Methylene Blue Photophysics

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Enhancing Intersystem Crossing in Phenotiazinium Dyes by Intercalation into DNA**

Juan J. Nogueira, Markus Oppel, and Leticia González**

Abstract: *Phenothiazinium dyes are used as photosensitizers in photodynamic therapy. Their mode of action is related to the generation of triplet excited states by intersystem crossing. Therefore, rationalizing the factors that influence intersystem crossing is crucial to improve the efficacy of photodynamic therapy. Here we employ quantum mechanics/molecular mechanics calculations to investigate the effect of aqueous and nucleic acid environments on the intersystem crossing mechanism in methylene blue. We find that the mechanism by which the triplet states are generated depends strongly on the environment. While intersystem crossing in water is mediated exclusively by vibronic spin–orbit coupling, it is enhanced in DNA due to a second pathway driven by electronic spin–orbit coupling. Competing charge-transfer processes, which are also possible in the presence of DNA, can therefore be suppressed by a suitable structural functionalization, thereby increasing the efficacy of photodynamic therapy.*

Photodynamic therapy (PDT) is a well-recognized process for the treatment of cancer and microbial infections[1] that could become an alternative to conventional antitumor therapies because of its advantageous[2] non-invasive character. The generation of a triplet state through intersystem crossing (ISC) is the key process that starts oxidative damage and can lead to cell death, either by electron transfer to biomolecules in the vicinity (type I) or by transferring energy to molecular oxygen present in the environment (type II; Figure 1). Therefore, the therapeutic effect of a photosensitizer is correlated with the ISC quantum yield and this, in turn, depends strongly on the intermolecular interactions between the photosensitizer and the target biomolecules.

Photosensitizers based on phenothiazine dyes (Figure 2a) are used in PDT against tumor cells[3–5] and microbial organisms.[6–8] The planar structure of these dyes allows binding to DNA by intercalation with the nucleic bases of DNA which are arranged in an almost coplanar configuration with respect to the photosensitizer (Figure 2b). This has been confirmed by several spectroscopic studies,[9–12] which have concluded that intercalation is the most favorable binding mode for several phenothiazine derivatives. Of all the phenothiazines, methylene blue (MB) is by far the most

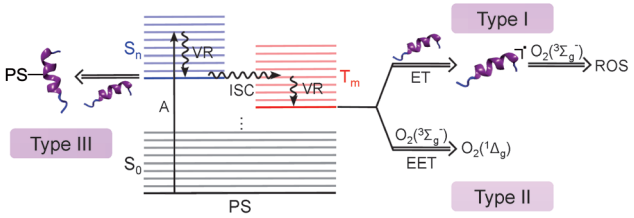

Figure 1. Photosensitization mechanisms: A photosentizer (PS) is excited to the bright state S_n after absorption (A) of radiation. Vibrational relaxation (VR) deactivates the PS to the vibrational ground state. Then, two types of processes can take place: intersystem crossing (ISC) to the triplet manifold T_m (type I and II) or chemical reactions with biomolecules in the vicinity (type III). In the former case, the PS in the triplet state can initiate electron transfer (ET, type I) or excited energy transfer (EET, type II) to the environment, thereby leading to cell death.

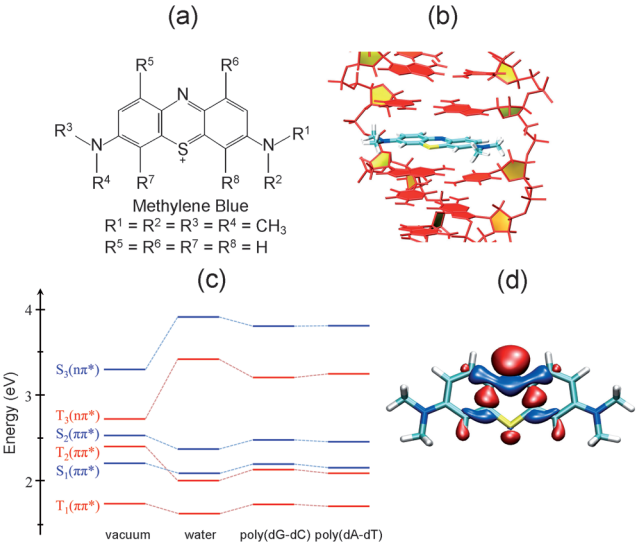

Figure 2. Structure, energies, and orbitals of methylene blue (MB). a) General chemical structure of phenothiazine derivatives and substituents of MB. b) MB intercalated into poly(dG-dC), where the aromatic rings of the dye and the nucleobases are aligned. Color code for the atoms: cyan: C, blue: N, yellow: S, and white: H. Color code for the residues: red: guanine and cytosine, and yellow: sugars. c) Vertical energies and character of the three lowest singlet and triplet excited states of MB in vacuo, in aqueous solution, in poly(dG-dC), and in poly(dA-dT), see also Table S1. d) The nonbonding orbital involved in the $S_3(n\pi^*)$ and $T_3(n\pi^*)$ states.

[*] Dr. J. J. Nogueira, Dr. M. Oppel, Prof. L. González
Institute of Theoretical Chemistry, University of Vienna
Währinger Strasse 17, 1090 Wien (Austria)
E-mail: nogueira.perez.juanjose@univie.ac.at
leticia.gonzalez@univie.ac.at
Homepage: http://www.theochem.univie.ac.at

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investigated derivative in PDT experiments.[2] MB initiates type II processes, producing singlet molecular oxygen $O_2(^1\Delta_g)$ with a quantum yield of 0.52 in water.[13] Singlet $O_2(^1\Delta_g)$ inside the cell has a short lifetime (0.01–0.02 μs);[14] hence, the contact between photosensitizers and biomolecules is very important for the efficacy of the PDT treatment.

Since MB, and phenothiazine dyes in general, are prone to bind noncovalently to DNA, we have performed electronic structure calculations on MB embedded in the intercalative pocket of two different nucleic acid strands: solvated dodecamers consisting of alternating guanine-cytosine [poly-(dG-dC)] and adenine-thymine [poly(dA-dT)] sequences. The results obtained for DNA were compared with the results of MB in vacuo and in aqueous solution. Our analyses show that the photophysics of MB dramatically changes depending on the environment, with striking consequences for PDT. On the basis of these results, we suggest the possibility of modifying MB to increase the ISC yield, and hence the PDT efficiency, when the dye is intercalated into DNA.

The calculations were performed using a hybrid quantum mechanics/molecular mechanics (QM/MM) approach. The dye was treated at the multistate complete active space second-order perturbation (MS-CASPT2)[15] level and the environment was described by the Amber force field.[16] The vertical excitation energies were obtained by averaging over 50 frames of a 40 ns ground-state classical trajectory[17] (see Section S1 in the Supporting Information for details). Figure 2c shows the vertical energies and characters of the three lowest singlet and triplet excited states (see also Table S1 in the Supporting Information). These energies are strongly altered by the environment. Placing the dye in an aqueous solution red-shifts the ππ* states by about 0.1–0.4 eV and blue-shifts the nπ* states by approximately 0.6–0.7 eV relative to those in vacuo. The same trend is observed when the dye is embedded into the DNA strands, although the shifts are less pronounced, which means that MB inside the intercalative pocket of the double strand is partially shielded from the effect of the solvent molecules. The agreement of the energy of the bright state $S_1(\pi\pi^*)$ in aqueous solution, 2.09 eV, with the experimental value[18] of 1.9 eV validates the results.

ISC is expected to be efficient if the singlet and triplet states involved are degenerate or nearly degenerate at some point of the phase space and their spin–orbit coupling (SOC) is non-negligible. According to the El-Sayed rule,[19] large SOC appears between states of different character. Based on these premises and considering that $S_1(\pi\pi^*)$ is the bright state of MB, ISC is expected to happen between the $S_1(\pi\pi^*)$ and $T_3(n\pi^*)$ states. As seen from Figure 2c, the gap between these states increases from 0.51 eV in the gas phase to 1.34 eV in explicit water, while intermediate values—1.00 eV for poly-(dG-dC) and 1.09 eV for poly(dA-dT)—are found in DNA. The increase in the energy gap in aqueous solution (and to a lesser extent in DNA) is a consequence of the blue-shift of the $T_3(n\pi^*)$ state and the red-shift of the $S_1(\pi\pi^*)$ state. The stabilization of the ππ* states in water can be explained with the dipole moments of the different electronic states. Table S2 shows that the dipole moments of $S_1(\pi\pi^*)$, $S_2(\pi\pi^*)$, $T_1(\pi\pi^*)$, and $T_2(\pi\pi^*)$ increase with respect to the S_0 value and, therefore, the electronic energies of these states decrease in going from the gas phase to aqueous media. In contrast, the dipole moments of the $T_3(n\pi^*)$ and $S_3(n\pi^*)$ states decrease and thus their energies increase. The large increase in the energy of the nπ* states hints at an additional effect: the formation of a hydrogen bond between the N atom of the central ring of MB (Figure 2a) and a neighboring water molecule. This interaction stabilizes the nonbonding orbital (Figure 2d) involved in the transitions from the ground to the nπ* states, thereby resulting in an additional blue-shift. When MB is intercalated into the nucleic acid strands, fewer hydrogen bonds are formed than in aqueous solution, because the dye in the binding pocket is shielded from the solvent molecules (see Section S1 in the Supporting Information for more details). The occurrence of fewer hydrogen bonds within the DNA causes a smaller blue-shift of the nπ* states compared to the aqueous solution case.

The resulting smaller $S_1(\pi\pi^*)$–$T_3(n\pi^*)$ energy gap for the MB embedded in DNA with respect to water suggests a more favorable ISC from $S_1(\pi\pi^*)$ to $T_3(n\pi^*)$ in DNA than in aqueous solution. To consolidate this hypothesis one has to investigate structures beyond the Franck–Condon region. Thus, the potential energies and the electronic SOCs along a linearly interpolated path between the $S_1(\pi\pi^*)$ and $T_3(n\pi^*)$ optimized geometries have been calculated. Figure 3 shows that the energy profiles for the dye in vacuo and in DNA are very similar to each other, but drastically different from the curves in water. There is a crossing between the $S_1(\pi\pi^*)$ and $T_3(n\pi^*)$ states, both in vacuo and in DNA, that promotes ISC. However, this crossing disappears in water because of the increase in the energy of the $T_3(n\pi^*)$ state as a result of

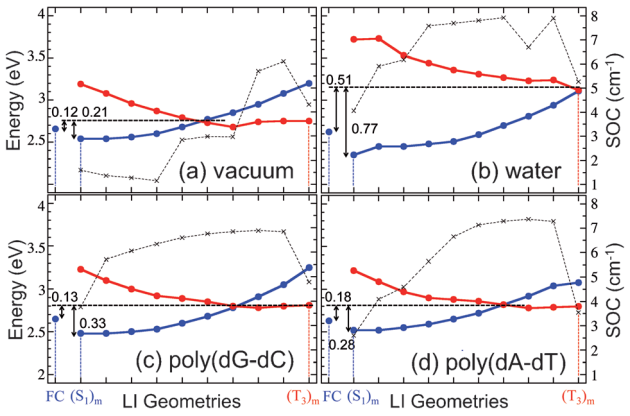

Figure 3. Linearly interpolated (LI) paths between the minimized $S_1(\pi\pi^*)$ geometry, $(S_1)_m$, and the minimized $T_3(n\pi^*)$ geometry, $(T_3)_m$. The vertical lines correspond to the geometries of the Franck–Condon (FC) point, the minimum of the S_1 state ($(S_1)_m$) and the minimum of the T_3 state ($(T_3)_m$), respectively. The energies of $S_1(\pi\pi^*)$ and $T_3(n\pi^*)$ are represented by blue and red lines, respectively. The electronic SOC along the LI paths is represented by the black line. The horizontal dotted line represents the energy at the crossing between the S_1 and T_3 states. Numbers inside the plots are the energy barriers in eV from the $S_1(\pi\pi^*)$ state in the Franck–Condon region and at the minimum of the potential well. The results for the photosensitizer in vacuo, in aqueous media, in poly(dG-dC), and in poly(dA-dT) are displayed in panels (a), (b), (c), and (d), respectively.

hydrogen bonding with a water molecule. These results confirm that the intercalative pocket of DNA creates a microenvironment similar to the vacuum protecting the dye from the influence of the external solvent. A similar dye protection by binding to DNA was also observed for 4′,6-diamidino-2-phenylindole in a recent theoretical study.[20] The electronic SOCs along the linearly interpolated paths shown in Figure 3 are not very large (1–8 cm^{-1}), but they can trigger ISC in vacuo and in nucleic acid environments when $S_1(\pi\pi^*)$ and $T_3(n\pi^*)$ are energetically close to each other. SOC values between 0.1 and 5.0 cm^{-1} are considered large enough to induce ISC on a nanosecond time scale.[21]

After photoexcitation to the bright state $S_1(\pi\pi^*)$, some of the dye molecules relax to the vibrational ground state of the $S_1(\pi\pi^*)$ by dissipating their vibrational energy to the environment, while others have enough vibrational energy to reach the barrier observed at the $S_1(\pi\pi^*)$–$T_3(n\pi^*)$ crossing, thus making ISC favorable. The barrier heights from both the bottom of the $S_1(\pi\pi^*)$ potential well and the Franck–Condon point are indicated in Figure 3. In aqueous solution, the energy barriers from the $S_1(\pi\pi^*)$ minimum and from the Franck–Condon region are large (0.77 and 0.51 eV, respectively). These barriers are significantly lower in vacuo and within the intercalative pocket of DNA, with values ranging from approximately 0.15 eV (from the Franck–Condon region) to 0.30 eV (from the $S_1(\pi\pi^*)$ minimum). This makes one assume that ISC from $S_1(\pi\pi^*)$ to $T_3(n\pi^*)$ is energetically feasible in vacuo and in DNA, while being virtually impossible in water solution. However, the $O_2(^1\Delta_g)$ generation quantum yield for MB in water is rather high (0.52).[13] This apparent contradiction is resolved by realizing that the interaction between the electronic and vibrational motions (vibronic SOC) for states of the same character can also contribute to ISC.

A recent theoretical publication[22] concluded that the generation of the triplet states of the closely related thionine dye in aqueous solution is exclusively due to the vibronic SOC between the $S_1(\pi\pi^*)$ and $T_2(\pi\pi^*)$ states, especially for geometry vibrations along out-of-plane motions. Considering the structural similarity between MB and thionine, it is very likely that the large ISC observed experimentally for MB in water[13] is also promoted by the vibronic coupling between the $S_1(\pi\pi^*)$ and $T_2(\pi\pi^*)$ states, as represented in Figure 4. The similar vertical energies of $S_1(\pi\pi^*)$ (2.09 eV) and $T_2(\pi\pi^*)$ (2.02 eV) in aqueous media (Figure 2c) support this hypothesis. This effect should also be present when MB is intercalated in DNA, since our molecular dynamics simulations[17] show out-of-plane vibrations and that the $S_1(\pi\pi^*)$ and $T_2(\pi\pi^*)$ states are energetically very close to each other (Figure 2c).

The results obtained indicate that ISC is enhanced when the dye is intercalated in DNA because both pure electronic SOC between $S_1(\pi\pi^*)$ and $T_3(n\pi^*)$ and vibronic SOC between $S_1(\pi\pi^*)$ and $T_2(\pi\pi^*)$ contribute to the generation of the triplet states (Figure 4). However, this conclusion contradicts laser flash spectroscopic experiments, which have shown that the triplet generation quantum yield of MB decreases upon addition of different amounts of poly(dG-dC).[23] The result of the experiment is explained by the quenching of the $S_1(\pi\pi^*)$ state of the dye by electron transfer from the guanine residues.[23] Since the reduction potential of MB in the $S_1(\pi\pi^*)$ state (1.89 V) is larger than that of guanine (1.53 V), the reduction of the singlet excited state of the dye is favorable.

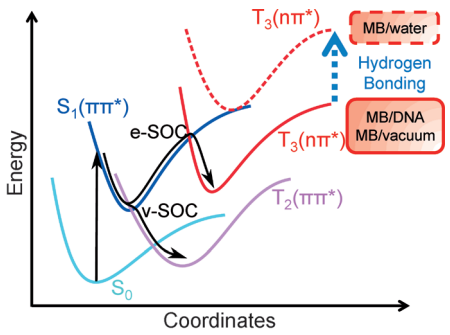

Figure 4. Mechanism for the generation of triplet methylene blue (MB) in vacuo, in water, and in DNA. In vacuo, as well as in a nucleic acid environment, MB can undergo ISC from the bright state $S_1(\pi\pi^*)$ to the triplet states $T_2(\pi\pi^*)$ and $T_3(n\pi^*)$ induced by vibronic and pure electronic spin–orbit coupling (v-SOC and e-SOC), respectively. However, when MB is in aqueous solution, the $T_3(n\pi^*)$ state is destabilized by hydrogen-bond formation and only the vibronic route is accessible.

Therefore, to increase the triplet generation quantum yield upon binding to DNA (and thus the efficacy of PDT), we suggest, based on our electronic structure analysis, to functionalize MB so that its reduction potential decreases and thus electron transfer from the nucleobases is blocked, for example by placing electron-donating substituents onto the rings. Two important factors should be taken into account when functionalizing MB. First, the chemical groups employed should not increase the energy gap between $S_1(\pi\pi^*)$ and the triplet states $T_2(\pi\pi^*)$ and $T_3(n\pi^*)$. Second, the functional groups should not be bulky because the intercalative binding mode is favorable only for small and planar drugs. Larger molecules prefer to bind into the minor and the major groves of DNA. In the latter case, hydrogen bonding between the nucleobases and the functionalized MB could occur, thereby leading to an increase in the energy gap between $S_1(\pi\pi^*)$ and $T_3(n\pi^*)$, as observed in aqueous media.

In conclusion, our QM/MM analysis shows that the ISC mechanism is different in water and in DNA. In aqueous solution, electronic SOC is quenched due to formation of hydrogen bonds with the surrounding water molecules and ISC is only mediated by vibronic SOC. In DNA, the dye is shielded from the solvent, thereby leaving both the electronic and vibronic SOC mechanisms operative, and thus enhancing ISC. Experimentally,[23] this effect is not seen due to the quenching of the $S_1(\pi\pi^*)$ state by electron transfer from the nucleobases to the dye. Thus, we suggest that the PDT efficacy of MB can be significantly improved by structurally functionalizing the dye with small electron-donating substituents, so that electron transfer is blocked.

Keywords: hydrogen bonds · intersystem crossing · photochemistry · photodynamic therapy · QM/MM

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