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Effective control of the intrinsic DNA morphology by photosensitive polyamines

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Non-viral vectors for gene therapy such as DNA-cationic probe complexes offer important bio-safety advantages over viral approaches, due to their reduced pathogenicity, immunogenicity and cytotoxicity. In the present study we examine two polycationic water-soluble azobenzene derivatives (bis-Azo-2N and bis-Azo-3N) containing different linear unsubstituted polyamine moieties and we demonstrate the ability of such photochromes to destabilize the intrinsic B-DNA secondary structure in a concentration-dependent manner. Furthermore, through a detailed series of biophysical experiments, varying the photochrome conformation, temperature, salt and DNA concentration, we provide a detailed insight into the azobenzene-DNA binding pathway (K: bis-Azo-2N(trans)-DNA = 5.3 ± 0.3 × 10⁶ M⁻¹, K: bis-Azo-2N(cis)-DNA = 2.6 ± 0.2 × 10⁶ M⁻¹; K: bis-Azo-3N(trans)-DNA = 7.1 ± 0.4 × 10⁶ M⁻¹ and K: bis-Azo-3N(cis)-DNA = 5.1 ± 0.4 × 10⁶ M⁻¹) establishing the versatility of such materials as promising candidates for use in non-viral gene delivery systems.

Introduction

Gene-based therapy is a pivotal method to treat pathological conditions by altering directly a patient’s genome. It provides a unique strategy to cure not only inherited genetic disorders but also a wide range of inflammatory, infectious and metabolic diseases by delivering therapeutic gene material and its associated regulatory elements into nuclei. It turns out that the development of safe, specific, effective and biocompatible carrier vehicles represents a fundamental engineering challenge to ensure success in clinical trials. Although viral vectors have substantially advanced the field of gene therapy, severe drawbacks, including immunogenicity, carcinogenesis, broad tropism, limited DNA packing capacity and issues in vector production have limited their effective application. Non-viral vectors possess the potential to address and circumvent many of these limitations, particularly with respect to pathogenicity, bio-safety, low cost and ease of production.

Within this framework, chemical vectors, mostly including cationic molecules, have emerged as one of the major non-viral DNA delivery tools and have been widely used to transfet various cell types and deliver cancer vaccines. DNA-cationic molecule complexes involving electrostatic and hydrophobic forces form uniquely compacted structures called cationplexes and the transfection efficiency depends on overall geometric shape, number of positive charges and linker bondage. Moreover, complexation between the vectors and DNA plays a crucial role in determining the efficiency of gene transfection since the nature of the physicochemical and biophysical response of the adducts determines the modes of cellular entry. These features prompted us to investigate whether the concept of innovative and smart carrier vehicles suitable for non-viral gene transfer could be enriched with an additional advantage by application of new photoactive cationic materials. In this framework, polyamines play a pivotal role since their structure is easily amenable to chemical modification through insertion of reactive moieties and their ability to induce DNA condensation to nanoparticles is well-known. Polyamines have been shown to possess reduced toxicity and to facilitate the transport of oligonucleotides in breast cancer cells. Moreover, it is known that the presence of high concentrations of polyamines leads to resolvulization of the complexed DNA in its free form through osmotic stress or charge reversal mechanisms. We have recently revealed the DNA binding mechanism of two mono-substituted azobenzene based-polyamine units and demonstrated that the changes in shape occurring upon photo-isomerization permitted to control, in a reversible way, the intrinsic DNA morphology (B-to-A transition). Along this line, the photoisomerization process of the azobenzene structure was investigated in deep by our group through nonlinear optics (NLO) and quantum mechanics in both solutions and liquid crystalline phases. In this study, we report on the synthesis and characterization of their photoswitchable DNA binding of...
two bis-substituted polycationic, water-soluble azobenzene
derivatives, denoted as bis-Azo-2N and bis-Azo-3N (Figure 1),
containing different linear unsubstituted polyamine moieties
and we demonstrate that their binding leads to stronger
changes in DNA structure if compared to the mono-substituted
compounds. In particular, we show that the changes in the
intrinsc DNA morphology are strongly dependent on the
[Azobenzene]/[DNA] ratio used and that, in sharp contrast to
our previous results[1], the bis-substituted photochromes, once
bound, lack the ability to reversibly modulate the chirality of the
biopolymer. Moreover, a detailed series of biophysical
experiments varying the photochrome conformation,
temperature, salt and DNA concentration provided an
exhaustive description of the azobenzene-DNA structure-
activity relationship. Our structural analysis of the
photochrome-DNA interactions should help to gain insights
into the nature of this biologically important in vitro
complexation serving as a proof of concept in the design of
multivalent smart photoswitchable vectors for targeted gene
delivery.

![Figure 1. Structures and interconversion pathways of the photosensitive polyanines bis-Azo-2N and bis-Azo-3N in their both relevant forms: trans and cis.](image)

**Experimental**

**Apparatus**

The UV-Vis absorption spectra were recorded on a Perkin
Elmer Lambda 20 UV-Vis spectrometer. An electronic
thermostated water bath (PTP-1 Peltier system DBS) was used
for controlling the temperature. Fluorescence analyses were
carried out with a Hitachi F-4500 spectrofluorometer equipped
with a xenon lamp and a thermostated bath. Circular
dichromism spectra were recorded with a Jasco J-815
spectropolarimeter (Jasco Inc, USA) equipped with the Jasco
Peltier-type temperature controller (CDF-426S/15). The
infrared spectra were collected on the diamond crystal surface
under vacuum (< 1 hPa), using a Bruker Vertex70v FT-IR
spectrometer.

**Reagents and preparation of stock solutions**

Common reagent-grade chemicals were used without further
purification. The stock solution of deoxyribonucleic acid
sodium salt from salmon testes (DNA ≈ 2000 bp), purchased
from Sigma Aldrich Chem. Co., was prepared by dissolving an
appropriate amount of solid DNA powder in 10 mM sodium
cacodylate buffer (pH 7.2). Stock solution was stored at 4 °C
for 24 hours with occasional stirring and was used after no
more than 3 days. The appropriate DNA solution
concentrations were determined by absorption spectrometry
according to the absorbance at 260 nm and expressed in bp
(ε260 = 13200 M⁻¹ cm⁻¹). The purity of the DNA was checked by
monitoring the ratio of the absorbance at 260 and 280 nm and
at 260 and 230 nm giving values higher than 1.8 and 2.2,
respectively, thus showing the DNA to be sufficiently free from
protein impurities. The bis-Azo-2N and bis-Azo-3N stock
solutions were prepared by dissolving appropriate amounts of
the azobenzene derivatives in double distilled water to a final
concentration of 0.5 mM. The stock solutions were stored
protected from light by wrapping the vials with aluminium foil.
The reported data are at least the average values of three
trials. The quantitative data analysis reported throughout the
whole paper took into account the trans/cis (80:20) and
cis:trans (25:75) ratio derived for each photochrome.

**UV-Vis measurements**

The UV-Vis absorption spectra were recorded at 298, 301, 305
and 309 K keeping the concentration of the azobenzene
derivatives constant and adding incremental amounts of DNA.
After addition of DNA to the azobenzene solutions, the
resulting system was subjected to UV-Vis analysis in the 200-
600 nm range. In order to resolve the contribution from
exclusively bis-Azo-2 and bis-Azo-3 the spectrum of the
equimolar solution of DNA added was subtracted in the full
measured range. The photoinduced isomerization reactions of
bis-Azo-2N and bis-Azo-3N were performed by using a high
pressure Oriel Hg lamp equipped with interference filters at
313 and 436 nm. The resulting light power density was ~ 0.41
mW/cm² at 313 nm (trans-cis) and ~ 1.04 mW/cm² at 436 nm
(cis-trans).

**Competition experiment**

The competitive interaction between Ethidium bromide (EtBr)
and bis-Azo-2N and bis-Azo-3N with DNA was studied by
adding different amounts of bis-Azo-2N and bis-Azo-3N to the
EtBr-DNA solution. Fluorescence spectra of the mixture were
recorded at 298, 301, 305 and 309 K in the range of 540-800
nm using an excitation wavelength of 530 nm.

**Circular dichromism measurements**

CD spectra were recorded at 298 K in the wavelength range of
200-600 nm at different azobenzene / DNA molar ratios and
keeping constant the DNA concentration. Before use, the
optical chamber of the CD spectrometer was deoxygenated
with dry nitrogen and was held under nitrogen atmosphere
during the measurements. Each spectrum was averaged from
five successive accumulations and the buffer contribution has
been subtracted.

**FT-IR spectroscopic measurements**

The infrared spectra were recorded, after incubation of the
salmon sperm DNA / azobenzene solution, via the Attenuated
Total Reflection (ATR) method, in the spectral range 4000-400 cm\(^{-1}\) with a resolution of 4 cm\(^{-1}\) and accumulation of 64 scans, and transformed into absorbance spectra using OPUS software. Spectra subtraction ([ds-DNA solution + Azobenzene] – Azobenzene solution) was performed to make sure that the observed changes in the DNA shift peak position were attributable exclusively to ligand interactions. The plots of the relative intensity (R) of several bands of DNA caused by in-plane vibrations of base pairs and the stretching vibration of the PO\(_2\) versus the azobenzene concentrations were obtained by carrying out normalization of bands using R = I\(_i\) / I\(_962\) where I\(_i\) is the intensity of absorption band at i cm\(^{-1}\) for pure salmon testes DNA and its complex with different concentration of azobenzene, and I\(_962\) is the intensity band of the 962 cm\(^{-1}\) internal reference band. \(^5\)\(^6\)

**Results and discussion**

**UV-Vis absorption spectra of azobenzene-DNA adducts**

The absorption spectra of the azobenzene derivatives consisted mainly of two main charge transfer (CT) bands: one of strong intensity located in the UV (\(\lambda_{\text{max}} \approx 330\) nm) and the other, of weaker intensity, in the visible spectral region (\(\lambda_{\text{max}} \approx 450\) nm).

When DNA was added to bis-Azo-2N and bis-Azo-3N, in both their isomeric forms, a noticeable hypochromic effect was detected (Figure 2 upper panel). This behaviour is typical of molecules intercalating between DNA bases, the magnitude of the effect being related to the strength of the intercalative interaction. \(^5\)\(^6\)\(^8\) The π-π* transition band of bis-Azo-2N and bis-Azo-3N in trans conformation in the presence of DNA undergoes 36 and 39% hypochromicity and a red shift of 2 and 4 nm, respectively. The differences in the intercalative binding strength between the two conjugates may be related to the regiochemical distribution of charges along the polyamine backbone. Indeed, the increase in the number of positive charges at nitrogens as well as the difference in the number of methylene spacers has an impact on the azobenzene derivatives capacity to intercalate DNA. \(^5\)\(^6\)\(^7\) bis-Azo-2N and bis-Azo-3N in the cis form, under the same experimental conditions, gave rise to 23 and 28% hypochromicity and a bathochromic effect of 4 and 6 nm, respectively. The observed changes can be ascribed to the planarity of the trans form which, having a marked hydrophobic character, makes it suitable as a DNA intercalator, whereas the non-planarity of the bent cis form being more hydrophilic decreases to some extent its intercalative ability. It is worth noting that these findings are in good agreement with those previously reported by us for the interaction of the monosubstituted Azo-3N with DNA.

Figure 2. Upper panel: (A) Absorption spectra of bis-Azo-3N trans, (B) bis-Azo-2N trans, (C) bis-Azo-3N cis and (D) bis-Azo-2N cis in the free (black line) and bound form (red line) at the saturation point. The concentration of bis-Azo-3N and bis-Azo-2N was 2 \(\times 10^{-5}\) M in 10 mM sodium cacodylate trihydrate (pH 7.2). The DNA concentration was 6 \(\times 10^{-5}\) M. Central panel: Benesi-Hildebrand plots of 1/(A-A\(_0\)) against 1/[DNA] for (A) bis-Azo-3N(trans)-DNA, (B) bis-Azo-2N(trans)-DNA, (C) bis-Azo-3N(cis)-DNA and (D) bis-Azo-2N(cis)-DNA systems at 298 K. A\(_0\) and A are the absorbance values of the azobenzene derivatives in absence as well as in presence of DNA, respectively.
DNA concentration was, 0, 10, 20, 40, 50 and 60 µM. Lower panel: Scatchard plots for (A) bis-Azo-3N(trans)-DNA, (B) bis-
Azo-2N(trans)-DNA, (C) bis-Azo-3N(cis)-DNA and (D) bis-Azo-
2N(cis)-DNA systems at 298 K. r is the number of moles of
ligand bound per mole of nucleic acid (referred as the concentration of the complex formed) and D is the molar
concentration of the free ligand (concentration of the free
photochrome in solution corrected by the trans/cis ratio upon
duplex complexation). The DNA concentration was, 0, 10, 20,
40, 50 and 60 µM.

UV-Vis quantitative data analysis

Exploting the absorption changes arising upon DNA addition
to bis-Azo-2N and bis-Azo-3N, it was possible to quantitatively
compare the binding affinity of the conjugates in both their
isomeric forms with DNA. Thus a multi binding data analysis
was performed allowing calculation of the equilibrium
association binding constants (K_a) using the Benesi-Hildebrand
equation : \[ \frac{1}{\Delta A} = \frac{1}{\Delta A_{\text{max}}} + \frac{1}{K_a(\Delta A_{\text{max}})} \times \frac{1}{[\text{DNA}]} \] (1)

where \( \Delta A \) is the difference between the absorbance of the
conjugates in the absence and in the presence of DNA and
\( \Delta A_{\text{max}} \) is the final absorbance of the Azo-DNA adducts which
indicates saturation of interaction.

When small molecules bind to a set of equivalent sites on a
macromolecule, the equilibrium association binding constant
(K_a) and the number of molecules bound per polynucleotide
(n) can be examined according to the Scatchard analysis based
on the following equation: \[ \frac{r}{D_f} = nK_b - rK_b \] (2)

where r is the number of mole of ligand bound per mole of
macromolecule (i.e. the relative concentration of the complex
formed between each photochrome isomer and the duplex
taking into account the trans/cis ratio), D_f is the molar
concentration of the free ligand (i.e. the absolute
concentration of the photochrome in its peculiar configuration
(+/- UV) free in solution upon duplex complexation) and n is the
apparent number of the binding sites. The corresponding
results from Equation 1 and 2 are shown in Table 1.

Table 1. Association constants and number of interacting sites
for the photochrome-DNA complexes at 298 K.

|          | \( K_a \) M^{-1} (eq. 1) | \( K_b \) M^{-1} (eq. 2) | n (eq. 1) | n (eq. 2) |
|----------|---------------------------|---------------------------|----------|----------|
| bis-Azo-2N trans | 5.3 ± 0.3 \times 10^4 | 9.0 ± 0.1 \times 10^4 | 0.96   | 1.03   |
| bis-Azo-2N cis   | 2.6 ± 0.2 \times 10^4 | 3.4 ± 0.2 \times 10^4 | 0.93   | 0.99   |
| bis-Azo-3N trans | 7.1 ± 0.4 \times 10^4 | 1.3 ± 0.1 \times 10^4 | 0.99   | 1.00   |
| bis-Azo-3N cis   | 5.1 ± 0.4 \times 10^4 | 6.4 ± 0.2 \times 10^4 | 0.95   | 0.99   |

The association constant values calculated by using different
binding data analysis show a relatively high binding affinity of
the azo compounds to duplex DNA (Figure 2 central and lower
panels). The order of affinity of Azo-DNA complexes was found
as: bis-Azo-3N trans > bis-Azo-2N trans > bis-Azo-3N cis > bis-
Azo-2N cis. It is important to stress that the magnitude of the
binding constants found for the Azo-DNA adducts match well
with those found for well-known intercalators indicating that
the intercalation process is most likely the dominant mode
of interaction between the azobenzene derivatives and
DNA.\(^7e,11\) Furthermore, the binding isotherms, obtained by
Scatchard analysis (Figure 2 lower panel), were linear
indicating no deviations from Clark’s model and the single
binding site n pointed out the relative high binding selectivity
of the photochromes towards the biopolymer reactive sites.\(^12\)

It is worth noting that the reported values of n must be
considered apparent for the following reasons:
(i) the association constants are calculated by a single-site
binding model,
(ii) the observable physical quantity used, i.e. absorption, is
proportional to the concentration (extensive variable) and not
to the mole fraction (intensive variable e.g. fluorescent
polarization) which do not allow for an overall calculation of
the binding parameters.

In order to better elucidate if cooperative binding is taking part
in the association process a Hill analysis was performed using
Equation 3.\(^12\)

\[ \log \left( \frac{B}{(B_{\text{max}} - B)} \right) = n \left( \log(L_f) \right) - \log(K_d) \] (3)

where B and \( B_{\text{max}} \) are the bound ligand and the total receptor
concentration, respectively, \( L_f \) is the free ligand concentration and
\( K_d \) is the equilibrium dissociation constant. The obtained
values are listed in Table 1.

The straight line achieved using Equation 3 (Supplementary
Figure S1 A-D) confirms the applicability of this model to our
data. As shown in Table 1, the average number of interacting
sites was found to be ~ 1 (Hill coefficient n = 1) suggesting that
only a single class of binding sites was involved and non-
cooperative binding phenomena obeying neighbour exclusion
principle ruled the bis-Azo-DNA interaction.

Determination of the binding mechanism by displacement
assay

No luminescence was observed for bis-Azo-2N and bis-Azo-3N
upon excitation either in aqueous solution or in the presence
of DNA at the concentrations used. Therefore, a displacement
assay was performed using Ethidium Bromide (EtBr), an
archetypical intercalator, to characterize the Azo-DNA binding
mode.\(^12\) If the azobenzene derivatives compete for the same
DNA binding sites as EtBr, a decrease of the fluorescence
intensity of the latter would be observed. As shown in Figure
3, the fluorescence of the EtBr-DNA complex is efficiently
reduced until stoichiometry is reached.
Additional quantitative information of the fluorescence quenching data was provided by using the Stern-Volmer equation:

\[
\frac{F_0}{F} = 1 + K_q \tau_0 [Q] = 1 + K_{sv} [Q]
\]

where \( F_0 \) and \( F \) denote the steady-state fluorescence intensities in the absence and in the presence of the quencher (azo-benzene derivatives), respectively. \( K_q \) is the Stern-Volmer quenching rate constant, \([Q]\) is the azo concentration, \( K_{sv} \) is the apparent quenching rate constant of the biomolecules which reflects the efficiency of quenching or in other words the accessibility of the fluorophore to the quencher and its absolute value can be calculated using the Smoluchowski equation (for further background reading the interested reader is referred to Lakowicz’s book referenced as 14 in the current manuscript) and \( \tau_0 \) is the average excited-state lifetime of biomolecules without a quencher taken as \( 10^{-8} \) s.\(^{14} \)

From the plot of Equation 4 (Figure 4), the values of \( K_{sv} \) and \( K_q \) were obtained at different temperatures and listed in Table 2.

![Figure 3. Fluorescence emission spectra of the competition between EtBr-DNA complex (λexc: 530 nm) and the photochromes treated with: 0, 2.5, 5, 7.5, 10, 15, 20, 25, 30, 35 and 40 µM (curves 1-11) of azobenzene derivatives. [EtBr]: 10 µM and [DNA]: 40 µM.](image)

![Figure 4. Stern-Volmer plots for the fluorescence quenching of EtBr-DNA system by (A) bis-Azo-3N trans, (B) bis-Azo-2N trans, (C) bis-Azo-3N cis and (D) bis-Azo-2N cis at 298, 301, 305 and 309 K. \( F_0 \) and \( F \) are the fluorescence intensity values of the EtBr-DNA complex in absence as well as in presence of different concentrations of photochromic molecules, respectively.](image)

### Table 2. Stern-Volmer (\( K_{sv} \)) and quenching rate constant (\( K_q \)) of the interaction between the photochromes and DNA at various temperatures.

| T(K) | \( K_q \) M\(^{-1} \) | \( K_{sv} \) M\(^{-1} \) s\(^{-1} \) | \( K_q \) M\(^{-1} \) | \( K_{sv} \) M\(^{-1} \) s\(^{-1} \) |
|------|-----------------|-----------------|-----------------|-----------------|
| 298  | \( 6.3 \pm 0.2 \times 10^6 \) | \( 6.3 \pm 0.2 \times 10^{12} \) | \( 4.5 \pm 0.1 \times 10^8 \) | \( 4.5 \pm 0.1 \times 10^{12} \) |
| 301  | \( 5.5 \pm 0.4 \times 10^6 \) | \( 5.5 \pm 0.4 \times 10^{12} \) | \( 3.8 \pm 0.3 \times 10^8 \) | \( 3.8 \pm 0.3 \times 10^{12} \) |
| 305  | \( 4.3 \pm 0.2 \times 10^6 \) | \( 4.3 \pm 0.2 \times 10^{12} \) | \( 3.2 \pm 0.2 \times 10^8 \) | \( 3.2 \pm 0.2 \times 10^{12} \) |
| 309  | \( 3.5 \pm 0.3 \times 10^6 \) | \( 3.5 \pm 0.3 \times 10^{12} \) | \( 2.5 \pm 0.3 \times 10^8 \) | \( 2.5 \pm 0.3 \times 10^{12} \) |
therefore confirming the static nature of the process and the involvement of specific interactions between the molecular photoswitches and the biopolymer.

Static complex formation can be further confirmed by the modification of the absorption spectra due to stacking interactions arising from the insertion of the azobenzene moiety among the duplex base pairs (vide supra), thus ruling out any fluorescence quenching process through dynamic collisions.

Fluorescence binding data analysis

By exploiting the fluorescence titration data and assuming static quenching the association constant ($K_a$) and the number of excluded binding sites ($n$) were analysed according to the following equation:\textsuperscript{15}

$$\log \left( \frac{[F_0-F]}{F} \right) = \log K_f + n \log [Q]$$  \hspace{1cm} (5)

The plots of $\log ([F_0-F]/F)$ versus $\log [Q]$ were linear (Figure 5) and the values of $K_f$ and $n$, shown in Table 3, were calculated at four different temperatures.

**Table 3.** Association constants ($K_a$) and number of excluded binding sites ($n$) for the interaction between the photochromes and DNA at various temperatures.

| Photochrome (M) | $K_f$ (M$^{-1}$) | $n$ | $K_f$ (M$^{-1}$) | $n$ |
|----------------|-----------------|----|-----------------|----|
| bis-Azo-2N trans | 2.4 ± 0.2 × 10$^5$ | 1.1 | 6.1 ± 0.3 × 10$^8$ | 1.0 |
| bis-Azo-2N cis | 2.5 ± 0.2 × 10$^5$ | 1.1 | 6.3 ± 0.3 × 10$^8$ | 1.0 |
| bis-Azo-3N trans | 9.1 ± 0.4 × 10$^5$ | 1.3 | 3.1 ± 0.3 × 10$^8$ | 1.0 |
| bis-Azo-3N cis | 7.9 ± 0.1 × 10$^7$ | 1.2 | 2.9 ± 0.3 × 10$^8$ | 0.9 |

The values of apparent association constants and number of binding sites obtained by fluorescence displacement assay show a strong and specific binding affinity of the photochromes for double stranded DNA. The magnitudes of these values, as expected, were higher for the stable trans isomer than for the metastable cis conformer, in good agreement with the UV-Vis data. Interestingly, the $K_f$ and $n$ temperature trend was found to be different for the two isomers. The decrease of these values, observed for the cis isomers, was in accordance with the $K_f$’s temperature dependence due to the reduction of the stability of the azobenzene-DNA complexes. It is well known that an increase in temperature can enhance the thermal stability of the planar trans form, which may be responsible for the increase in the magnitude of the association constants. The apparent unitary values calculated in the fluorescent intercalator displacement assay (FID) suggested that one molecule of Et-Br was displaced upon binding of one molecule of photochrome in good agreement with the size of the binding site discussed below.

Figure 5. Plots of log ([F0-F]/F) versus (A) log [bis-Azo-3N trans], (B) log [bis-Azo-2N trans], (C) log [bis-Azo-3N cis] and (D) log [bis-Azo-2N cis] at 298, 301, 305 and 309 K. $F_0$ and $F$ are the fluorescence intensity values of the EtBr-DNA complex in absence as well as in the presence of different concentrations of photochromic molecules, respectively.

Elucidation of the binding parameters

The binding site size of the azobenzene derivatives to ds-DNA, in both conformations, was evaluated based on luminescence titration using the mole ratio method, keeping constant the concentration of EtBr and DNA and changing that of the photochromes. Plots of variations in fluorescence intensity at 590 nm vs. the Azobenzene / DNA mole ratio are shown in Figure 6. From the inflection point the molar ratios Azobenzene / DNA were found to be ~ 0.4, and the number of base pairs involved in the association process are listed in Table 4.

**Table 4.** Binding size for the photochromes-DNA complexes.

| Photochrome (M) | Binding size (bp) |
|----------------|------------------|
| bis-Azo-2N trans / DNA | 2.56 |
| bis-Azo-2N cis / DNA | 2.38 |
| bis-Azo-3N trans / DNA | 2.94 |
| bis-Azo-3N cis / DNA | 2.63 |

The size of the binding sites of the azobenzene derivatives agrees with those found for classical intercalators in accordance with the neighbour exclusion principle.\textsuperscript{16} The number of base pairs involved in the association process seems to be also related to the size of the photochromes and
to their spatial configuration. Moreover, from the slope of the binding isotherm, the DNA binding affinity of the azobenzene derivatives followed the order bis-Azo-3N trans > bis-Azo-2N trans > bis-Azo-3N cis > bis-Azo-2N cis (data shown only for bis-Azo-3N trans and bis-Azo-2N trans). These findings are in accordance with results found using various spectroscopic approaches.

Figure 6. Plots of (A) bis-Azo-3N (trans)-DNA and (B) bis-Azo-2N (trans)-DNA fluorescence intensity versus the mole ratio.

Thermodynamic studies
Thermodynamic parameters, such as the entropic and enthalpic contributions to the whole binding process, are good indicators of the type of interaction that regulates such macromolecular interactions. Positive values of both enthalpy and entropy can be ascribed mainly to short-range interactions and dehydration effects, whereas negative enthalpy and positive entropy values indicate mainly long-range interactions of ionic nature. If the enthalpy change (ΔH) does not vary significantly in the temperature range studied, the thermodynamic parameters, listed in Table 6, using the Kx values determined at four different temperatures listed in Table 5, can be evaluated by applying the Van’t Hoff equation:

\[ \log K_a = -\frac{\Delta H^0}{2.303RT} + \frac{\Delta S^0}{2.303R} = -\frac{\Delta G^0}{2.303RT} \]

\[ -RT \ln(K_a) = \Delta G = \Delta H^0 - T\Delta S^0 \]

Table 5. Values of the intrinsic binding constant (Kx) for the photochromes-DNA systems calculated at different temperatures 298, 301, 305 and 309 K by using Eq. 1.

| Temperature (K) | bis-Azo-2N trans | bis-Azo-2N cis | bis-Azo-3N trans | bis-Azo-3N cis |
|----------------|------------------|----------------|------------------|----------------|
| 298            | 5.3 ± 0.3 x 10^4 | 2.6 ± 0.2 x 10^4 | 7.1 ± 0.4 x 10^4 | 5.1 ± 0.4 x 10^4 |
| 301            | 5.6 ± 0.3 x 10^4 | 2.4 ± 0.2 x 10^4 | 7.8 ± 0.1 x 10^4 | 4.5 ± 0.3 x 10^4 |
| 305            | 6.5 ± 0.2 x 10^4 | 2.1 ± 0.4 x 10^4 | 8.5 ± 0.3 x 10^4 | 4.0 ± 0.1 x 10^4 |
| 309            | 7.2 ± 0.5 x 10^4 | 1.9 ± 0.2 x 10^4 | 9.5 ± 0.3 x 10^4 | 3.5 ± 0.3 x 10^4 |

The values of ΔH^0 and ΔS^0 were determined from the slope and the intercept of the linear plot between log Kx and the reciprocal absolute temperature (Supplementary Figure S2 A-D).

The negative values of ΔG^0 clearly indicate the spontaneity of the process. A different trend of the thermodynamic parameters (ΔH^0 and ΔS^0) was observed for the two conformers. The negative enthalpy (ΔH) and the positive entropy (ΔS) found for the metastable cis conformation, indicates that complex formation is driven and stabilized mainly by long-range forces including electrostatic, hydrogen bonding and van der Waals interactions. The positive value of both enthalpy and entropy observed for the stable trans isomers, suggests that the free-energy contribution to adduct stabilization arises mainly by short-range contacts of a hydrophobic character even though long-range interactions cannot be excluded. The substantial difference, for the trans and cis isomers, in stabilizing the DNA adduct, seems to be related to conformational changes of the photochromes occurring upon light irradiation. The reduced free volume of the planar trans form allows the azobenzene core an easy transition among the non-polar DNA bases resulting in favourable hydrophobic contacts, whereas the larger molecular size of the bent, non-planar cis form displaces to some degree the ligand from the stacked position, allowing an additional surface binding with the functional groups positioned on the edge of DNA bases and/or with the phosphate backbone of the duplex.

Since the thermodynamic studies show positive values of entropy for all the photochrome-DNA adducts the binding may entail the release of bound ions and therefore additional ionic strength investigations were carried out and are presented below.
Azo-2N upward shifted to 1534 and 1420 cm\(^{-1}\). It is noteworthy that, unlike the other bands related to the nitrogenous DNA bases, at higher molar ratios no shift was observed for the bands at 1701, 1417 cm\(^{-1}\) and 1488 cm\(^{-1}\). All the changes of the bands related to the nitrogenous DNA bases. At higher molar ratios, the bands at 1701 and 1417 cm\(^{-1}\) were not further perturbed, while the bands at 1652, 1527, 1488 cm\(^{-1}\) were shifted to 1656, 1533 and 1483 cm\(^{-1}\), respectively. All the shifts of the DNA bases are also associated with changes in their relative intensity (Supplementary Figure S5 A-D). It is important to stress that no shift was observed for the bands at 1606, 1579, 1294, 1068, and 962 cm\(^{-1}\) for each DNA / Azobenzene (trans) molar ratio considered, thus ruling out their involvement in the DNA interaction.

Similarly, the azobenzene derivatives in cis conformation act as their trans isomers, however, with some remarkable differences. In particular, in the system with a low DNA / bis-Azo-2N (cis) molar ratio (1/20, 1/10 and 1/6.6) the bands related to the bases at 1701 cm\(^{-1}\) (G), 1527 cm\(^{-1}\) (C) and 1488 cm\(^{-1}\) (C) were shifted to 1684, 1531, 1484 and 1419 cm\(^{-1}\), respectively. By increasing the concentration of the ligand \((r = 1/5\) and \(r = 1/4\)) no additional shift of the bands at 1701, 1527 and 1488 cm\(^{-1}\) was noticed while the bands at 1527 and 1417 cm\(^{-1}\) were further upward shifted to 1534 and 1420 cm\(^{-1}\), respectively. Similar shifts of these bands were also found for the bis-Azo-3N (cis) DNA systems. It is noteworthy that, unlike the trans isomers, the bent cis conformers induced also shifts of the bands at 1606 and 1579 cm\(^{-1}\). The spectral changes observed for such bands are indicative of a possible binding of the cis-photochromes either to N7 guanine and adenine reactive sites or O6 on guanine atoms as well as to adjacent adenine and guanine N3 residues located in the DNA grooves. As expected, the interaction of the azobenzene derivatives in the cis form seems to involve the same binding sites as the trans isomer, with additional surface contacts through the edge of DNA in agreement with the thermodynamic analysis.

**Interaction with phosphate-sugar backbone**

At low DNA / bis-Azo-2N (trans) mole ratio, the bands related to asymmetric and symmetric stretching vibration of the phosphate groups at 1370, 1242, 1093 and 730 cm\(^{-1}\) were shifted to 1366, 1238, 1085 and 725 cm\(^{-1}\), respectively. The same bands related to the DNA / bis-Azo-3N (trans) systems were shifted to 1373, 1237, 1085 and 725 cm\(^{-1}\), respectively. At higher molar ratios for the former system, the bands at 1370 and 1093 cm\(^{-1}\) were shifted to 1364 and 1084 cm\(^{-1}\), respectively and no further shift of the bands at 1242 and 730 cm\(^{-1}\) was observed. At high DNA / bis-Azo-3N (trans) mole ratio systems, the bands at 1370, 1242 and 1093 cm\(^{-1}\) were further shifted to 1374, 1235 and 1080 cm\(^{-1}\), respectively and no additional perturbation of the band at 730 cm\(^{-1}\) was detected. The shifting of the PO\(_4\) bands observed for the azobenzene (cis)-DNA systems were found to be similar to those reported above for the trans isomers and no or weak shifts of the bands at 1294, 962 and 781 cm\(^{-1}\) were detected for each DNA / Azobenzene molar ratio considered. The spectral changes observed may be due to the occurrence of external binding interactions probably of ionic nature between the positive multi charged ligands and the negative duplex backbone. The insertion of the ligand among the base pairs can also destabilize the B-DNA form to some degree, therefore leading to distortion and perturbation of the sugar-phosphate backbone, which can be an additional explanation for the shifting of those bands.

Nevertheless, it is interesting that the major perturbation of the deoxyribose sugar vibration band at 1068 cm\(^{-1}\), arising upon interaction with the non-planar cis conformer, provides direct evidence of a possible stronger outside binding contribution with the sugar-phosphate backbone of the DNA double helix than with respect to the planar isomer; which can instead face the hydrophobic cavity of the duplex.

**Changeover in the duplex DNA conformation**

The B-DNA marker bands, for both the azobenzene derivatives in trans conformation, at 833 cm\(^{-1}\) assigned to S-C\(_2\) endo/anti sugar pucker-phosphodiester mode and that at 889 cm\(^{-1}\) assigned to sugar phosphate-stretch\(^{19}\), were overall shifted upward and downward to 839 and 881 cm\(^{-1}\), respectively. Moreover, the band at 1456 cm\(^{-1}\) assigned to C-N glycosyl bond, also responsible for the B-DNA form\(^{19}\), was overall shifted downward to 1445 cm\(^{-1}\). The DNA-azobenzene (cis) systems were also characterized by the perturbation of the DNA marker bands at 833 and 889 cm\(^{-1}\) but unlike the trans derivative, the cis isomer weakly perturbs the band at 1456 cm\(^{-1}\) at all the molar ratios considered. Those results indicated that even if the bent non-planar cis form affected the DNA secondary structure the B-DNA conformational transition is less likely to occur, compared to the elongated planar trans form. These findings are in accordance with the CD spectroscopic results discussed below.
Circular dichroism spectral studies

In order to better assess and understand the ability of the photochromes to induce conformational changes in the DNA secondary structure, circular dichroism (CD) spectra were studied (Figure 7 and Supplementary Figure S6). The canonical B-DNA form is characterized by four major characteristic CD bands: 211 nm (negative), 224 nm (positive), 245 nm (negative) and 275 nm (positive).20 The azobenzene derivatives being object of the present study do not possess a chiral centre and thus they are CD inactive. However, upon association with DNA, a bisignate induced circular dichroic band (ICD) appears for both their isomeric forms. The appearance of ICD signals in the region between 310 and 540 nm can be attributed to degenerate coupling of the electric transition moment of the azobenzene derivatives with those of the chirally arranged DNA base pairs. It is important to stress that excitonic coupling of the azobenzene chromophores may be excluded since the ICD magnitude is linear with respect to the concentration of bound molecules.21 Intercalators usually display a weak negative or bisignate ICD signal, as found for some anthracene-spermine derivatives and for the mono-substituted photosensitive polyamine Azo-3N, whereas larger positive ICD signals are attributable to the groove-binding geometry.20,27 It turns out that the changes observed can be ascribed to the ability of the photochromes to intercalate the nucleobases, even if this behaviour seems to be smaller for the hydrophilic cis form than for the hydrophobic trans isomer. In order to better elucidate the association mechanism, the intrinsic positive and negative B-DNA bands were analysed.17 At low DNA / azobenzene molar ratio (r = 5), an increase in ellipticity of the bands at 275, 224 and 211 nm along with a decrease (shifting towards zero) of the CD band at 245 nm was observed upon interaction of the photochromes in both their conformations. The presence of isodichroic points in the CD spectra is indicative of a perturbation of the duplex secondary structure providing evidence for the formation of the fast, two state and highly cooperative partial B-to-A DNA transition.23 On increasing the concentration of bis-Azo-2N (r = 1.66; r = 1; r = 0.55 and r = 0.42) and bis-Azo-3N (r = 1.66; r = 1 and r = 0.71) in both their isomeric forms, a similar trend of the intrinsic DNA CD bands changes was noted. The bands at 275 and 245 nm gradually decreased with increasing ligand concentration and an overall red shift of the negative band of 12 nm was observed. Moreover, both the bands at 224 and 211 nm shown an overall decrease of intensity at high DNA / bis-Azo-2N ratio (r = 0.55 and r = 0.42) and at medium DNA / bis-Azo-3N ratio (r = 1 and r = 0.71). CD changes here can be ascribed to the slow A-to-Z DNA transition, which is in good agreement with polyamine induced interconversion: B-DNA, A-DNA and Z-DNA.23,24 Interestingly, progressive addition of bis-Azo-3N in both conformations (r = 0.55) resulted in a decrease of the intensity of the positive B-to-A DNA band at 275 nm which may be ascribed to the formation of the condensed multi molecular Ψ-DNA form.25 Further ligand addition (r = 0.42) gave rise to a complete DNA precipitation. It should be noted that at equal amounts of ligand bound to the duplex the ability of the ligands to induce DNA conformational changes grows on increasing the number of methylene spacers and positive charges at the nitrogens (bis-Azo-3N > bis-Azo-2N) which is consistent with our spectroscopic results discussed above. These findings unambiguously differ from those reported for the mono-substituted Azo-3N (trans) in which a monotonic trend of changes occurring on the DNA chirality was observed.26 We speculate that the number of charges distributed along the photosensitive polyamines are not only deeply responsible for the changes occurring on the duplex morphology but they also exert a pivotal role in conferring reversibility to the process once the photochrome is bound to the biopolymer.

Figure 7. Circular dichroism spectra of ds-DNA (red line) at different azobenzene / DNA molar ratio (r). r = 5 (blue line), r = 1.66 (green line), r = 1 (pink line), r = 0.71 (yellow line), r = 0.55 (violet line) and r = 0.42 (brown line). The optical inactivity of the photochromes is shown by the black line. (A) bis-Azo-3N trans-DNA system, (B) bis-Azo-2N trans-DNA system, (C) bis-Azo-3N cis-DNA system and (D) bis-Azo-2N cis-DNA system.

Effect of varying concentration of monovalent salt on affinity of azobenzene derivatives to ds-DNA

Analysis of the effect of changes in salt concentration on the equilibrium binding constant, K_a, for the azobenzene-DNA complexes was carried out according to the theory of Record et al.26 In the presence of a monovalent salt MX, the number of ionic interactions and counterions released involved in a ligand-nucleic acid adduct can be estimated by measuring the derivative δlog (K_a) / δlog [M^+] of the association constant on the ionic strength is:27

\[
\frac{\delta \log K_a}{\delta \log [M^+]} = -Z\Psi
\]

where, \(\Psi\) is a constant (0.88 for B-DNA form) and the linear plot of \(\log K_a\) on \(\log [M^+]\) allows to determine Z.25a
Table 7. Apparent association constant (K_a) of binding of the azobenzene derivatives to DNA at various salt concentrations calculated by using Eq. 1. ZΨ is the slope of the dependence log K_a vs. log [NaCl] for each azobenzene-DNA system considered.

| [NaCl] mM | K_a (M^-1) bis-Azo-2N trans | K_a (M^-1) bis-Azo-2N cis | K_a (M^-1) bis-Azo-3N trans | K_a (M^-1) bis-Azo-3N cis |
|-----------|-------------------------------|---------------------------|-------------------------------|---------------------------|
| 2.5 × 10^4 | 5.0 ± 0.3 × 10^4             | 1.4 ± 0.2 × 10^4          | 6.3 ± 0.3 × 10^4             | 3.2 ± 0.4 × 10^4          |
| 10 × 10^3  | 1.0 ± 0.3 × 10^3             | 3.2 ± 0.4 × 10^3          | 2.0 ± 0.1 × 10^4             | 1.4 ± 0.2 × 10^4          |
| 20 × 10^3  | 4.0 ± 0.2 × 10^3             | 2.0 ± 0.2 × 10^3          | 1.3 ± 0.4 × 10^4             | 1.0 ± 0.1 × 10^4          |
| 50 × 10^3  | 1.6 ± 0.3 × 10^3             | 1.0 ± 0.2 × 10^3          | 5.0 ± 0.4 × 10^3             | 6.3 ± 0.2 × 10^3          |
| 2Ψ         | 1.7                           | 1.8                       | 2.6                           | 3.1                       |

The least squares slopes (Supplementary Figure S7 A-D) were 1.7, 1.8, 2.6 and 3.1 for the bis-Azo-2N and bis-Azo-3N in trans and cis form, respectively, implying that ~ 2 and ~ 3 ions are released in the interaction of each monomer of the photochrome with the biopolymer in the salt range studied. Consequently the number of phosphate groups on the DNA involved in the ionic interactions with the positively charged ligands were ~ 2 and ~ 3 for bis-Azo-2N and bis-Azo-3N, respectively, which is in good agreement with the intrinsic positive charges of azo compounds (Supplementary information p. S12). The slightly higher number of ions released and consequently neutralized phosphates found for the cis isomers further confirms the prediction that the bent non planar form is hindered to fit entirely the intercalation site resulting in an end-stacking mode with the substituent arms more faced to the external edges of the duplex. The overall net positive charge distribution along the azobenzene substituent groups exerts a pivotal role in the photochrome-DNA stabilization and seems to be the major driving force for the resulting conformational changes occurring on the intrinsic B-DNA morphology.

Conclusions

In summary, the biophysical DNA-binding properties of two water-soluble polycationic azobenzene derivatives (bis-Azo-2N and bis-Azo-3N) have been comprehensively investigated as functions of the photochrome states and temperature, salt and DNA concentration. The qualitative and quantitative binding data analysis obtained by the various spectroscopic approaches indicates that the order of stability of the photochroms bound to DNA was: bis-Azo-3N trans > bis-Azo-2N trans > bis-Azo-3N cis > bis-Azo-2N cis. These results unequivocally establish that the number and magnitude of positive charges along the surface of the molecules as well as the intrinsic isomeric states have profound effect on DNA modification. Results of fluorescence displacement assays clearly establish the major ability of the hydrophobic planar azobenzene derivatives to displace the archetypical intercalator Ethidium Bromide compared to the hydrophilic non-planar bent cis form. These findings hint at the possibility that the different hydrophobic/hydrophilic character possessed by the two conformers may be exploited during the well-known resolubilization process of DNA occurring at high polyamine concentration since the disruption of the liquid crystalline order of the biopolymer is regulated by the ability of polyamions to enter into the hydrophobic environment of the DNA helix. Evaluation of the thermodynamic parameters, beyond confirming the spontaneity of the process, highlights that the increment of entropy in the system mainly arises from the release of monovalent counterions from the duplex backbone which is further supported by the results of the ionic strength analysis. FT-IR results showed that both the phosphonate backbone and the functional groups located at the edges of the DNA were involved in the complexation process. The circular dichroism data clearly demonstrated that the conformational changes induced by the photochroms on the ds-DNA were indicative of B-to-A-to-Z-like partial transitions and their relative modulation could be accomplished through a concentration-dependent manner. The overall changes occurring on the intrinsic DNA chirality upon binding of the bis-substituted compounds were in sharp contrast to those observed for the mono-substituted photoswitch Azo-3N indicating that the number of positive charges along the azobenzene template play a key role in destabilizing the B-DNA morphology as well as in conferring photo-reversibility to the binding process. Nevertheless, the relative ease of production makes these new photochromic materials attractive models for future vehicle-based gene therapy applications.

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Graphical abstract

Changes occurring on the intrinsic B-DNA morphology upon binding to the molecular photoswitches.