Transient photochemistry of naphazoline in a protein environment

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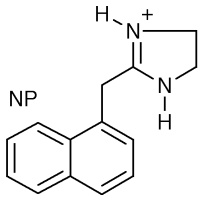
The photoreactivity of naphazoline (NP), 2-(1-naphthylmethyl)imidazoline, in the presence of bovine serum albumin (BSA) is investigated. The protein microenvironment affects markedly the efficiency of the photochemical deactivation pathways of the NP transient intermediates photogenerated upon laser excitation. The triplet state of the drug associates with BSA and its lifetime increases by more than one order of magnitude. Hydrated electrons and nitrogen-centered radicals formed in the NP photolysis react efficiently with protein sites, behaving as sources of reductive and oxidative protein damage, respectively. NP photoinduced protein structural modification is also observed.

The topic of drug photochemistry has always received a considerable amount of attention but recently the interest has markedly increased due to the awareness among the scientific community of an increase in the UV portion of the sun spectrum reaching the earth. The majority of the work done in the last few years concerning the mechanisms of both drug photodegradation and photosensitization has appeared in recent reviews.[1–3]

For an appropriate correlation between the phototoxicity and the photochemical properties of a drug, the ideal approach consists in studying the photochemistry of the photosensitizer in the presence of the biological target. Indeed, parameters such as hydrophobicity, geometry, size, shape and charge lead often to the formation of supramolecular aggregates between photosensitizer and biomolecule. As already reported for a large variety of systems,[4–6] the nature of the lowest excited states, the efficiency of the photophysical and photochemical pathways, the redox potentials, the fate of the reaction intermediates and the opening of new photoreactive channels, are some of the parameters of the sensitizer that can be modulated by its compartmentalization in a biological microenvironment. Laser flash photolysis techniques represent a powerful tool to get much of this information and, as a consequence, to contribute to the understanding of the relationship between drug photochemistry and phototoxicity.

Naphazoline (NP), 2-(1-naphthylmethyl)imidazoline, is a drug belonging to the vaso-regulator class, present in the market as eye drops. It is widely used to relieve redness due to minor eye irritation caused by cold, dust, wind, smog, pollen, swimming, or wearing contact lenses. Due to the protonation of the imidazoline ring (pK_a *ca.* 10), NP is present in its cationic form at physiological pH.

Despite some reports in the literature concerning the *in vivo* toxicity of NP,[7–9] only recently has the *in vitro* phototoxic effect of NP been pointed out through its ability to promote DNA cleavage upon light irradiation.[10] This is not suprising given the presence of the naphthalene chromophore. Indeed, other drugs bearing this moiety have been extensively reported as being phototoxic both *in vivo* and *in vitro*.[11–16]



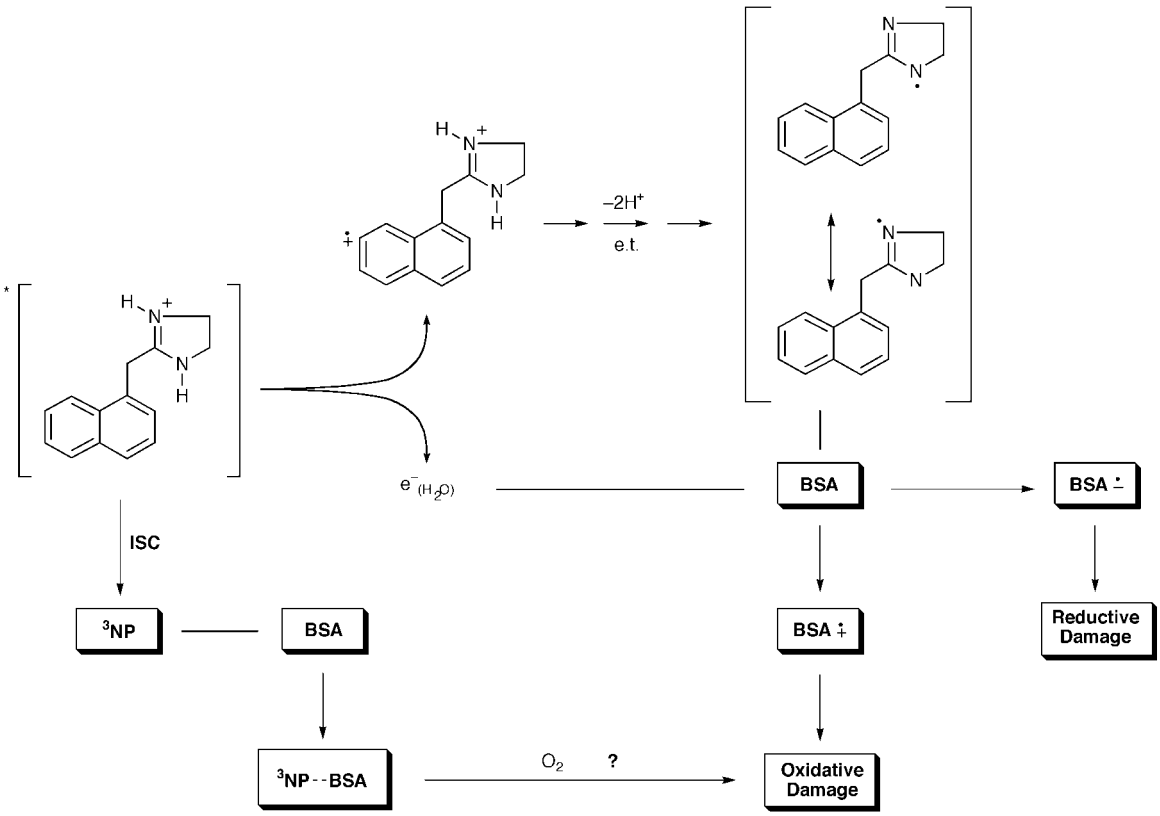
A detailed study concerning the transient photochemistry of NP[17] has shown that the photoreactivity of the drug is characterized by a photoionization process occurring through a mixture of mono- and biphotonic pathways. An intramolecular electron transfer involving both the imidazoline ring and the naphthalene radical cation, followed by two deprotonation steps, lead to the formation of nitrogen-centered radicals (see Scheme 1). Photosensitization of singlet oxygen from the lowest excited triplet state of NP was also observed. These results demonstrated the potential for NP to act both as a Type I and Type II photosensitizer.

In the present work, we have examined the effect of a protein (bovine serum albumin) environment on the transient species generated upon NP laser excitation in order to get direct evidence of the role played by these intermediates in their reaction with the biosubstrate. Some preliminary photosensitization experiments by monitoring NP-photoinduced protein structural modification through HPLC analysis have been also performed to support the laser flash photolysis data.

Experimental

Naphazoline hydrochloride (molecular weight 246.7) and bovine serum albumin were purchased from Sigma Chemical Company (St. Louis MO, USA). Water was purified through a Millipore Milli-Q system. Phosphate buffer 10^{-2} M, prepared from reagent grade products, was used in the experiments at pH 7.4.

All the transient spectra and kinetics were recorded by employing a flow system with a 7×7 mm^2 Suprasil quartz cell with a 2 mL capacity, and were purged in a storage tank with N_2 for 30 min before as well as during the acquisition. The samples were excited with a Lumonics EX-530 laser with Xe–HCl–Ne mixtures generating pulses at 308 nm of ~ 6 ns and $\leqslant 60$ mJ per pulse. Care was taken to renew the solution after each laser shot. The signals from the monochromator/photomultiplier system were initially captured by a Tektronix



Scheme 1

2440 digitizer and transferred to a PowerMacintosh computer that controlled the experiment with software developed in the LabView 3.1.1 environment from National Instruments. Further details have been previously described.[18,19]

In the photosensitization experiments, the irradiation was performed using a Rayonet photochemical reactor equipped with 16 'black light' phosphor lamps with an emission in the 310–390 nm range with a maximum at 350 nm. The flux at the irradiation position was about 800 μW cm^{-2}. The incident photon flux on a 3 mL solution in the quartz cuvettes (10 mm optical path) was 3×10^{16} quanta s^{-1}. A "merry-go-round" irradiation apparatus was used to ensure that all the samples received equal radiation. The experimental irradiation procedures and the light intensity measurements have been described previously.[20]

High performance liquid chromatography (HPLC) analysis was performed on a Hewlett Packard 1100 chromatograph equipped with an on-line photodiode array detector (DAD). The UV traces were monitored at 230 nm. The quantitative separation of the three forms of BSA was achieved on a SEC column by eluting with 0.05 M phosphate buffer (pH 7) in the presence of 0.13 M NaCl and 0.02 M KCl.

Results and discussion

Fig. 1 shows the transient spectra recorded in a nitrogen saturated solution containing NP in the presence of BSA (1 mg mL^{-1}) upon 308 nm laser excitation at different delay times with respect to the laser pulse. The spectrum taken 0.1 μs after the laser pulse shows a main absorption band characterized by two peaks centered at 390 and 410 nm, a shoulder around 330 nm and a broad absorption band extending beyond 700 nm. These spectroscopic features were very similar to those observed in the absence of protein,[17] suggesting that no new NP transient species are generated in the BSA environment during the initial light excitation, besides those observed in homogeneous aqueous medium. In light of this, we can safely assign the broad absorption extending beyond 700 nm to the hydrated electrons formed after NP photoionization, the peak at 410 nm to the triplet state of NP and the 330 nm band to the nitrogen-centered radicals.[17]

While the spectroscopic behavior may suggest a lack of BSA effect on the photochemistry of the drug, the kinetic analysis performed at the main wavelengths indicated some remarkable differences compared to what is observed in aqueous medium.

Fig. 2 shows the decay of the NP triplet monitored at 410 nm in the presence of 1 mg mL^{-1} of BSA. It can be noticed that the kinetic trace obtained in the presence of BSA shows a clear biexponential trend fitted well by the relation:

$$\Delta A(t) = a_1 \exp(-k_1 t) + a_2 \exp(-k_2 t) + a_3$$

with rate constants $k_1 = 1 \times 10^5$ s^{-1}, $k_2 = 6 \times 10^3$ s^{-1} and normalized pre-exponential factors $a_1 = 0.85$, $a_2 = 0.15$. This behavior reflects the fact that two different populations of

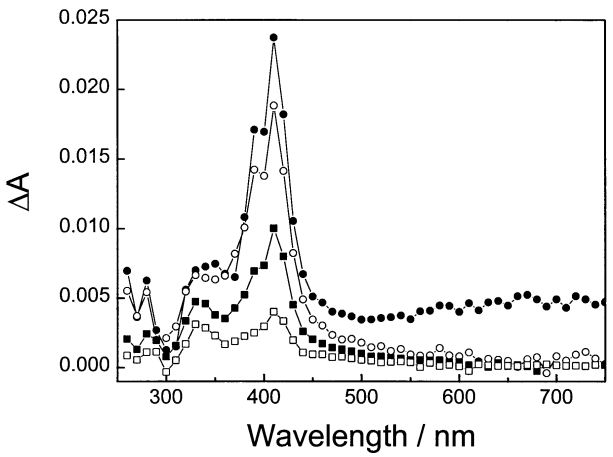


Fig. 1 Transient absorption spectra observed in a 4×10^{-4} M NP N_2 saturated solution in 10^{-2} M phosphate buffer at pH 7.4 upon 308 nm laser excitation in the presence of 1 mg mL^{-1} of BSA: (●) 0.1, (○) 1, (■) 8 and (□) 17 μs after the pulse.

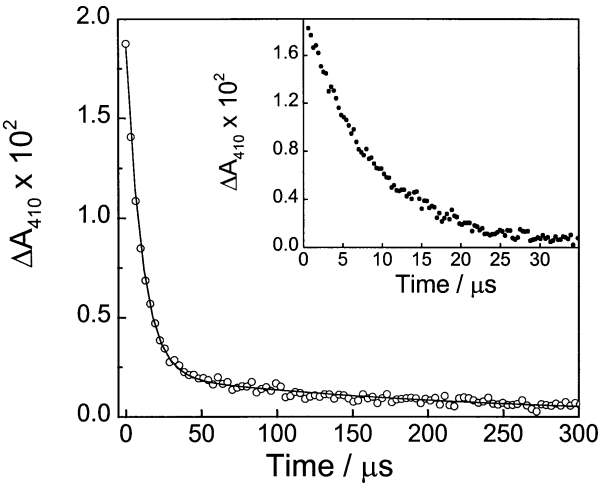


Fig. 2 Time profile and related fitting of the absorbance changes of the NP triplet decay monitored at 410 nm after 308 nm laser excitation of a 4×10^{-4} M NP nitrogen saturated solution in the presence of 1 mg mL^{-1} of BSA in 10^{-2} M phosphate buffer, pH 7.4. Inset: NP triplet decay monitored at 410 nm related to an optically matched solution in the absence of BSA.

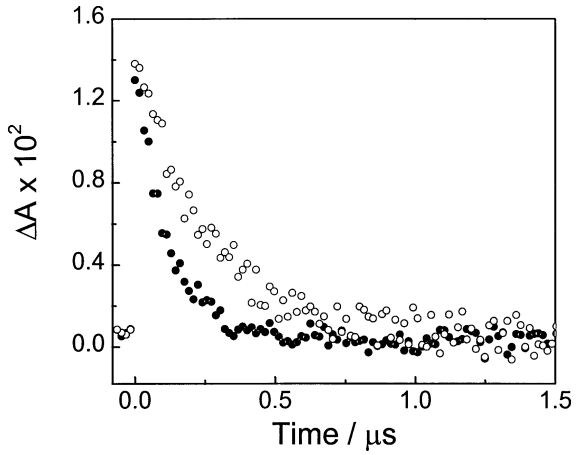


Fig. 3 Time profile of the absorbance changes related to the hydrated electrons monitored at 720 nm after 308 nm laser excitation of a 4×10^{-4} M NP N_2 saturated solution (○) in the absence and (●) in the presence of 1 mg mL^{-1} of BSA in 10^{-2} M phosphate buffer, pH 7.4.

triplets are monitored in the presence of protein. Several reasons allow us to argue this hypothesis. The faster component is the same as that observed in the absence of BSA for an optically matched solution of NP (inset, Fig. 2). This result suggests that in these experimental conditions, 85% of the NP triplet does not interact significantly with the protein, thus ruling out potential energy or electron transfer processes involving BSA residues (*i.e.* tryptophan, cysteine, tyrosine). In contrast, the long-lived component accounts for a strong interaction of 15% NP triplet with the protein. We think that the presence of this second population of triplets can not be totally attributed to a decrease of the triplet self-quenching bimolecular rate constant due to localization of the naphthalene chromophore in deep regions of the microenvironment. Indeed, the value of $k_2 = 6 \times 10^3$ s^{-1} was even smaller than the low value found for the NP triplet decay in aqueous medium (2×10^4 s^{-1}).[17] We believe that the observed lifetime could reflect an intrinsic new property of the NP triplet associated to BSA. This hypothesis is in good agreement with literature data, which report good binding affinities of drugs bearing the naphthalene-like chromophore with BSA.[16,21,22] In particular, in the case of nabumetone[16] we found a long-lived triplet component characterized by a rate constant and a pre-exponential factor very similar to those observed for NP. Unfortunately, we could not determine the value of the association constant for either ground or excited states of NP with BSA. In fact, protein concentrations higher than 1 mg mL^{-1} lead to direct absorption of the excitation wavelength (308 nm) by BSA with consequent photogeneration of new transient species, which interferes with the analysis of the data. Even though the amount of NP triplet bound to BSA is not very high under these experimental conditions, its long lifetime confers great kinetic significance. The fraction of this triplet quenched by oxygen can indeed be significantly large and lead to formation of singlet oxygen near protein sites.

Fig. 3 shows the kinetic traces, monitored at 720 nm, related to the hydrated electrons both in the absence and in the presence of 1 mg mL^{-1} of BSA. Note that the decay is faster in the presence of protein, suggesting that a new deactivation channel of this transient species is opened. Our previous study[17] showed that hydrated electrons are trapped by the NP ground state with a bimolecular rate constant $k_3 = 9 \times 10^9$ M^{-1} s^{-1}. This high value is consistent with an efficient reaction of the hydrated electrons with the positively charged imidazoline ring of NP. We do not think that the presence of BSA can be responsible for an increase of this quenching constant. Indeed, due to either compartmentalization of the drug in hydrophobic sites of the protein or electrostatic binding involving NP with negatively charged sites of BSA, a decrease of this value would have been expected. More likely, the behavior observed accounts for a direct participation of the protein in the electron trapping process. Among the several potential protein sites that are candidates for the scavenging of the photoejected electrons, the carbonyl group of the peptide chain appears likely. Trapping of hydrated electrons by the carbonyl moiety is in fact a common process, and it is expected to occur with very high efficiency, leading to the formation of radical anions.[16,23] In our case, electron trapping by carbonyls in peptide chains would lead to the formation of radical anions, which are expected to be transparent in the wavelength range monitored. This hypothesis is in accord with the spectral changes observed. Indeed, as showed in Fig.1, no new bands form concurrently with the decay of the solvated electrons.

As mentioned earlier, the transient absorption at 330 nm was attributed to nitrogen-centered radicals formed by an efficient intramolecular electron transfer involving both the imidazoline ring and the naphthalene radical cation followed by two steps of deprotonation.[17] Fig. 4 shows the decay traces monitored at 330 nm in the presence and, for comparison, in

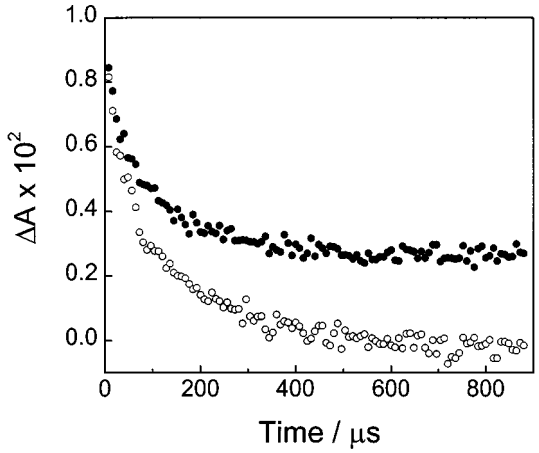


Fig. 4 Time profile of the absorbance changes related to the nitrogen-centered radicals monitored at 330 nm after 308 nm laser excitation of a 4×10^{-4} M NP N_2 saturated solution (○) in the absence and (●) in the presence of 1 mg mL^{-1} of BSA in 10^{-2} M phosphate buffer, pH 7.4.

the absence of 1 mg mL^{-1} of BSA. As was assessed in ref. 17, the decay kinetics at 330 nm in aqueous solution appears due to two mono-exponential components with rate constants of $k_4 = 1 \times 10^5\ s^{-1}$ and $k_5 = 4 \times 10^3\ s^{-1}$. The faster component was due to the triplet state of NP, which still absorbs at this wavelength, whereas the nitrogen-centered radicals were responsible for the slower decay. As observed in Fig. 4, the triplet decay in the presence of BSA is followed by a very long-lived transient. We believe that this species can be triggered by an electron transfer reaction involving the nitrogen-centered radicals and protein amino acid residues. Thermodynamic arguments can be advanced in support of this hypothesis. As already reported[17] the nitrogen-centered radicals are characterized by a very high oxidizing character. Taking into account that $E_{N\cdot/N^-}$ for this species is expected to be higher than 1 V, whereas for tryptophane, tyrosine and cysteine the reduction potential $E_{R\cdot+/R}$ is 1.01, 0.93 and 0.63 V, respectively,[24] the thermodynamic balance

$$E_{N\cdot/N^-} - E_{R\cdot+/R} > 0$$

accounts for the energetically favorable quenching of the 330 nm transient, *via* an electron transfer mechanism.

Despite the fact that the products of the quenching are predicted to be other long-lived oxidized heterocyclic species, probably also absorbing around 330 nm, we cannot rule out that follow-up reactions can be responsible for the persistence of the 330 nm absorption beyond the microsecond time scale.

The consequence of the reactivity of the NP transient species with BSA sites is a NP photoinduced protein structural modification. Fig. 5 shows the results of preliminary tests of NP photosensitization performed on BSA. It can be noticed that irradiation of the drug in the presence of protein leads to significant changes in the distribution of the fraction of oligomer, dimer and monomer forms of the biosubstrate, accounting for preferential damage of the quaternary structure of the protein. This result is not surprising in light of the effects of BSA on the photochemical properties of the drug, and confirms that NP represents a potential source for protein photosensitized damage. We are continuing to explore the NP photosensitization of BSA.

In conclusion, this study has provided an example of how the efficiency of photochemical deactivation pathways of a drug can be different in the presence of a biological target compared to those observed in aqueous solution. While the spectral characteristics of NP are not affected by BSA, accommodation of the guest molecules in specific regions near reactive sites of protein, and/or compartmentalization in regions characterized by different polarity, affect markedly the kinetic behavior of the transient species photogenerated upon excitation. In fact, all the NP intermediates interact efficiently with BSA. The triplet state of NP associates with BSA and its lifetime increases remarkably, conferring great kinetic significance. As a consequence, involvement of a Type II mechanism is expected. The other two main transient species generated in the NP photolysis, hydrated electrons and nitrogen-centered radicals, are characterized by a high reactivity with structural elements of protein and are good candidates for the involvement of Type I mechanisms. In particular, owing to their electrochemical properties, the former can be involved in reductive damage whereas the latter may be able to trigger oxidative damage. The overall situation is summarized in Scheme 1.

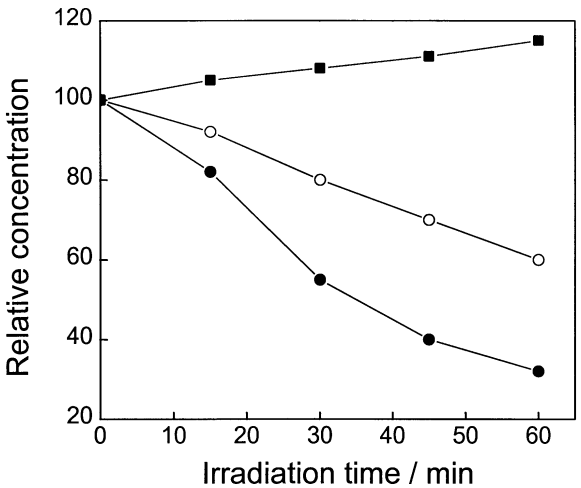


Fig. 5 NP photoinduced changes in the relative distribution of the fraction of (●) oligomer, (○) dimer and (■) monomer forms of BSA as a function of the irradiation time in 10^{-2} M phosphate buffer, pH 7.4 in N_2 saturated solution. [NP] = 1×10^{-3} M; [BSA] = 3 mg mL^{-1}. The composition of BSA at zero irradiation time is: monomer = 80.3%, dimer = 15.2%, oligomer = 4.5%.

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