# Hoechst 33258 as a pH-Sensitive Probe to Study the Interaction of Amine Oxide Surfactants with DNA

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*The use of Hoechst 33258 (HO) as a fluorescent probe to characterize the interactions between DNA and pH-sensitive amphiphiles is discussed. In the case of amine oxide amphiphiles dodecyldimethylamine oxide (DDAO) and* p*-dodecyloxybenzyldimethylamine oxide (pDoAO), the decrease in fluorescence emission, which signals DNA–amphiphile association, is accompanied by a large hypsochromic shift in the emission maximum of the bound probe; this eventually reaches a value characteristic of HO in a neutral or slightly basic environment. These findings are compared to results obtained by using the more common ethidium bromide (EB) probe, which shows no such shift. Circular dichroism and fluorescence depolarization experiments indicate that fluorescence emission only occurs from the DNA-bound probe, and the observed shift in emission maximum when using HO as a fluorescent probe is due to a variation in the local pH in the vicinity of the probe.*

## Introduction

Amphiphiles interact with macromolecules thorough various nondirective intermolecular forces, including hydrophobic and hydrophilic interactions and, in the case of charged species, electrostatic forces. Such interactions form the basis of numerous applications, ranging from chemistry to material science and biology.[1,2] In the case of biopolymers, much work has focused on the use of DNA/amphiphile systems for the purpose of efficient transfection processes, and synthetic DNA-delivery agents are a promising alternative to viral vectors in gene therapy due to the absence of the risks associated with immunogenicity and propagation.[3,4] Additionally, synthetic amphiphilic delivery systems can, in principle, be tailored to suit specific needs, for example the controlled release of vectored material upon external stimuli, such as light, heat, or a variation in pH. In the last case, the interaction between an amphiphile and DNA is modulated by altering the pH of the surrounding environment; this represents a simple approach to the site-directed release of the transported DNA in specific cell locations where strong pH gradients are present (e.g. in proximity to the cell nucleus). In this context, zwitterionic amine oxide surfactants, such as dodecyldimethylamine oxide (DDAO), are interesting given the strong pH-dependence of their interaction with DNA. In the case of DDAO, the pH-controlled micelle- or vesicle-induced condensation of DNA has been recently investigated.[5,6]

In view of the increased interest in the development of new synthetic DNA delivery agents, methods capable of rapidly screening DNA–surfactant interactions are essential for future progress in this area, and the use of ethidium bromide (EB) as a fluorescent probe is very common.[7,8] It is generally assumed that the association of cationic lipids to the DNA strand induces a release of bound EB into the aqueous phase, and this results in a decrease of the observed fluorescence emission.[9] However, this simple model is only valid over a limited range of DNA/EB/surfactant ratios,[10] and the interactions between the intercalated dye and surface-bound surfactants have proven to be considerably more complicated than what was initially proposed. Herein, we propose the use of a pH-dependent probe, Hoechst 33258 (HO), to monitor interactions between DNA and pH-sensitive amine oxide amphiphiles. An advantage of using a DNA-binding agent with preference for binding to the exterior of the double helix[11,12] (in contrast to intercalating agents such as EB) is that it might be more sensitive to DNA–surfactant interactions. Moreover, HO is a pH-dependent probe,[13,14] and should be very useful in detecting the interaction between DNA and pH-dependent delivery systems.[15]

In the present study, we compared the behaviour of HO bound to DNA in the presence of two pH-sensitive surfactants: DDAO and *p*-dodecyloxybenzyldimethylamine oxide (pDoAO), a new amine oxide surfactant possessing an aromatic residue in the hydrophobic moiety. It has already been shown that small variation in the surfactant structure can induce significant changes in the aggregation properties.[16,17] In particular, it was recently observed that modification of the hydrophobic

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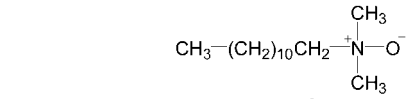
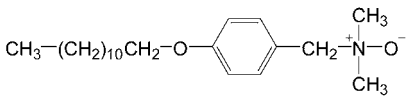


DDAO



pDoAO

moiety, as for pDoAO, induces a great decrease in the critical micellation concentration (c.m.c.) value with respect to DDAO ($1.6\times10^{-5}$ M vs. $7.4\times10^{-4}$ M, for pDoAO and DDAO, respectively) and allows the formation of aqueous gels at high surfactant concentration (data not shown). The results obtained for DDAO and pDoAO are compared to those obtained for a conventional cationic surfactant, cetyltrimethylammonium bromide (CTAB), and when using EB as probe. The findings support the intriguing possibility that HO can "read" local pH variations in the vicinity of the DNA–solution interface that occur upon surfactant binding; this is of considerable interest in the development of pH-sensitive DNA-transport agents. The association between the surfactants and DNA was further characterized by UV-visible and circular dichroism (CD) studies.

## Results

### Fluorescence measurements

Interactions between surfactants and DNA have been mainly studied by using cationic surfactants. In particular, the interaction between cetyltrimethylammonium halides (bromide and chloride) and DNA has been extensively studied by fluorescence spectroscopy with ethidium bromide as probe. In the presence of increasing amounts of the cationic surfactant CTAB, the fluorescence intensity of EB or HO bound to dsDNA decreased as shown in Figure 1. The DNA–CTAB interaction occurs at low concentrations of surfactant, which is most likely present in its monomeric form (c.m.c. value in water: $8.0\times10^{-4}$ M).[18] The results obtained when using EB or HO as a probe are very similar, and support the use of HO as an alternative probe to EB. We thus studied the interaction of DNA with pH-sensitive amine oxide surfactants DDAO and pDoAO using HO as a probe. In the case of the amine oxide surfactants, the studies were undertaken at two pH values: 7.5 and 5.8. At pH 7.5, DDAO and pDoAO exist mainly in the unprotonated, zwitterionic form ($pK_a \approx 5$),[19,20] and no changes in fluorescence were observed upon addition of DDAO or pDoAO. At higher concentrations of surfactant (above the c.m.c.), pDoAO induced a small increase in the fluorescence of the probe (Figure 2). No quenching of fluorescence by such systems was observed when using EB as probe (data not shown). Under acidic conditions (pH 5.8), DDAO and pDoAO are partially protonated, and, consequently, cationic monomers are present in solution. Under these conditions, their capability to induce a decrease in the fluorescence intensity of EB and HO is high, comparable to that of a conventional cationic surfactant such as CTAB. The emission spectra of HO–DNA in the presence of increasing amounts of DDAO or pDoAO at pH 5.8 is shown in Figure 3. It can be seen that the decrease in fluorescence intensity of HO is accompanied by a hypsochromic shift of the emission maximum; this is indicative of a change in the immediate environment of the probe. No such shift was observed in the case of CTAB or when using EB as the fluorescent probe (data not shown). Moreover, it seems that a concentration of zwitterionic surfactant above its c.m.c. in water is needed, in agreement with what was observed by Mel'nikova and Lindman in the case of DDAO.[5] The gradual shift of the HO emission eventually reached a value that is identical to that observed for DNA-bound HO at pH 7.5 at a concentration of

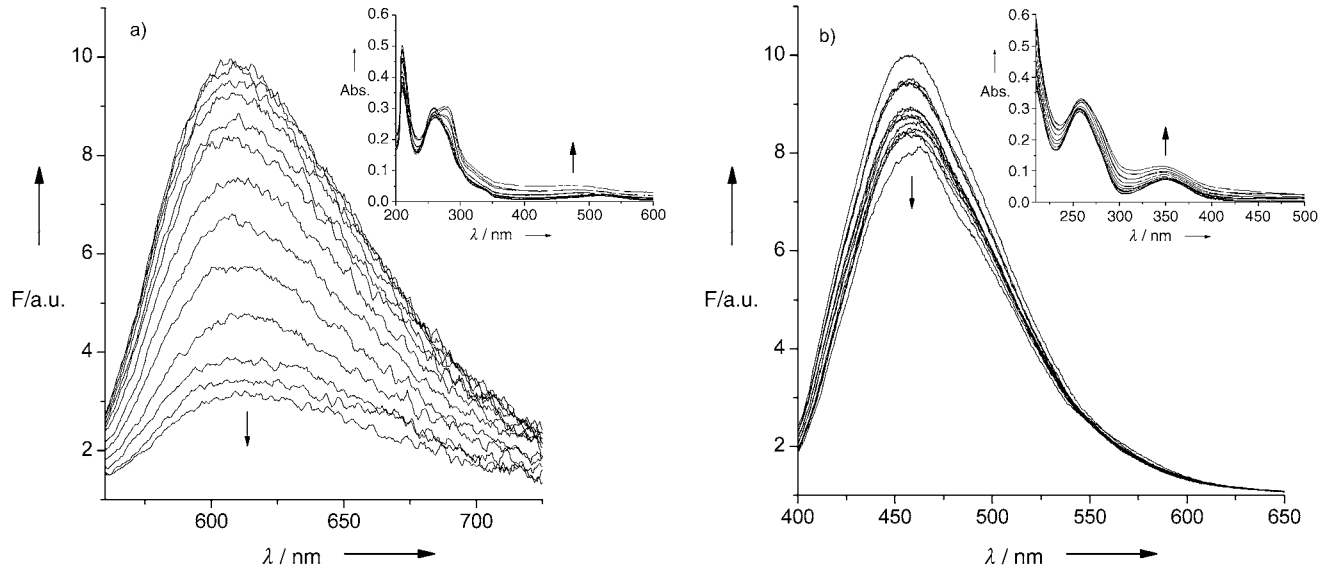



*Figure 1. Effect of addition of CTAB on a) DNA-bound EB and b) HO fluorescence emission (expressed in arbitrary units). The correspondent absorption spectra are reported as insets.*

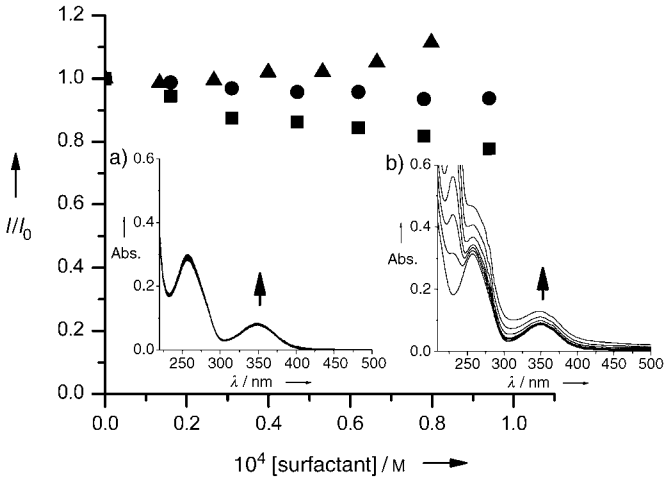



***Figure 2.*** *Fluorescence emission intensity at the maximum of the curve ($\lambda_{max}$ 457 nm) for DNA-bound HO against additive concentration for CTAB (■), DDAO (●) and pDoAO (▲) at pH 7.5 in Tris-HCl buffer. The correspondent absorption spectra for a) DDAO and b) pDoAO are reported as insets.*

amine oxide of about 1.2 mM for DDAO and 0.32 mM for pDoAO (no changes in the bulk pH of the solution were observed).

### Circular dichroism

To unambiguously attribute the observed changes in fluorescence intensity of the probe to binding of the surfactant molecules to the DNA strand, the surfactant–DNA systems were investigated by circular dichroism. CD spectra were registered in a range of surfactant concentration above and below their c.m.c. value in water. The CD spectra of dsDNA in the presence of increasing amounts of DDAO or pDoAO at pH 5.8 are shown in Figure 4. The presence of surfactant induced a shift in the maximum of the DNA CD spectra due to structural variations in the DNA's double-helix architecture.[21] As could be expected, the interaction of the pH-sensitive amine oxide surfactants with DNA was dependent on the pH of the solution. As shown in Figure 5, at pH 7.5 the interactions between DDAO

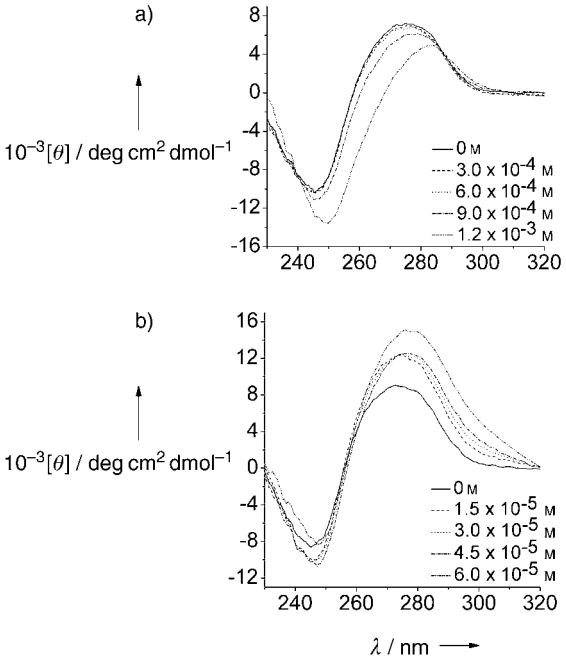



***Figure 4.*** *Effect of the addition of a) DDAO and b) pDoAO on the CT-DNA CD spectrum in a 50 mM Tris-HCl solution at pH 5.8. The surfactant concentrations are reported in the legend.*

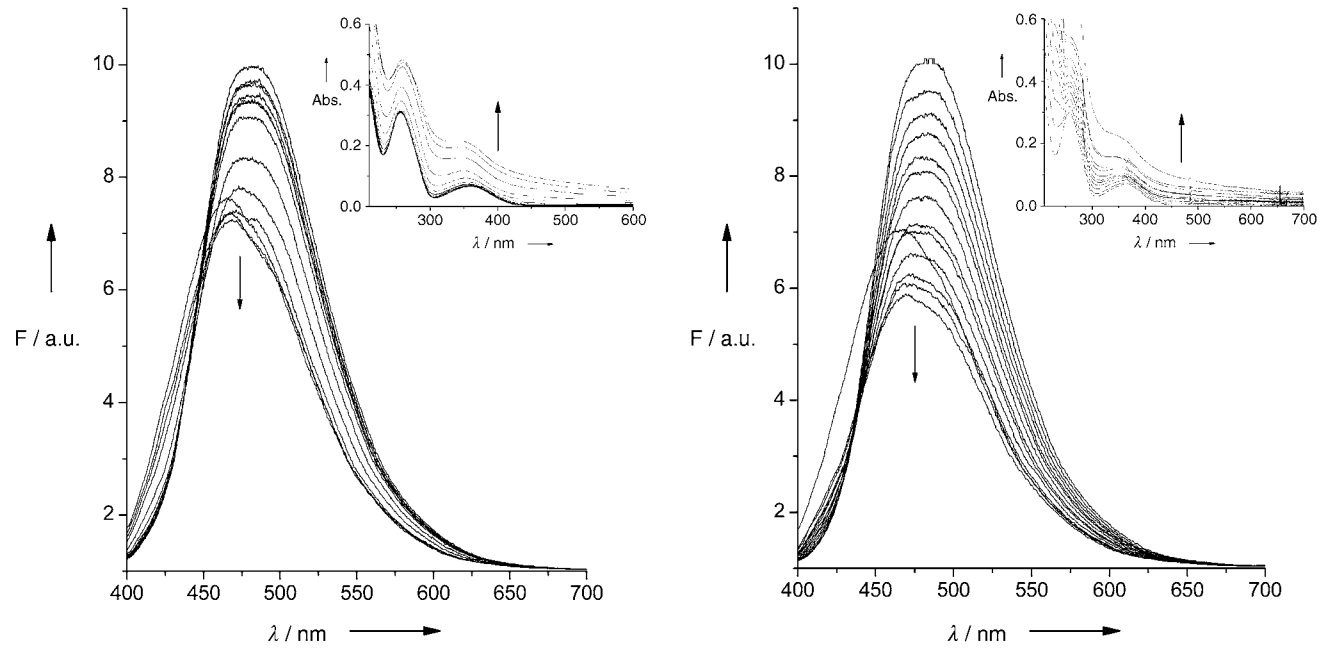



***Figure 3.*** *Effect of the addition of a) DDAO and b) pDoAO on DNA-bound HO fluorescence emission (expressed in arbitrary units) at pH 5.8. The correspondent absorption spectra for a) DDAO and b) pDoAO are reported as insets.*

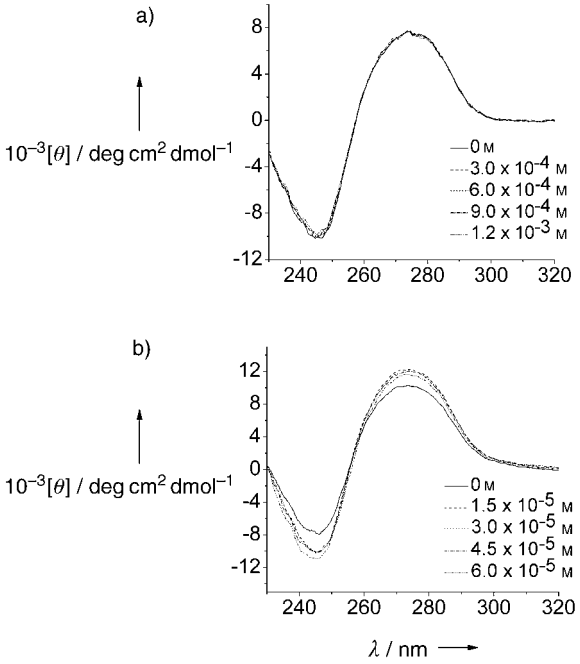



***Figure 5.*** *Effect of the addition of a) DDAO and b) pDoAO on the CT-DNA CD spectrum in a 50 mM Tris-HCl solution at pH 7.5. The surfactant concentrations are reported in the legend.*

and pDoAO were suppressed; this is in agreement with the results from fluorescence experiments. From the data, it is possible to conclude that a concentration of surfactant higher than the c.m.c. in water is indeed necessary for interaction with DNA.

#### Absorption measurements

The absorption spectra of the probe–DNA complex in the presence of increasing amounts of surfactant is shown as insets in Figures 1–3. In the case of CTAB (Figure 1), even at surfactant concentrations below the c.m.c., substantial scattering was observed at longer wavelengths. This feature is attributed to the formation of macroparticles in suspension and is only observed in the presence of DNA. Moreover, the scattering seems to appear at a concentration of surfactant equivalent to the concentration of the phosphate groups of DNA. The same behaviour was observed irrespective of whether HO or EB was used as the probe and is probably due to precipitation of the DNA–cationic surfactant complex induced by the neutralization of the negative charges of DNA. However, because of the very small absorptivity of EB, a special optical cell (path length = 10 cm) has to be used in order to accurately follow the changes in the absorption spectrum. In this respect, HO presents the advantage that its absorption spectrum can be measured in a conventional 1 cm cuvette, thereby allowing easy monitoring of any fluctuations in the optical density of the solution. In the case of the amine oxide surfactants considered, no scattering was observed at neutral pH values (Figure 2), in agreement with the absence of interaction with DNA. At pH 5.8 (Figure 3), scattering was observed at concentrations above $7\times10^{-4}$ M for DDAO and above $\sim4\times10^{-5}$ M for pDoAO, whether HO or EB is used.

## Discussion

Fluorescence spectroscopy has been recently proposed as a useful tool for performing a rapid screening of DNA–surfactants interactions. Whereas EB is insensitive to changes in the pH of the surrounding solution, HO is a pH-sensitive probe that could be used to monitor pH in the microenvironment of the DNA strand. However, unlike EB, the association of HO to dsDNA is known to be dependent on the ratio of probe to DNA base pairs. At low binding ratios ([HO]/[DNA bp] = 0.1), HO exclusively binds in the minor groove of the DNA strand, with a preference for AT-rich regions. At higher probe loadings (> 0.1), a nonspecific, weaker binding mode is observed that induces a modest increase in fluorescence intensity.

The complex behaviour of the amine oxide surfactants' interaction with DNA is a result of multiple equilibrium processes that involve both protonation/deprotonation and aggregation. The interaction between DNA and neutral aggregates of amine oxide surfactants in the zwitterionic state is negligible, and no significant variation in the HO fluorescence was observed. Acidification of the solution results in protonation of the amine oxide, which induced association to the DNA. Because the amine oxide functionality is only partially protonated at pH 5.8, the aggregates that are formed under these conditions are composed of a mixture of both zwitterionic and protonated surfactants.[22]

The hypsochromic shift in the emission maximum of HO upon addition of DDAO or pDoAO that is observed at pH 5.8 is indicative of a change in the probe's environment. A shift in the emission maximum of a fluorescent probe is indicative of a change in the relative energies of the Frank–Condon states involved in the transition and can be attributed to i) a change in the local pH or ionic strength in the immediate vicinity of the probe, ii) a significant structural change in the conformation of the DNA or iii) association of the probe expelled from the DNA with surfactant micelles. The first hypothesis is supported by considering that, both for DDAO and pDoAO, the emission maximum ($\lambda_{max}$) finally coincided with the emission maximum of HO at neutral or slightly basic pH (Table 1). This implies that the addition of amine oxide surfactants induces an increase in the pH in the vicinity of the DNA, in agreement with the formation of aggregates in which only a portion of the surfactants are protonated. The overall effect is thus to increase the

***Table 1.*** *Emission maximum wavelength ($\lambda_{max}$) of DNA-bound EB and HO in the absence and presence of surfactants.*

| | HO | | EB | |
|---|---|---|---|---|
| | pH 5.8 | pH 7.5 | pH 5.8 | pH 7.5 |
| No Surfactant | 480 | 458 | 601 | 607 |
| CTABr (0.94 μM) | | 458 | | 607 |
| DDAO (1.2 mM) | 466 | 460 | 601 | 607 |
| pDoAO (0.32 mM) | 469 | 460 | 601 | 607 |

local pH due to the presence of unprotonated amine oxide molecules in the surfactant aggregates.

It is very important to note that the DNA–amine oxides interaction occurred at a concentration of surfactant above a limit value that is specific for each surfactant and that can be defined as its critical concentration of aggregation ($C_{cr}$).[5] Such a value generally differs from the c.m.c. in water; indeed it is well known that surfactant c.m.c. usually decreases in the presence of a polymer due to a self-assembly in the polymer vicinity.[23,24] In any case, the $C_{cr}$ is always related to the aggregation properties of the surfactants, as confirmed by results for DDAO and pDoAO reported here. In fact, the pDoAO c.m.c. in water is about 40-fold lower than the value for DDAO, and a smaller amount of pDoAO was necessary to have the same effect as that with DDAO.

It is reasonable to assume the coexistence of DNA–surfactant complexes and surfactant aggregates in solution. In the presence of micelles, a portion of the probe could be sequestrated within the surfactant micelles, thus leading to observation of fluorescence emission from HO/micelle aggregates. In fact, enhancements in the fluorescence emission of EB have been observed upon inclusion into micelles,[25] and similar effects were observed for HO.[14] As showed in Figure 2, the hypsochromic shift of the fluorescence curves was accompanied by a small increase in the fluorescence emission so that the hypothesis of an inclusion of the probe into a micellar environment could be also possible.

Fluorescence depolarization is a technique that is sensitive to the mobility of the emitting species and has been previously used to distinguish between EB bound to DNA and EB included into lipid liposomes and micelles.[26] Polarization ($p$) is defined as the ratio of the linearly polarized component's intensity divided by the natural light component's intensity, and was calculated according to Equation (1):

$$p = \frac{I_{VV} - I_{VH}}{I_{VV} + I_{VH}} \quad (1)$$

The maximum value of polarization that can be observed depends on the relative orientation of the electronic oscillators involved in the absorption and emission processes and is between $+0.5 \leq p \leq -0.3$ for vertically polarized light and an ensemble of randomly distributed chromophores.[10] Table 2 presents the polarization of HO and EB bound to DNA in the presence of DDAO or pDoAO at pH 5.8, as well as previously reported data on EB bound to plasmid DNA in the absence and presence of CTAB, even if at a concentration below its c.m.c. value. No polarization was observed in the absence of DNA; this indicated that rotational depolarization of the probe in solution is fast. In contrast, binding of the probe to DNA results in a large increase in the polarization of the fluorescence emission. As shown in the table, the polarization of EB in the presence of DNA increases upon addition of CTAB, and this effect can be related to the more compact structure of the complex of DNA with the surfactant monomers. In the case of the amine oxide surfactants, we could expect that addition of DDAO or pDoAO causes an increase of polarization if the probe remains associated to DNA, or a decrease in the value of $p$ if the probe is transferred from DNA to the bulk solution or into surfactant aggregates. An increase in $p$ is observed upon addition of surfactants; this increase is attributed to increased rigidity of the DNA strand, commonly associated with DNA–surfactant interactions.[5] These results clearly indicate that the fluorescence emission observed in the presence of DDAO or pDoAO arises from probe molecules that are still associated with the DNA strand. This, in turn, supports the assertion that the observed shift in the emission of HO in the presence of DDAO or pDoAO is due to a change in the environment in the vicinity of the HO–DNA complex and not to expulsion of HO from the DNA.

***Table 2.*** *Polarization values for the DNA-bound probes EB and HO in the absence and presence of surfactants.*[a]

| | EB (601 nm) | HO (460 nm) |
|---|---|---|
| Solution | 0.001[b] | |
| DNA | 0.110 | 0.23 |
| CTABr (20 μM) | 0.000[b] | |
| DNA + CTABr (20 μM) | 0.223[b] | |
| DNA + DDAO (1.2 mM) | 0.306 | 0.325 |
| DNA + pDoAO (76 μM) | 0.277 | 0.358 |

[a] CT-DNA, $2 \times 10^{-5}$ M. [b] Data reported from ref. [26].

## Conclusion

The use of minor-groove-binding agents as fluorescent probes to assess DNA–surfactant interactions presents several advantages over that of intercalating agents, such as EB, which are buried deep inside the double helix. An expected benefit from locating the probe in the vicinity of possible locations for association between a surfactant and the DNA is that probe will be sensitive to variations in the local environment of the DNA–surfactant aggregates. In this respect, the observation that HO is capable of both reporting the interaction of the surfactant with the DNA strand, as well as the local pH in the vicinity of the probe is remarkable and of importance for possible applications in the area of pH-stimulated DNA vectorization processes for gene delivery. In this work, conclusions derived from the faster and more convenient steady-state fluorescence measurements of HO–DNA complexes in the presence of pH-sensitive amine oxide surfactants were confirmed by in-depth investigation with circular dichroism and fluorescence depolarization experiments. Preliminary results suggest that changes in the hydrophobic moiety of the amine oxide surfactants can drastically modify the concentration of surfactant needed for the interaction. This is a further sign that the hydrophobic interactions have an important role in the DNA–surfactant interactions. Further investigations on the use of HO as a pH-dependent probe for high-throughput screening of DNA–surfactant interactions are in progress.

## Experimental Section

**Materials**: The sodium salt of highly polymerized calf thymus DNA (CT-DNA, ≈10000 base pairs) was purchased from SIGMA (USA) and used without further purification. Ethidium bromide was purchased from SIGMA (USA). Hoechst 33258 was obtained from Molecular Probes (Eugene, OR). CTAB was purchased from Aldrich and purified by crystallization from ethanol/diethyl ether (1:1).

**Synthesis and purification of DDAO**: In a 1 L round-bottom flask dodecyldimethylamine (43.1 g, 0.1 mol) was added to ethanol, and the mixture was heated to reflux. Hydrogen peroxide (33 wt.%, 16.5 g, 0.50 mol) was added over 1 h to the refluxing mixture, then the reaction was allowed to proceed for 9 h. Excess peroxide was removed by carefully adding solid $MnO_2$ to the hot mixture until no more oxygen evolvement was observed. The reaction mixture was then brought to room temperature, filtered through a paper filter (washing more than once with anhydrous EtOH) and evaporated to obtain a white solid. The solid was crystallized from acetone/$Et_20$ (30:70, *v/v*) and dried in vacuo over $P_2O_5$. As no minima were observed in the surface tension versus log[DDAO] plot, the surfactant was considered pure. Yield = 90%; c.m.c. = $7.0\times10^{-4}$ M (surface tension); $^1$H NMR (200 MHz, $CDCl_3$) $\delta = 0.88$ (t, 3H; $CH_3$), 1.31 (m, 18H; $CH_2$), 1.87 (m, 2H; $CH_2$), 3.18 (s, 6H; $CH_3$), 3.24 ppm (m, 2H; $CH_2$).

**Synthesis and purification of pDoAO**

*Preparation of* p-*dodecyloxybenzyldimethylamine*: *p*-Dodecyloxybenzylbromide (18.5 g, 0.052 mol), prepared as already reported,[27] was dissolved in a 500 mL flask in absolute EtOH (30 mL) and dry ethyl ether (30 mL), then $NHMe_2$ (33% *w/w* in EtOH, 42 mL, 0.234 mol) was slowly added under magnetic stirring at room temperature, and the reaction was left to run for 4 h. The reaction mixture was worked up by adding NaOH (10% *w/w* in water, 100 mL) and extracted with ethyl ether. The organic phase was washed with water until neutrality and evaporated; the yellow oil was separated from a fine white solid impurity by filtration on a short neutral alumina column by elution with petroleum ether. Yield: 90%. $^1$H NMR (200 MHz, $CD_3OD$) $\delta = 0.88$ (t, 3H; $CH_3$), 1.34 (m, 18H; $CH_2$) 1.76 (m, 2H; $CH_2$) 2.37 (s, 6H; $CH_3$) 3.47 (s, 2H; $CH_2$) 3.94 (t, 2H; $CH_2$), 6.87 (d, 2H; Ar), 7.28 ppm (d, 2H; Ar).

*Preparation of p-dodecyloxybenzyldimethylamine oxide*: *p*-Dodecyloxybenzyldimethylamine (6.7 g, 0.021 mol) was dissolved in anhydrous EtOH (15 mL) in a 100 mL flask, and $H_2O_2$ (33% *w/w* in $H_2O$, 3.4 mL, 0.033 mol) was added over about 1 h to the refluxing mixture, then the reaction was left to run for 14 h. The excess $MnO_2$ was removed as for DDAO, and the reaction mixture was filtered through a paper filter (washing several times with anhydrous EtOH) and evaporated. The yellow oil obtained was treated 3–4× with ethyl ether and evaporated until a white solid was obtained. The solid was dispersed in ethyl ether, sonicated, cooled to 0°C, filtered, rinsed with cold ethyl ether and dried over $P_2O_5$ in vacuo. As no minima were observed in the surface tension versus log[pDoAO] plot, the surfactant was considered pure. Yield: 98%; c.m.c. = $1.5\times10^{-5}$ M in water; $^1$H NMR (200 MHz, $CD_3OD$) $\delta = 0.78$ (t, 3H; $CH_3$), 1.24 (m, 18H; $CH_2$), 1.64 (m, 2H; $CH_2$), 2.95 (s, 6H; $CH_3$), 3.87 (t, 2H; $CH_2$), 4.23 (s, 2H; $CH_2$), 6.84 (d, 2H; Ar), 7.33 ppm (d, 2H; Ar).

**Methods**

*Determination of DNA concentration*: The DNA concentration in solution was determined by spectrophotometric measurements, by using a Hitachi U-3300 spectrophotometer. The wavelength of reference is 260 nm. A molar extinction coefficient of 13000 $M^{-1}cm^{-1}$ was used to obtain a DNA concentration expressed in molar base pairs.

*Fluorescence measurements*: A Hitachi F-4500 fluorimeter was used for the titration experiments. Samples were prepared at a DNA concentration of $2\times10^{-5}$ M (bp) in Tris-HCl buffer (2 mL). The pH of the solution was adjusted by using a HCl stock solution. Hoechst 33258 was added to the solution to have [DNA]/[Hoechst] = 10, whereas for measurement performed by using ethidium bromide a [DNA]/[EB] ratio of 4.6 was used. The experiments were carried out by recording the fluorescence emission spectra of the probe–DNA complex in the absence of surfactants and after each one of subsequent additions of 10 μL of stock solutions of surfactant ($1.56\times10^{-3}$ M for CTAB, 0.02 M for DDAO and $1.33\times10^{-3}$ M for pDoAO) into the probe–DNA solution. Excitation wavelength: 360 nm (HO) or 520 nm (EB).

For the fluorescence depolarization experiments, a SPEX FLUOROLOG 2.1.2 instrument fitted with polarizers was used.

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**Keywords:** aggregation · amine oxide · amphiphiles · DNA · fluorescent probes

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