Amplified nitric oxide photorelease in DNA proximity†

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A novel bichromophoric conjugate integrating a NO photodonor and a DNA intercalator in its molecular skeleton, allows the light-controlled NO delivery nearby DNA and amplifies NO release *via* effective photoinduced energy transfer mechanism.

Exciting discoveries in biochemical research have demonstrated that nitric oxide (NO), an ephemeral diatomic molecule, is much more than a mere environmental pollutant. It is well established that NO is a pleiotropic bioregulator of important physiological processes encompassing neurotransmission, vasodilatation and hormone secretion in living bodies.[1] Besides, NO has proven to be an excellent chain breaking antioxidant in the free radical-induced lipid oxidation[2] and an efficient anticancer agent that inhibits key metabolic pathways to block growth or kills cells outright.[3] In this regard, DNA seems to be the main target of NO, which can induce deamination of the nucleobases leading to strand breaks.[4]

This scenario has stimulated fervent research activity devoted to developing NO delivery compounds for potential therapeutic applications.[5] In this perspective, *light* is an appealing external on/off trigger to accurately regulate the NO dosage. This is crucial for biomedical purposes, in view of the opposite effects NO may exert either inhibiting or encouraging the tumor proliferation depending on the dose.[6]

On the basis of the above considerations, the achievement of NO donors exhibiting binding affinity for DNA and, at the same time, generating NO under appropriate light inputs, would allow the NO delivery to be spatially and temporally controlled, offering great advantages for bio-applications.

Prompted by our ongoing interest in developing functional NO photodonors,[7] we pursued this objective by designing and synthesizing the conjugate **1** (Fig. 1). It integrates two chromophoric units in its molecular skeleton: an anthracene moiety, a typical DNA intercalator binder (unit **i**), and a commercial nitroaniline derivative that we have recently discovered to be a suitable NO photodonor under visible light irradiation (unit **p**).[8] Furthermore, it includes a secondary amino group which is protonated at physiological pH. The presence of this cationic charge is expected to improve the water solubility of **1** and to further encourage its DNA binding affinity by coulombic attraction with the opposite charged phosphates backbone. Here, we demonstrate that (1) the NO release from unit **p** of the conjugate can be significantly amplified through a photoinduced energy transfer mechanism when both the chromophores absorb the excitation light and (2) the conjugate effectively binds to double strand DNA and enables NO photodelivery nearby.

Compound **1** was easily synthesized in two steps (see ESI†) and its spectroscopic features are illustrated in Fig. 2. The absorption spectrum (a in Fig. 2A), shows bands that reflect fairly well those of the equimolar mixture (d in Fig. 2A) of the single components (b and c in Fig. 2A), accounting for only a weak interaction between the two chromophoric units in the ground state. On the other hand, the typical fluorescence emission of the anthracene moiety observed for the model compound **2** (b Fig. 2B) is totally quenched in the case of the conjugate **1** (a Fig. 2B), suggesting a remarkable interaction in the excited state.

As far as the possible quenching mechanism is concerned, a thermodynamically favored photoinduced energy transfer from unit **i** (donor) to unit **p** (acceptor) seems to be the most likely. The bichromophoric conjugate presents two indispensable pre-requisites for this energy transfer to occur, that are: (1) the lowest excited singlet state of **i** is *ca.* 0.5 eV higher than that of **p**,[9] (2) the emission spectrum of **i** considerably overlaps the absorption of **p**.[10] This hypothesis is well-supported by the NO photorelease experiments reported below, which rules out feasible photoinduced electron transfers involving the two chromophoric units of the conjugate as responsible for the fluorescence quenching.[11]

The most convenient methodology to demonstrate the NO generation is the direct and in real-time monitoring of this radical species. To this end, we used an ultrasensitive NO electrode, which directly detects NO concentration by an amperometric technique (see ESI†). Fig. 3 shows the results of two experiments carried out by exciting the conjugate **1** with either 420 or 380 nm light, respectively. These wavelengths were chosen such that the excitation light is absorbed

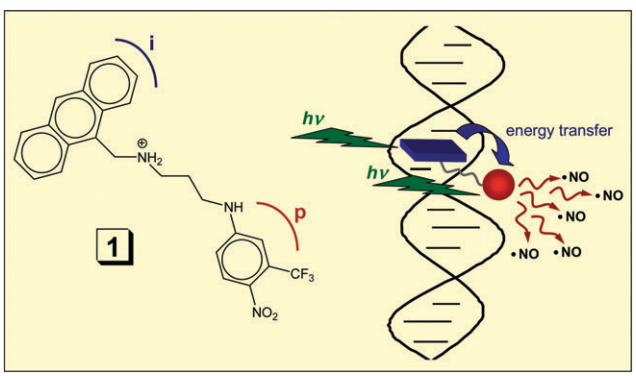

Fig. 1 The molecular structure of **1** (protonated form) and its working principle.

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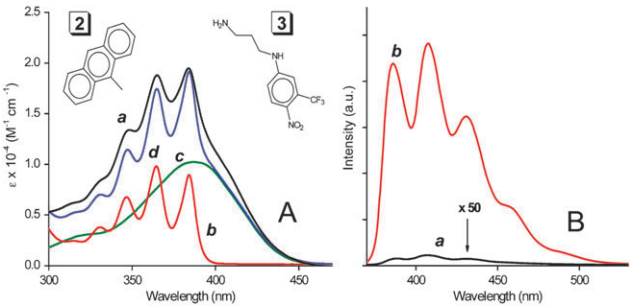

Fig. 2 (A) Absorption spectrum of **1** (a), the model compounds **2** (b) and **3** (c) and their equimolar mixture **2** + **3** (d). (B) Fluorescence spectra (λ_{exc} = 365 nm) of **1** (a) and the model compound **2** (b). T = 25 °C, phosphate buffer (pH 7.4, 1 mM): MeOH 2 : 1. The fluorescence intensities have been corrected for the exact fraction of absorbed photons by the anthracene moieties at the excitation wavelength.

selectively by the NO photodonor unit **p** in the former case and by both the chromophoric units, **p** and **i**, in comparable amounts, in the latter (see sketch in Fig. 3 and absorption spectra in Fig. 2, for sake of clarity). In both experiments we observed linear photogeneration of NO which promptly stopped when the light was turned off and restarted as the illumination was turned on again, obtaining unambiguous evidences for the exclusively light-controlled generation of NO from the conjugate **1**.

Despite the similar qualitative behavior, the rate of the NO released in the two experiments was significantly different, being *ca.* 0.3 nM s^{-1} and *ca.* 0.8 nM s^{-1} at 420 and 380 nm, respectively. Such a difference cannot be ascribed to trivial effects caused by a different number of either *incident* or *absorbed* photon by the photodonor unit **p** at the two wavelengths. In fact, (1) care was taken to ensure the same photon flux in the two experiments and (2) due to the larger extinction coefficient of **1** at 380 nm, the fraction of absorbed photons by the unit **p** at this wavelength (*ca.* 18%) is comparable to that absorbed when the same unit is selectively excited at 420 nm (*ca.* 20%). Furthermore, control experiments carried out by irradiating solutions of the model compound **3** absorbing the same fraction of photons at the two excitation wavelengths (420 nm or 380 nm), did not show any significant difference in

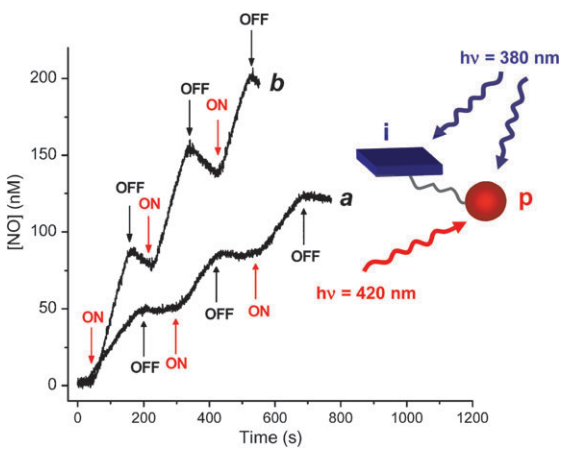

Fig. 3 NO released upon irradiation of **1** (10 μM) at 420 (a) and 380 nm (b). T = 25 °C, phosphate buffer (1 mM, pH 7.4): MeOH 99 : 1.

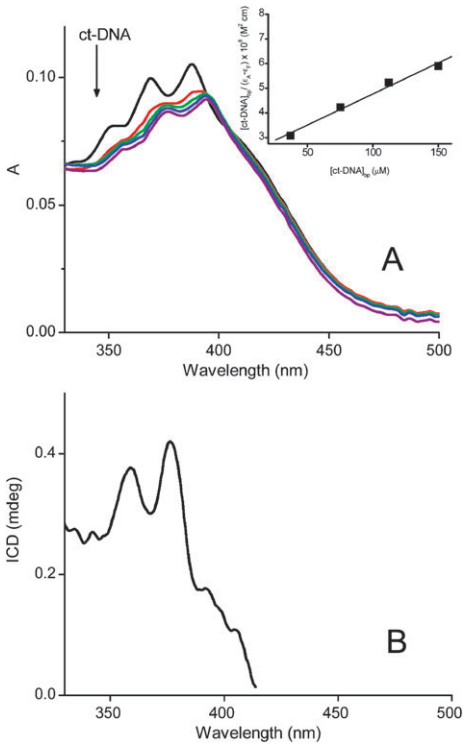

Fig. 4 (A) Absorption spectra of **1** (6 μM) in 1 mM phosphate buffer pH 7.4, in the presence in increasing amounts of ct-DNA in the range 0–150 μM. Cell path: 1 cm. The inset shows the half-reciprocal plot of **1** binding with ct-DNA determined at 388 nm. (B) Induced circular dichroism of the **1**-ct-DNA complex in 1 mM phosphate buffer pH 7.4; $[\text{ct-DNA}]_{bp}$ = 10 mM; [**1**] = 6 μM. Cell path: 1 cm.

the rate of the NO photogenerated, suggesting that the primary photochemical process leading to NO by unit **p** does not depend by the excitation energy in the range explored. Therefore, on the basis of these findings, the higher NO release observed upon 380 light irradiation can be unequivocally interpreted on the basis of an amplification effect due to an effective photoinduced energy transfer from unit **i** to **p**, in very good agreement with the fluorescence quenching results discussed before.

Interaction of compound **1** with double strand DNA was proven by means of absorption and induced circular dichroism (ICD) spectroscopy. Fig. 4A shows that the absorption bands of **1** undergo both hypochromism and red-shift upon addition of increasing amounts of calf-thymus DNA (ct-DNA). Besides, a tight isosbestic point at 404 nm is observed, suggesting homogeneity of the binding process. These spectral changes are very similar to those typically observed for other anthracene derivatives and are accepted as a confirmation of proximity of the polycyclic hydrocarbon to the DNA core.[12] Hypochromism is in fact due to a strong interaction between the electronic states of the intercalated chromophore and those of the DNA bases.[13]

The binding constant, K_b, related to association complex was calculated through the half-reciprocal plot of the absorption spectral changes, according to eqn (1):[14]

$$[\text{ct-DNA}]_{bp}/(\varepsilon_A-\varepsilon_F)=[\text{ct-DNA}]_{bp}/(\varepsilon_B-\varepsilon_F)+1/K_b(\varepsilon_B-\varepsilon_F) \quad (1)$$

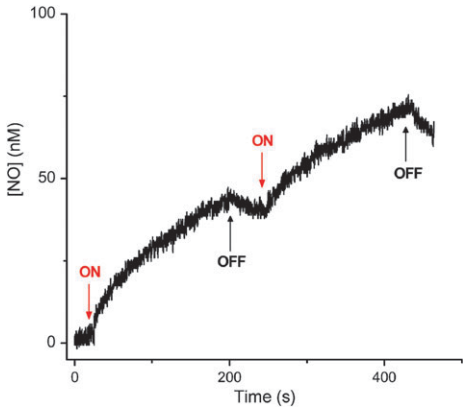

Fig. 5 NO released upon 420 nm light irradiation of the complex **1**-ct-DNA phosphate buffer (1 mM, pH 7.4) at 25 °C. $[\text{ct-DNA}]_{bp}$ = 10 mM; [**1**] = 6 μM.

where $[\text{ct-DNA}]_{bp}$ is the concentration of the polynucleotide in base pairs, ε_A, ε_F and ε_B correspond to $A_{obs}/[\mathbf{1}]$, the extinction coefficient for the free **1** and the extinction coefficient for the totally bound form of **1**, respectively.[14] By the ratio of the slope to intercept of the linear plot reported in the inset of Fig. 4A, a K_b value of $1.1 \pm 0.3 \times 10^4$ M^{-1}, in excellent agreement with those reported for anthracene derivatives including cationic functions,[12] was obtained.

More insights into the binding mode were provided by ICD spectroscopy. Due to the absence of chiral centers, compound **1** is not optically active by itself. As shown in Fig. 4B the presence of ct-DNA induces optical activity as a consequence of its close proximity with the asymmetric environment of the biopolymer helix. In particular, it can be noted that the ICD absorption maxima correspond fairly well to those of the anthracene unit of **1** whereas no relevant ICD signals are noted in the correspondence of the main absorption region of the photodonor unit of **1** (dominant above 380 nm). Since ICD signals are dependent on the cube of the distance of the two interacting partners[15] our findings seem to be consistent with a binding fashion involving the intercalation of unit **i** of the conjugate and the localization of the unit **p** mainly at the DNA periphery, as pictorially sketched in Fig. 1.

The suitability of **1** to generate NO when bound to ct-DNA was demonstrated by NO photorelease experiments carried out in the presence of 10 mM ct-DNA (**1** complexation > 90%). As shown in Fig. 5, NO photogeneration strictly depending on the illumination conditions was observed. A remarkable point of interest of this work is the comparable efficiency of the NO photodelivery in the absence and in the presence of DNA. This excludes potential photoinduced electron transfer between the excited **1** and the DNA bases as competitive pathways to the NO generation.

In summary, we report a photoactivated, multifunctional conjugate that, to our knowledge, represents the first example of an NO donor that effectively bind to DNA and enables generation of this radical species in its close proximity, under the exclusive control of visible light stimuli, with the additional advantage of amplifying the NO delivery *via* an efficient photoinduced energy transfer mechanism.[16] These features, together with the ease of preparation, make the conjugate **1** an appealing candidate to be tested in biological experiments. Studies addressed to evaluate its capability to induce DNA photocleavage are underway. We believe that the extension of the present approach to the preparation of multicharged conjugates integrating more intercalator and photodonor units in their molecular skeleton may hopefully pave the way for the development of novel classes of NO donors for potential applications in biomedical research where NO release with precise spatiotemporal control is required.

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Notes and references

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8 The photochemical mechanism for NO photorelease involves a nitro-to-nitrite rearrangement followed by the rupture of the O–N bond to generate a phenoxyl radical and NO.
9 Estimated by the end of the absorption spectra of the model compounds **2** and **3**.
10 The overlap integral J was numerically calculated to be 4.68×10^{-11} M^{-1} cm^3.
11 A potential photoinduced electron transfer involving the excited anthracene and the adjacent secondary amino group as electron donor is, of course, out of question in light of the protonation of this latter under our experimental conditions ($pK_a > 8$).
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16 An example of NO photodonor incorporating a potential DNA intercalator in its molecular skeleton has been recently reported by Miyata and colleagues.[4c] No investigations demonstrating affinity for DNA and ability to generate NO nearby were reported, however.