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REGULAR ARTICLE

A theoretical investigation on the interaction of a new gene vector with DNA

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Abstract A new neutral gene vector, based on a lipopolythiourea *N*-(2-(3-[2-(2-(3-[2-(3-methyl-thioueido)-ethyl]-thioureido)-ethylamino)-ethyl]-thioureido)-ethyl)-*N*′, *N*′-ditetradecyl-succinamide (DTTU) has recently been synthetized but its behavior is difficult to study at the experimental level. Density functional theory (DFT) calculations have thus been performed to predict its interaction mode with B-DNA. Its acidic properties are first computed and suggest that DTTU should be non-charged when interacting with DNA. Different ways of DTTU/DNA associations based on hydrogen bonding–namely external and groove-binding interactions—are then investigated. Our calculations clearly point out that external interaction is preferred with respect to groove-binding, due to three bifurcated hydrogen bonds between DTTU thiourea groups and DNA phosphates. Such results can be explained by the absence of charged groups in groove-binding whereas the negative charge of DNA phosphates deeply strengthens hydrogen bonds.

Keywords Density functional theory calculations · Interaction with DNA · Gene therapy · Hydrogen bonding · Thiourea · Acidic constant

Contribution to the Nino Russo Special Issue.

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1 Introduction

Within the past decade, gene therapy has been amazingly developed, being the topic for numerous studies [1,2]. Its basic principle rests on the fact that the absence or the defective structure of a gene may cause numerous diseases, hereditary (e.g. mucoviscidose) or not (e.g. cancers). One promising way of treatment is then to insert a normal gene directly into the genome to replace absent or disease-causing ones. The main interest is indeed related to the possibility to act at the very origin of the dysfunction, whereas drugs are only effective downstream, on biological functions. However, this implies to be able to carry over DNA to targetcells, to cross the cell membrane and finally to deliver genes into the nucleus [3]. Synthetic gene vectors based on cationic lipids or polymers are commonly used to protect DNA while conveying [2]. It is assumed that the vector enters into the cell and is then encircled by an intracellular vesicle called endosome. The acidity of the endosome decreases progressively. To be effective, the DNA/vector complex must then be destabilized to go out of the endosome and release DNA into the nucleus. Otherwise, it is destroyed by enzymes. Synthetic

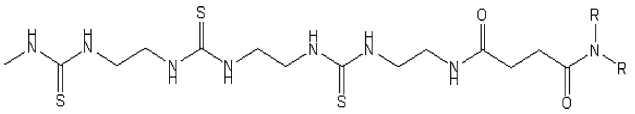


Fig. 1 Scketch of *N*-(2-(3-[2-(2-(3-[2-(3-methyl-thioueido)-ethyl]-thioureido)-ethylamino)-ethyl]-thioureido)-ethyl)-N',N'-ditetradecyl-succinamide, DTTU. In the real system, R = $C_{14}H_{29}$, whereas simplified models in our study correspond to R=H, CH_3 or C_3H_7

cationic vectors have already proved their efficiency [2,4]. Yet, recent studies have shown that phenomena of aggregation may occur with anionic blood proteins due to electrostatic interactions and their positive charge could also be toxic for the organism [5–7].

Such observations have thus led Tranchant et al. [8] to synthesize a new neutral gene vector, the *N*-(2-(3-[2-(2-(3-[2-(3-methyl-thioueido)-ethyl]-thioureido)-ethylamino)-ethyl]-thioureido)-ethyl)-*N'*, *N'*-ditetradecyl-succinamide (hereafter refered to as DTTU, see Fig. 1). This molecule is composed by three thiourea groups which are supposed to act as the binding part of the system, thioureas being well-known for establishing strong hydrogen bonds with their relatively acidic NH protons [9,10]. The first in vivo experiments have nevertheless revealed a very low level of gene expression. Biologists suspect this failure to result from a very strong association between DTTU and DNA, thus leading to the destruction of the DDTU/DNA adduct in the endosome. Yet, DTTU binding-mode is still unknown and thus prevents experimentalists from any improvement.

In this context, modeling studies could be relevant and helpful to shed light on the DTTU/DNA association, but difficult to characterize at the experimental level. Due to both the complexity of the phenomena and the size of the systems under study, a method combining a good accuracy with fast calculations is required and the density functional theory (DA) route was applied in this perspective. Even so, simplified models of DTTU and DNA were considered. In particular, DTTU ending alkyl chains were replaced with smaller groups (R=H, methyl or Propyl, see Fig. 1). The DNA/DTTU interaction was also described with models that will be specified as one goes along.

The first step was to investigate the bare DTTU, i.e., its structural features and acido-basic properties (these latter via p*K*a calculations). The aim was to give some indications on how DTTU behaves at physiological pH and in the endosome, and thus to identify its chemical form when complexing DNA. Its association with B-DNA was examined next. Basically, three interaction modes with double-strand DNA are commonly considered in the literature [11]:

- External electrostatic interaction with negatively charged DNA phosphates
- Groove-binding interaction
- Intercalation between two DNA bases.

These three interactions are intrinsically different. Intercalation is experimentally observed with planar conjugate systems and is thus not relevant for DTTU/DNA association. In contrast, external and groove-binding interactions can occur through H bondings. The S-acceptor and NH acidic groups consequently make DTTU a good candidate for these two kinds of association with DNA. They were therefore successively examined in a second step.

2 Computational details

All calculations were performed with the Gaussian 03 package [12]. The correct treatment of hydrogen bonds being fairly tricky [13,14], the DFT together with the non-parametric hybrid functional PBE0 [15] were applied as they have already proved their efficiency for such interactions while keeping a reasonable computational cost [16–18]. Here, although large basis sets are mandatory for a proper description of H-bonding, all DFT optimizations (apart from p*K*a calculations) were conducted with the 6-31G(d,p) basis set. Actually, this limitation does not significantly affect our results due to the large energy differences computed between the different species (see below).

The p*K*a calculations are based on the thermodynamic cycle reported in Fig. 2 [19]. Following this Born-Haber cycle, several basis sets were tested on a thiourea representing an acido-basic DTTU group (Fig. 3). Even though compensation of errors still occur for small basis sets, this graph steadily tends to the reference value of −1.19 [20]. The following four-steps methodology was thus adopted:

1. Optimization and frequencies calculation on B and BH^+ species in vacuum with the 6-31G(d) basis set.
2. Single point energy evaluation on the optimized structures in vacuum, 6-31+G(2d,2p) basis set.
3. Optimization of the former structures in aqueous phase with the 6-31G(d) basis set.
4. Single point on the resulting geometry in the aqueous phase, with 6-31+G(2d,2p) basis set

The free energy of all the species, but H^+, G°_{gas}, is obtained by adding the energy in the 6-31+G(2d,2p) basis set to the thermal correction obtained in the frequencies calculation output. Not all the quantities needed for ΔG_{aq} can be directly computed since the proton solvation energy, $\Delta G_{solv}(H^+)$ and its gas-phase free energy, $G(H^+_{gas})$, cannot be easily determined theoretically. For these two, experimental values are usually considered [19].

Solvent effects were assessed using the conductor-like approach within the framework of the polarizable continuum model (PCM) [21,22].

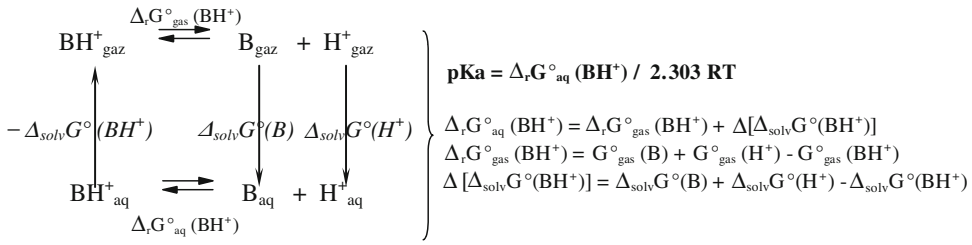


Fig. 2 Thermodynamic cycle for pKa calculations. Data for H^+ solvation ($\Delta_{solv}G°(H^+)$) are taken from Ref. [53,54]

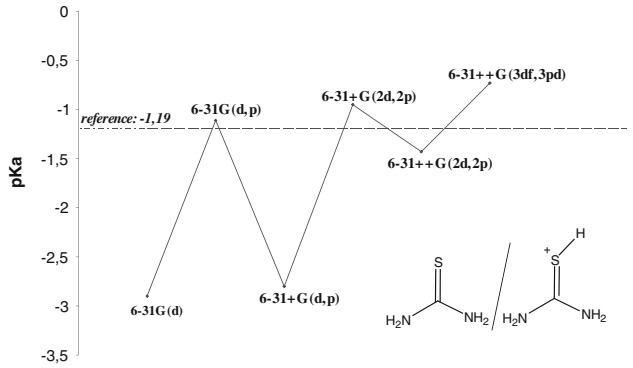
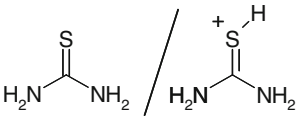
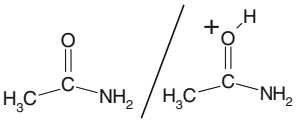


Fig. 3 Evolution of p*K*a values for the $NH_2CSNH_2/NH_2(CSH)NH_2^+$ pair as function of the basis set

Table 1 Comparison between theoretical and experimental pKa values for DTTU model systems

	Theoretical p*K*a	Experimental p*K*a
	−0.93	−1.19[a]
	1.2	−0.49[b]

[a] Ref. [20]
[b] average of Ref. [51], [52]

Binding energies were each time corrected accordingly to the basis set superposition errors (BSSE) using the counterpoise procedure [23]. They were assessed at the DFT level but Moller–Plesset (MP2) calculations [24,25] were also performed to confirm these values.

Starting DNA structures were taken from the PDB database [28]. Note that DTTU/DNA model adducts were preoptimized at the semi-empirical PM3 level.

3 Results and discussion

3.1 Characterization of bare DTTU: geometry and p*K*a

Because of its hydrophobicity, DTTU acido-basic properties are quite difficult to obtain experimentally and were thus examined first so as to determine which form should be considered when studying its interaction with DNA. Unfortunately, even from a theoretical point of view, such an approach is made difficult by the lack of experimental data on thiourea acidic constants, necessary for assessing calculations accuracy. In fact, even the site of protonation is a source of controversy, either the amonium or the sulfonium form being considered [20,27–36]. All the same, despite some data suggesting the N-protonation [27–29], the sulfonium structure was identified by several spectroscopic, conductivity or even semi-empirical studies [30–36].

The first step was thus to determine the best protonation site for thiourea and amide functions, by testing both possibilities, i.e. S or O protonation. Model systems, namely protonated thiourea and methylamide, were thus optimized in aqueous phase (using CPCM) considering the amonium and sulfonium/hydroxonium forms for each model. Corresponding energies show that the S- or O-protonation gives the most stable species notably thanks to resonance stabilization, with respective discrepancies of 13.0 and 14.8 kcal/mol with respect to the amonium form. Therefore, only sulfonium and hydroxonium forms were examined next. Their acidic constants were calculated following the cycle given in Fig. 2 and previously discussed (see Sect. 2). Corresponding values, reported in Table 1, are globally in good agreement with experimental data. The computed deviations between the experimental and theoretical values (0.3 and 1.7 for S and O, respectively) were nevertheless applied as a correction to the p*K*a values computed for the larger DTTU model (R=H). Such a model introduces an ending amine group which was not considered since the right DTTU formula exhibits alkyl chains instead. Likewise, DTTU acts at physiological pH which is around 7 or in a more acidic environment in the endosome. Consequently, cases of deprotonation were not taken into account. On the whole, five sites of protonation were studied and their p*K*a are reported in Fig. 4. Interestingly, wide discrepancies are observed for equivalent functions, ranging from −9.2 to −1.3 for thiourea groups. They can be explained by the important flexibility of the molecule which is made easier by possible intramolecular hydrogen bonds when being protonated. Yet, regarding such low values, DTTU was considered as non-protonated for the

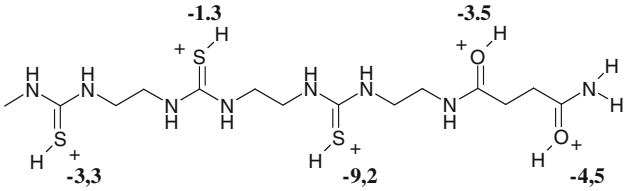


Fig. 4 Protonation sites and corresponding computed values of p*K*a for DTTU in *bold*

rest of the study since the physiological pH is around 7. Actually, protonation might occur inside the endosome but this analysis should be much more complicated because of potential multiprotonation at very low pH [37].

3.2 DTTU/DNA association

3.2.1 • Groove-binding interaction

DNA grooves are the binding sites of many drugs and proteins. The so-called netropsin represents one of the archetypes of such groove-binding systems [38–42] and interacts with DNA in spots rich in adenine-thymine (AT) bases. Its nitrogen groups can be indeed involved in hydrogen bonds with both DNA strands [42]. Such interactions are particularly strengthened at the very ends of netropsin thanks to its positively charged amides that have been proved to have a major role in netropsin/DNA adduct stability [39]. A similar behavior has been observed with the lexitropsin derivative that features hydrogen bonds with the guanide–cytosine (G–C) couple [43]. Two model systems—methylthiourea-adenine-thymine and methylthiourea–guanine–cytosine—were thus considered so as to represent these two interactions. Figure 5 shows their optimized geometries at the PBE0/6-31G(d,p) level of theory. The bifurcated hydrogen bonds described in the literature for netropsin do not appear with thioureas and they are replaced by one single interaction. Binding energies at the DFT level are, respectively, of −7.0 kcal/mol for the adenine–thymine couple and −2.1 kcal/mol for the guanine–cytosine one. A single point at the MP2 level respectively provides −11.7 and −7.5 kcal/mol. This slight difference between the two methods of calculation is consistent with the underestimation of the hydrogen-bond strength expected for DFT approaches (see for instance Ref. [44]).

3.2.2 • External interaction

External stacking often occurs with cationic systems interacting electrostatically with negatively charged DNA phosphates. Herein, cationic groups are expected to be replaced by thiourea functions establishing hydrogen bonds with phosphates oxygen atoms, their high affinity being already known

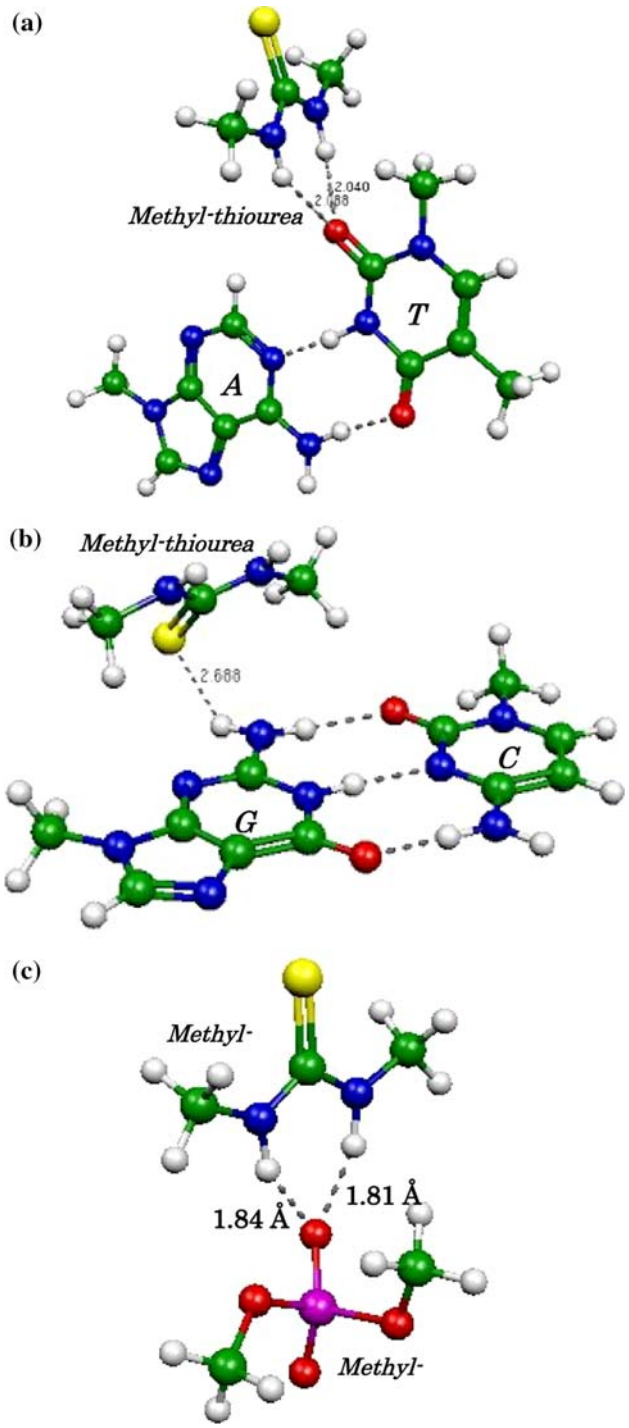


Fig. 5 Optimized structures (PBE0/6-31G(d,p)) of model systems for groove-binding (**a**, **b**) and external (**c**) interactions

[45]. Accordingly, to identify the most favored DTTU/DNA adduct, the external interaction was modeled by the methylthiourea–methylphosphate system. The phosphate group comes from DNA crystallographic data [26]. Its carbon and nearby oxygen atoms coordinates were kept constant to respect this structure of origin. The resulting PBE0/6-31G

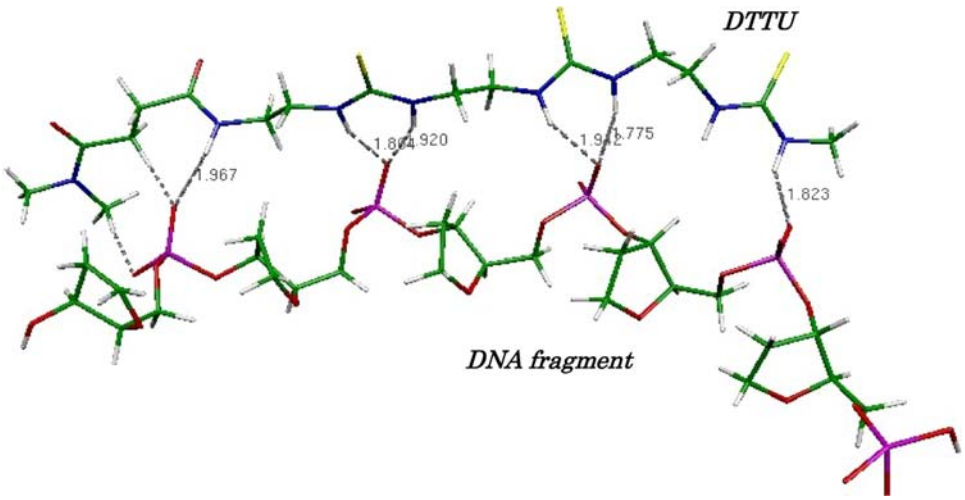


Fig. 6 Optimized structure of the DTTU (R=Me)/DNA external interaction at the PBE0/6-31G(d) level

(d,p) optimized structure is shown in Fig. 5. Two main hydrogen bonds (1.81 and 1.84 Å) are observed between the thioureas NH groups and one phosphate oxygen atom, thus forming a six-centered system. They are strengthened by two minor hydrogen bonds with further oxygen atoms (2.55 and 3.78 Å). Although the main hydrogen bonds distances are fairly low, their bicentric pattern induces a strong angular constrain. The N–H–O angle is indeed reduced to 155°, whereas the best electronic transfer should require almost 180° [13,46]. Even so, the binding energy, calculated in four different basis sets (6-31G(d), 6-31G(d,p), 6-311G(d,p) and 6-311+G(2d,2p), remains significant, with a mean value of −27.5 kcal/mol. This value even reaches −33.2 kcal/mol at the MP2 level of theory. It is worth noting that in commonly used classifications [46,47] strong hydrogen bonds feature binding energies below −15 kcal/mol, with distances as low as 1.7 Å when interacting with phosphate groups [48]. Such strength seems actually to stem from negative charge of phosphate groups and explains why groove-binding provides much lower binding energies (<10 kcal/mol).

As expected, solvent, counter-ions and the size of the system could play a non negligible role in tuning the interaction between phosphates and thiourea. As first step, bulk solvent effects have been modeled using a PCM [21,22] model at the PBE0/6-311+G(2d,2p) level. The obtained results suggest that solvent further strengths the interaction between the two moieties, since the energies decreases up to −31.5 kcal/mol, i.e., 3.7 kcal/mol lower than the gas-phase value.

The analysis of counterion effect is instead more involved the interaction between a sodium cation and the phosphate group is −143.0 kcal/mol, including BSSE correction, significantly larger than that obtained for thiourea at the same level of theory. This result suggests that the interaction of phosphate and thiourea could be tuned by a hydratation/dehydratation mechanism, involving also the counterion. A detailed study of such process would require a (quantum or classical) dynamical modeling to have detailed information on the solvent structure around the solute and on the dynamics of the dehydratation processes. Nevertheless cluster modeling, obtained by considering only the solvent molecules strongly bound to the solutes, could give valuable insights. To this end, the interaction energies of Na^+, thiourea and phosphate with some water molecules have been computed at the PBE0/6-311+G(2d,2p) level. Both thiourea and phosphate show interaction energies with one water molecule (−5.3 and −17.2 kcal/mol, respectively) lower than that obtained for the sodium cation. In fact, the water adduct of Na^+, $Na(H_2O)_5^+$, has a total dissociation energies of 76.4 kcal/mol. Even if only a partial dehydration of Na^+ is needed to have a strong interaction with the phosphate group, these results suggest that the interaction of thiourea and phosphate is favorite since the corresponding dehydration process is lower in energy and the reaction leads to a strong interaction.

Finally, to support these first conclusions, a more complete system (DTTU with R=Me) was then studied at the PBE0/6-31G(d) level, with a previous optimization at the semi-empirical (PM3) level (Fig. 6). The ending methyl substituant was chosen to avoid artificial hydrogen bonds. A small DNA fragment was only considered, where nitrogen bases are substituted by hydrogen atoms, heterocycles are kept constant while optimizing and five phosphate groups are only taken into account. The alternating sequence A–G–C–T–A was selected from PDB database so as to be as general as possible. Surprisingly, DTTU appears to be flexible enough to change its conformation while interacting with DNA (Fig. 6). In order to favor hydrogen bonds, the three DTTU thiourea groups, normally in *trans* from one another in the bare molecule (Fig. 1), move into the *cis* position. This noteworthy modification gives rise to six hydrogen bonds with a mean length of 1.87 Å. Five of them are due to the thiourea groups whereas the last one occurs with the amide nitrogen atom. As in the previous model system, they are strengthened by

seven minor interactions between 3 and 4 Å. The mean angle of interaction is also hardly modified, with an average of 155° for thiourea groups and 165° for the last hydrogen bond on the amide function. The binding energy remains quite high, up to −130.1 kcal/mol. The Mulliken charges analysis [49] enables to refine these observations. As expected, there is an electron transfer from the phosphate (the acceptor of hydrogen) to the thiourea group (the donor of hydrogen). In this manner, the highly electronegative sulphur atoms of DTTU thioureas retrieve a large amount of electrons (−0.18 |e−|) while a mean increase of 0.09 |e−| on the phosphate groups is calculated with respect to the bare DNA strand. Actually, the highly polarizable phosphor atoms act as the source of electrons. The rise of 0.09 |e−| is indeed only located on these atoms whereas the harder oxygen atoms manage to keep their charge constant.

In order to check the effect of DNA bases and of DTTU alkyl chains neglected on interaction energies, PM3 calculations have been carried out on larger systems. The role of nitrogen bases was first assessed considering a complete DNA strand (six phosphates, T–A–G–C–T–A) in interaction with DTTU (R=Me). As previously, the coordinates of heterocycles and bases are fixed. The optimized structure is hardly affected. Five hydrogen bonds are observed, with a mean length of 1.76 Å and a total binding energy of −129.6 kcal/mol. With respect to the former system at the same level of theory (PM3), the consideration of the nitrogen bases leads to a variation of only of 6.3 kcal/mol on the total binding energy. The influence of bases may be therefore neglected.

The influence of DTTU alkyl chains was then worked out. In cationic vectors, hydrophobic parts are organized in a bilayer pattern orthogonally to DNA [50]. In DTTU, the polar heads of cationic vectors are substituted by a long motif with three thioureas. It is thus quite difficult to predict how alkyl chains may influence the interaction and if the bilayer arrangement will be kept. Hence, propyl substituants were used to model DTTU 'R' groups. In the optimized structure (PM3), the ending amide group is slightly pulled away from DNA, in the order of 0.3 Å. Nevertheless, since hydrogen bonds are located far enough from this area, no variation is observed as indicated by the global binding energy (−131.7 kcal/mol with R=Pr vs. −129.6 kcal/mol with R=Me).

4 Conclusion

In this work, we have analyzed the behavior of a new lipid-based non-cationic gene vector called DTTU in the framework of the DFT. Its chemical properties, that is geometry and pKa, were first investigated. We have proved that the molecule is particularly flexible, which partially explains the large variation in pKa values among the five protonation sites. In particular, these values are very low and suggest that protonation at physiological pH is excluded. Yet, it could occur in the endosome where the acidity is strengthened.

The kind of association with B-DNA was then studied. Two ways of complexation, namely external and groove-binding interactions, were compared. Binding energies clearly highlight that external interaction is favored. We believe that this result is directly linked to the absence of charge on DTTU contrary to usual groove-binding systems where charged groups are involved in a strong interaction with DNA bases. In the external interaction, charges on DNA phosphate groups compensate the DTTU neutrality. Its flexibility may enable DTTU to adapt its geometry to that of the outer part of the DNA helix.

Numerous issues still remain on this association. We are currently checking our results analyzing the behavior of the thiourea/phosphate interaction in natural biological media, i.e., water. All these data should guide experimentalists in improving DTTU properties and even in designing new gene vectors.

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