction with DNA. In Hepes 20 mM (pH = 7.4), the Zeta potential of the 1a liposome is 21.4 mV and the Zeta potential of 1b liposome is 14.8 mV. The Zeta potential of the liposome of 1b is positive even if this lipid cannot present the positively charged iminothiol form of the thiourea. From the previous data, three different forms of the thiourea function (thioureas, iminothiols and charged iminothiols) in lipid 1a might be present which could eventually explain the differences in the measurement of zeta potential. Moreover, the compaction of DNA by the thioureas lipids occurs at a lower amount of thiourea lipid to phosphates in the case of 1a as compared to 1b. This difference could be explained by an interaction of the iminothiol function of 1a with the phosphates of DNA that can displace the tautomeric equilibrium towards the charged form of the iminothiol moiety. This is not the case with 1b which cannot present this equilibrium.

We could then evidence that the lipids of the "serinol" lipopolythiourea family involved a cationic charge for the interaction with DNA. However, this does not reflect all the mechanisms of interaction between thiourea lipids and DNA as compound 1b which only bears thiourea functions is able to interact with DNA although less efficiently. The compounds from the "lysine" lipopolythiourea family [16–18] which are also able to compact DNA do not undergo acidic conditions during their synthesis which implies that DNA compaction does not necessarily need electrostatic interactions. The lysine structure of this family of compounds might induce rigidity in the lipid structure which surely affects the DNA compaction. The structure of the thiourea bearing moiety seems therefore also very important to explain the interaction between the thiourea groups and DNA.

5. Conclusion

We improved the NMR analysis of a family of thiourea lipid by synthesizing the ^{13}C-enriched equivalent of our products. Thus we were able to prove that in acidic synthetic conditions, the obtained product could be a charged iminothiol, an iminothiol and a thiourea in a tautomeric balance. These iminothiol forms can remain present even in polar solvents when stabilized by a supramolecular effect in assemblies such as liposomes. As a consequence of the positive charge of the iminothiol form, the compaction of DNA was possible at a lower lipid/DNA ratio thanks to electrostatic interactions. In this case, hydrogen bonding surely also plays a role in the stabilisation of these complexes and the interaction with plasmids.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bpc.2009.08.003.

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