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Photophysical properties of 5-methylcytidine

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Cytosine methylation, which determines the hot spots for DNA photo-damage, is shown to induce a red-shift of the nucleoside absorption spectrum, making the chromophore more vulnerable to solar radiation, and a tenfold increase of the fluorescence lifetime, making excited state reactions more probable. A femtosecond investigation of the excited state deactivation reveals a quite complex mechanism.

Introduction

DNA methylation and demethylation, occurring on position 5 of cytosine bases, are natural processes which play a key role in biological functions such as regulation of gene expression and genome reprogramming.^{1,2} Moreover, cytosine methylation determines the hotspots of DNA damage³ which can be induced by sunlight.⁴

Despite the fact that 5-methylcytosine is a preferential target for carcinogenic mutation caused by UV radiation, the underlying photophysical and photochemical mechanisms are not understood. A few publications report the characterisation of the reaction products obtained upon exposure to the UV light of aqueous solutions in which 5-methylcytosine was present as a nucleobase⁵ or incorporated in dinucleosides and polynucleotides.⁶⁻⁸ But, so far, the properties of the excited states and in particular their relaxation dynamics remain unknown. Regarding the first singlet excited state, the lack of such information is due to its very short lifetime. Only very recently has it been possible to study the excited state dynamics of the four common DNA bases, nucleosides and nucleotides by absorption^{9,10} and fluorescence¹¹⁻¹⁴ spectroscopy with femtosecond resolution.

As a continuation of our work on the photophysical properties of DNA components,¹²⁻¹⁶ we have undertaken a study of 5-methyl-2'-deoxycytidine (5m-dC) (Fig. 1) using steady-state absorption and fluorescence spectroscopy as well as time-resolved fluorescence spectroscopy with femtosecond resolution based on the upconversion technique. We report here its behaviour in comparison with that of the non-methylated analogue (dC) studied previously.¹⁴

Experimental

5-Methylcytidine was purchased from Sigma Aldrich and used without further purification. It was dissolved in ultrapure water produced by a MILLIPORE (Milli-Q Synthesis) purification system.

Steady-state absorption and fluorescence spectra were recorded using a Perkin Lambda 900 spectrophotometer and a SPEX Fluorolog-2 spectrofluorometer, respectively. Fluorescence quantum yields (ϕ) were determined for 10^{-5} M solutions using quinine sulfate dihydrate in 0.1 M HClO₄ ($\phi = 0.59$).¹⁷

Time-resolved fluorescence measurements were performed by the upconversion set-up described in detail elsewhere.^{12,13} The third harmonic of a mode-locked Ti:sapphire laser

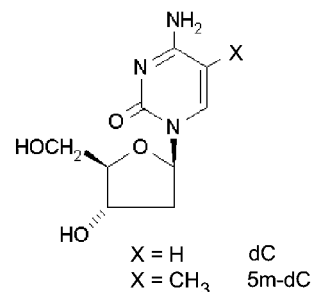


Fig. 1 DNA nucleosides.

(267 nm) was used for excitation and the spectral resolution at the detection wavelength was set to 8 nm. Parallel (I_{par}) and perpendicular (I_{perp}) excitation/detection configurations were realized by controlling the polarization of the exciting beam with a half-wave plate. The fluorescence decays presented here were calculated as $I_{\text{par}} + 2I_{\text{perp}}$. The apparatus function was 420 fs (fwhm). Temporal scans were made in a 30 ps time-window with 100 fs steps. The zero time was defined at the half maximum of the signal rise. Spectra were recorded directly by simultaneously changing the monochromator wavelengths, rotating the upconversion crystal, and changing the delay line position in order to compensate for group velocity dispersion.¹⁸ Subsequently, they were corrected for the spectral response of the detection system.

Solutions (2×10^{-3} M) were kept flowing in a 0.4 mm quartz cell, which also was kept in continuous motion perpendicular to the excitation beam. Successive recordings with the same solution gave reproducible signals, showing that photo-degradation does not affect the measurements.

All measurements were performed at room temperature (20 ± 1 °C) under aerated conditions.

Results

The absorption spectrum of 5m-dC in aqueous solution peaks at 278 nm (Fig. 2). It is somewhat less intense and shifted to longer wavelengths in comparison with the spectrum of dC. The shift, determined at the absorption maximum, is 1000 cm^{-1} .

The fluorescence spectra recorded for 5m-dC upon excitation at wavelengths ranging from 250 to 300 nm are all identical and peak at 342 nm (Fig. 3). They are also red-shifted with respect to the dC spectra (by 1200 cm^{-1} at the emission maxima). Neither the absorption nor the fluorescence spectra show any concentration dependence in the range $10^{-5} - 2 \times 10^{-3}$ M, except reabsorption of the emitted photons below 305 nm.

The fluorescence quantum yield of 5m-dC was found to be $(5.6 \pm 0.1) \times 10^{-4}$. This value is about six times higher than that determined by us for dC (0.89×10^{-4}).¹⁴

All the fluorescence decays recorded for 5m-dC aqueous solutions at various wavelengths upon excitation at 267 nm are

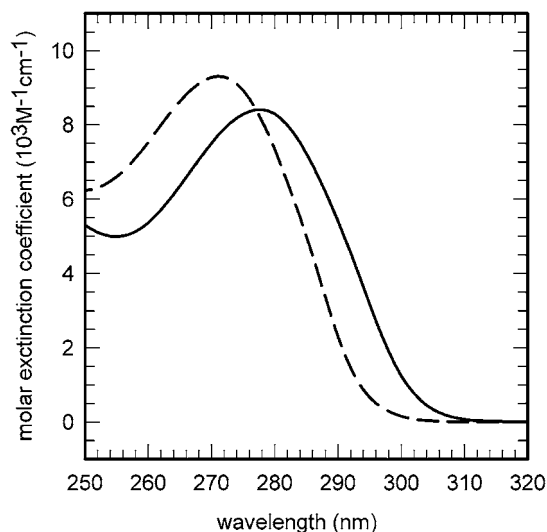


Fig. 2 Absorption spectra of 5m-dC (solid line) and dC (dashed line) in aqueous solution.

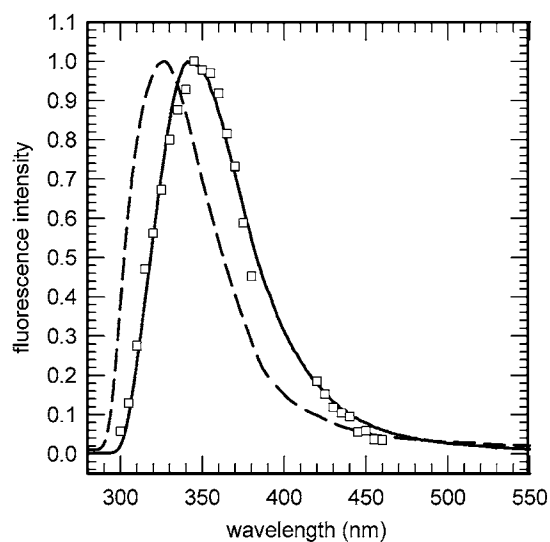


Fig. 3 Fluorescence spectra of 5m-dC (solid line) and dC (dashed line) in aqueous solution; $\lambda_{\text{ex}} = 255$ nm. The circles correspond to the total fluorescence recorded by the upconversion technique.

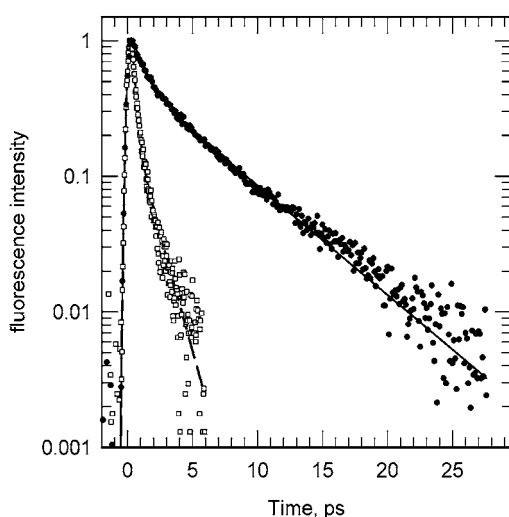


Fig. 4 Fluorescence decays recorded at 330 nm for 5m-dC (filled circles) and dC (squares) fitted by bi-exponential functions.

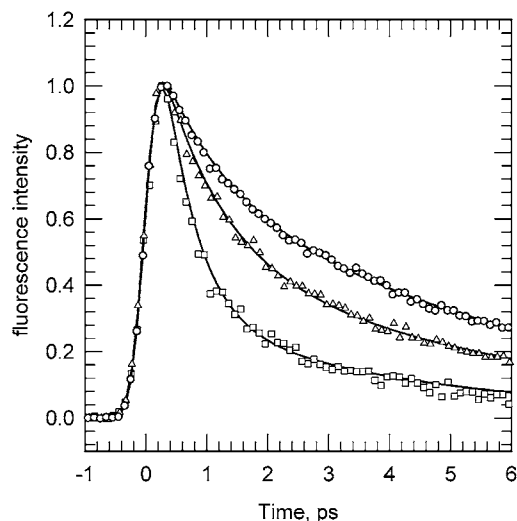


Fig. 5 Fluorescence decays recorded for 5m-dC aqueous solutions at 310 nm (squares), 330 nm (triangles) and 370 nm (circles) upon excitation at 267 nm.

Table 1 Characteristic times/ps of the 5-methyl-2'-deoxycytidine fluorescence decays resulting from fitting to bi-exponential functions $a \exp(-t/\tau_1) + (1 - a) \exp(-t/\tau_2)$

λ/nm	a	τ_1/ps	τ_2/ps	$\langle \tau \rangle/\text{ps}^a$
310	0.82 ± 0.01	0.56 ± 0.08	4.3 ± 0.4	1.2
330	0.6 ± 0.01	1.0 ± 0.1	5.4 ± 0.3	2.8
350	0.46 ± 0.01	1.1 ± 0.2	5.7 ± 0.3	3.6
370	0.38 ± 0.01	0.9 ± 0.1	5.7 ± 0.1	3.9
380	0.39 ± 0.01	1.3 ± 0.2	6.1 ± 0.3	4.2
420	0.36 ± 0.04	0.9 ± 1.1	5.8 ± 0.9	4.0

$$^a \langle \tau \rangle = a\tau_1 + (1 - a)\tau_2.$$

much slower than the dC ones. For example, it can be seen in Fig. 4 that 99% of the signal recorded for dC at 330 nm has disappeared at 5 ps, whereas for 5m-dC this happens only at 25 ps. In contrast to dC, a clear wavelength dependence can be observed for 5m-dC. Upon increasing the wavelength from 310 nm to 350 nm, the decays become drastically slower and slower (Fig. 5). Above 350 nm, the behaviour is still present although much less pronounced. The apparent rise of the signal observed in Fig. 5 is identical for all wavelengths and is due only to the instrumental response function.

The decays were fitted with bi-exponential functions $a \exp(-t/\tau_1) + (1 - a) \exp(-t/\tau_2)$ from which average decay times $\langle \tau \rangle = a\tau_1 + (1 - a)\tau_2$ were determined. The results of the fits are shown in Table 1. The τ_1 values vary from 0.6 to 1.3 ps and the τ_2 values from 4.3 to 6.1 ps. The average decay time is only 1.2 ps at 310 nm and reaches a rather constant value of 3.9 ± 0.3 ps at longer wavelengths. This is more than one order of magnitude higher than the average decay time found for dC (0.3 ps).¹⁴ It should be noticed that the relatively high uncertainties found for the values at 420 nm are due to the weak signal intensity at this wavelength.

The integrated fluorescence spectrum of 5m-dC recorded with the upconversion set-up compares quite well with the corresponding steady-state spectrum (Fig. 3). This means that the fluorescence decays do not contain any important long lived component having a different spectrum than that detected with the upconversion technique. During the first few picoseconds the time-resolved fluorescence spectra decrease in intensity, shift to the red and become narrower. This is illustrated in Fig. 6 where the spectra recorded at 200 fs, corresponding to the emission maximum, and at 5 ps are shown together. The spectrum at 5 ps is shifted to lower energies by 400 cm^{-1} and its width (fwhm) is reduced by 1300 cm^{-1} , compared to the spectrum at 200 fs.

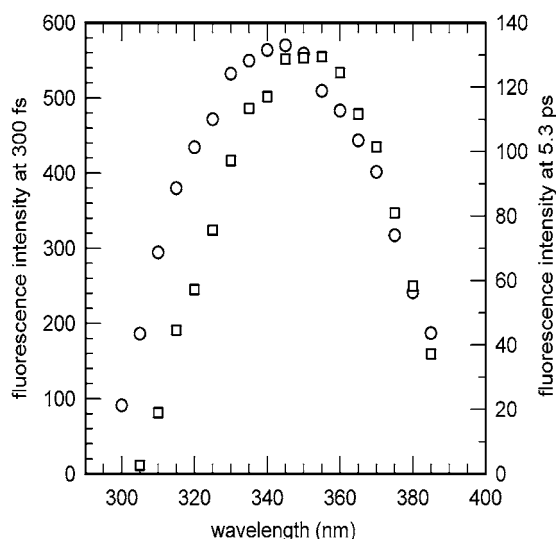


Fig. 6 Time-resolved fluorescence spectra recorded for 5m-dC aqueous solutions at 200 fs (circles) and 5 ps (squares).

Discussion

A red-shift of the absorption and fluorescence spectra, as well as an increase in the fluorescence quantum yield and the fluorescence lifetime found for 5m-dC as compared with dC, has also been observed upon methylation of other DNA components. For example, the fluorescence quantum yield of 7-methylguanosine is two orders of magnitude higher than that of guanosine.^{14,19} The important point here is that 5m-dC, which is naturally present in the human genome, can absorb more photons than the four common DNA nucleosides in the UVB region of the solar spectrum and the longer lifetime of its first excited state increases the probability for the photoreactions to occur.

Regarding singlet excited state relaxation, we have reported previously that the fluorescence decays of all DNA nucleosides in aqueous solution cannot be described by single exponentials.¹⁴ However, in contrast to 5m-dC, no clear wavelength dependence in the fluorescence decays could be detected for adenosine, cytidine, guanosine and thymidine. It should be kept in mind that the very short lifetimes, compared to the apparatus function, of these compounds makes it difficult to characterise the spectral dynamics.

The specific behaviour of 5m-dC may have various origins. The simplest one is that the ground state is a mixture of tautomers with different fluorescence spectra and fluorescence lifetimes. However, this hypothesis can easily be ruled out because the shape of the steady state fluorescence spectrum does not depend on the excitation wavelength. Thus, the observed inhomogeneity is likely to arise from the multiple processes involved in excited state relaxation. Emission from hot vibrational levels could be responsible for the relatively faster decay at the blue side of the fluorescence spectrum. Such a process should result in a spectral narrowing as a function of time which is in agreement with the experimental data (Fig. 6). Another effect which may interfere is solvent rearrangement causing a red shift of the fluorescence spectrum, which is also observed for 5m-dC (Fig. 6). A recent investigation carried out for aqueous solutions of 2-aminopurine, a modified DNA base, showed that solvation takes place at the time-scale probed in our experiments.²⁰ Finally, we cannot exclude complex deactivation mechanisms involving several electronic states or proton/hydrogen exchange between the chromophore and surrounding water molecules. A study of the fluorescence dynamics of 5m-dC as a function of the solvent should help to elucidate the various factors playing a role in excited state relaxation.

Acknowledgements

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