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Fluorescence properties of aza-helicenium derivatives for cell imaging

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ABSTRACT

In this work the optical properties of three N-methyl-aza-[5]-helicenium salts having the same organic moiety but different counterions (I^-, NO_3^- and $COOCF_3^-$) were investigated in different media. In particular, the absorption and fluorescence spectra of the compounds were strongly affected by the solvent properties. Fluorescence synchronous scans and time resolved fluorescence measurements indicated that in alcoholic solvents a dual emission behaviour occurred, likely due to aggregation phenomena which can be tuned by the counterion nature. The emission properties of the compounds were tested for cell labelling aims using Hude cells. The occurrence of aggregation processes controlled by the media properties resulted in a dual colour labelling in different cell compartments. In particular, the cell interior was visualized as a green fluorescence corresponding to a higher energy emission assigned to the dye molecules in monomeric form, while longer wavelength fluorescence was observed in cytoplasm, likely due to aggregation of the dye in this environment.

1. Introduction

Helicenes have extraordinary optical [1], electronic [2], and chelating properties [3], that have rekindled an interest in these nonplanar condensed aromatic compounds in applications as diverse as liquid crystals [4], sensors [5], dyes [5], asymmetric synthesis, molecular recognition, polymer synthesis, and materials science [6–8]. Moreover their complexes with transition metal ions exhibit peculiar behaviour when irradiated in the UV–vis region [9]. These helically shaped molecules possess interesting chiroptical properties such as large circular dichroism spectra and large optical rotations [10,11]. These polycyclic aromatic molecules, whose preparation strategies have recently reviewed [12], are known to intercalate in double stranded DNA [3] or to adsorb on the biomolecule grooves [10], leading to complex formation with alteration of DNA structure. This might result in a hidden or suppressed DNA function in physiological processes, such as DNA synthesis and/or transcription inhibition which can be desirable for the treatment of diseased cells.

However, drug–DNA interactions *in vivo* are strongly related to the accessibility of the nuclear envelope. The pharmacological activity of a drug and/or the labelling properties of the dyes are related to their chemico-physical properties, that allow the molecule to pass by cytoplasm and reach the inner nuclear membrane.

In this work the absorption/emission properties of three N-methyl-aza-[5]-helicenium salts having the same organic moiety but different counterions (I^-, NO_3^- and $COOCF_3^-$) have been investigated in different solvents in order to better understand their photophysical properties for cells labelling aims. The uptake and the localization of the helicenium derivative in eukaryotic cells were investigated by fluorescence confocal microscopy.

2. Experimental

N-methyl-aza-[5]-helicenium salts (Fig. 1) were prepared as described in the literature [13].

Acetonitrile, ethanol and butanol (Aldrich, spectroscopic grade) were used as received. MilliQ grade water was freshly prepared for each experiment.

Absorption spectra of the sample solutions were recorded by a Perkin–Elmer spectrophotometer (Lambda 800).

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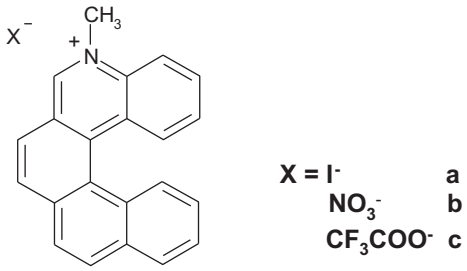

Fig. 1. Chemical structure of investigated compounds.

A spectrofluorimeter (Spex Fluorolog F112AI) was used to record corrected emission and excitation spectra and fluorescence synchronous scan measurements of the samples.

The luminescence decay times, τ_L (mean deviation of three independent measurements, ca. 5%), were measured by the single photon counting method using an Edinburgh Instrument 199S setup. A laser diode (Horiba Jobin Yvon, pulse width ca. 0.1 ns) emitting at 370 nm was used as excitation source and the signal was acquired by a Hamamatsu R7400U-03 detector.

The fluorescence images were recorded through a laser scanning confocal microscope (Nikon, PCM2000) by using a laser diode (λ_{exc} = 400 nm) as light source [14]. The light emitted from the samples was collected and equally splitted in two parts and analyzed by two acquisition channels in front of which two different emission filters (band-pass, $\lambda_{max} = 515 \pm 15$ and 600 ± 20 nm, respectively) were placed. The images were obtained with a 60×, 1.4 N.A. oil immersion objective (512 × 512 pixels) and setting the pinhole at 10 μm.

Hude fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) heat-inactivated Foetal Bovine Serum (FBS, Biokrom, Berlin, Germany), 2 mM glutamine, 10 U/ml penicillin, 10 μg/ml streptomycin.

Cells were plated in duplicate at 40–50% confluence in 6-well plates. Known volumes of helicenim salts in PBS were added to cell cultures and incubated for several hours.

The mitochondrial dehydrogenase activity of the cells after incubation with the organic compounds was determined through the MTT assay. This assay detects the conversion of 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide to formazan. For the experiment, 2.5×10^4 cells per well in a 24-well plate were seeded and let them grow for 24 h; then, different concentrations of helicenium salts were added to the cell cultures and incubated for 48 h. At the end of the treatment, the supernatant from each well was discarded and replaced with 200 μl fresh medium, then 20 μl of MTT (5 mg/ml) were added into each well for an additional 4 h incubation at 37 °C. The medium was discarded and the formazan formed by viable cells was solubilized with DMSO (100 μl/well), then the absorbance was measured at 570 nm with a microplate reader (Multiskan MK3, USA).

The number of viable cells was also determined by counting cells in a hemocitometer. The viability of the cells was estimated by examining their ability to exclude trypan blue (0.1% in 0.9% NaCl). All experiments were repeated at least twice and yielded close results.

For fluorescence imaging, the Hude cells were seeded at a density of 2×10^4 cells/well into 6-well plates. After 24 h adherence, the cells were treated with helicene-derivatives (5 μM) for different times in order to follow the uptake. After incubation, cells were washed with PBS and fixed for imaging. To monitor the clearence process, before the fixing step, the cells were incubated for increasing times in their medium.

3. Results and discussion

3.1. Photophysical characterization

The absorption spectra of the compounds under investigation (Fig. 1) were recorded in different solvents in the 250–500 nm range and are shown in Fig. 2. The absorption coefficients of helicene-derivatives are in the $4.6–18 \times 10^3\ M^{-1}\ cm^{-1}$ range, according to allowed $\pi \rightarrow \pi^*$ transitions.

The spectra in water were similar to those reported in the literature [10] for the helicenium salts in aqueous buffers and no dependency on the dye concentrations and counterion nature were observed. Different spectral features were observed in organic solvents (Fig. 2). In acetonitrile the spectra show two absorption bands with maxima at 315 and 380 nm whose positions are not strongly affected by the nature of the counterion [13]. In ethanol the absorption spectra were blue shifted and their structure depended on the counterion nature; in particular, for **1a** in EtOH the absorption at 300 nm appeared as a shoulder of the intense band at 250 nm (Fig. 2, dashed lines).

The emission spectra of the helicenium salts were recorded in the 400–700 nm range (Fig. 3). By exciting at 380 nm the compounds showed a broad fluorescence band centred at about 500 nm in water and acetonitrile, except for **1c** in acetonitrile where a hypsochromic shift of about 20 nm was observed. Instead, the excitation of helicene-derivatives in alcoholic solutions resulted in a broad emission spectrum whose maximum was shifted to lower energies. The data suggested that upon changing solvent, the energy of the fluorescent state was modified. In particular, the lack of observing changes on going from water to acetonitrile, compared to the marked shift observed in ethanol and butanol, indicated that the fluorescence properties are controlled by the solvent polarity. Taking into account that the chromophore unit is the same for all the compounds under investigation, the shift may be attributed to the counterion nature; this observation is different from what recorded in ETN buffer where the fluorescence band centred at 505–507 nm is not affected by the counterion nature [10]. This behaviour is likely due to the reduced solvation of the compounds under investigation which can lead to aggregate formation or solvatochromism of the ion-couple.

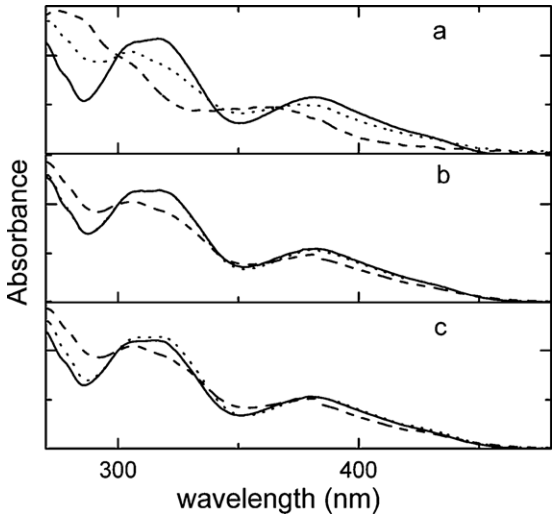

Fig. 2. Absorption spectra of (a) **1a**, (b) **1b** and (c) **1c** in water (solid line), ethanol (dashed line) and acetonitrile (dotted line).

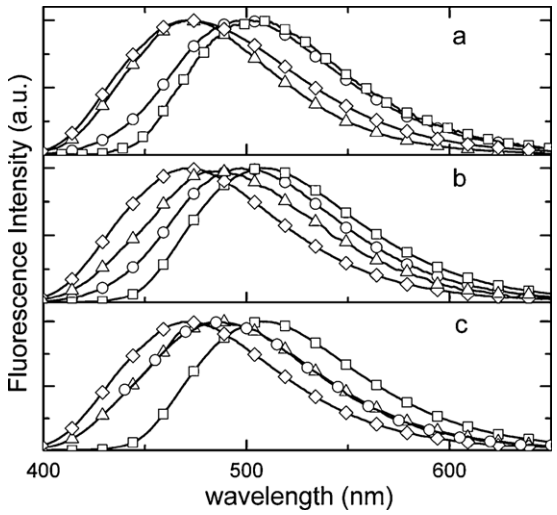

Fig. 3. Fluorescence spectra of (a) **1a**, (b) **1b** and (c) **1c** in water (square), acetonitrile (circle), ethanol (triangle) and butanol (diamond).

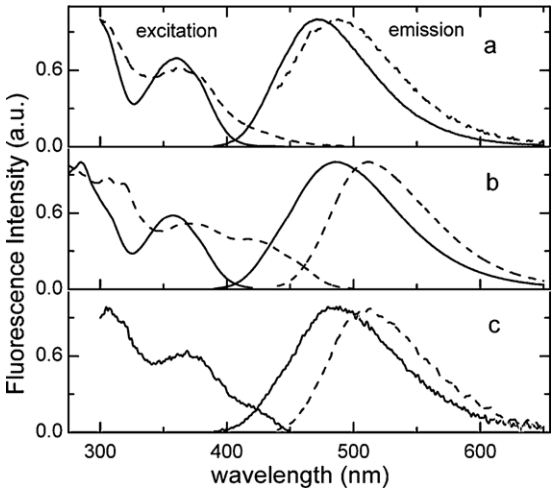

Fig. 4. Excitation and emission spectra of (a) **1a**, (b) **1b** and (c) **1c** in ethanol (λ_{exc} = 380 nm, solid line; λ_{exc} = 430 nm, dashed line; λ_{em} = 450 nm, solid line and λ_{em} = 560 nm, dashed line).

To get a deeper insight on the solvent effects on the optical properties of the helicenium salts, their absorption and the emission spectra were recorded in different solvents and the spectral maxima were collected in Table 1 and correlated with the Kamlet–Taft parameters. These empirical parameters correlate the capacity of the solvent to donate (α) or accept (β) a proton in a solute–solvent hydrogen bond and the ability of a solvent to stabilize a charge or a dipole by virtue of its dielectric effect (π) [15,16]. The analysis of the data reported in Table 1 indicates that the solvent effects are smaller on the absorption spectra compared to the changes observed in the emission spectra, suggesting that the excited state has a different electronic distribution on respect to the ground state. Furthermore, the spectral features appear to be mostly affected by the solvent polarizability (π-parameter) and the H-donating capacities (α-parameter) although a linear trend was not observed. Differences among the three compounds were observed, especially in alcoholic solvents, indicating that the counter ion might play a role in determining the emission properties of the helicenium salts in neat solvents.

In order to clarify the solvent-induced changes on the emission spectra, the effect of excitation and emission wavelengths on the emission and excitation spectra, respectively, was investigated in ethanol (Fig. 4) and butanol (Fig. 5). For all the three compounds in ethanol, upon increasing the excitation wavelength, the emission spectra were still broad but shifted at lower energies. The excitation spectra, recorded by monitoring the fluorescence on the blue and red edges of the emission spectrum, were different (Fig. 4) and did not overlap with the absorption spectra. The spectral differences were even more evident when the same experiment was carried out using butanol as solvent. In particular, the excitation spectra recorded from **1b** in butanol showed structured bands with maxima at 430, 315 and 275 nm and 350 and 285 nm for the bathochromic and hypsochromic emission, respectively (Fig. 5). The observed changes in the excitation spectra upon changing the emission wavelength led to rule out the hypothesis that the double emission behaviour is due to excited state dimer (excimer), since the excimer formation would not alter the excitation spectrum of the fluorophore. Thus the observed spectral changes led to the conclusion that helicenium salts are involved in interactions in the ground state which alter their electronic distribution and hence their emission properties. The fact that the absorption spectra do not show remarkable changes is likely due to the low absorption coefficients of the species.

These findings disagree with the literature data recorded in aqueous media [10]. Synchronous fluorescence scan measurements on **1b** in butanol and water were carried out to better understand the dual emission behaviour [18]. The scan obtained from water solutions presented one peak at 460 nm, indicating the presence of one emitting species, while the scan recorded from alcoholic solutions showed two peaks centred at 475 and 395 nm, thus giving strength to the hypothesis that in butanol the emission is due to two emitting species formed in the ground state.

Time resolved fluorescence measurements further confirmed the hypothesis of a double emission controlled by the solvent. In more polar solvents (water and acetonitrile) the fluorescence decay time was nicely correlated with a mono-exponential function leading to a decay time of 4.6 ± 0.2 ns for all the compounds under investigation. In butanol the fluorescence decay could be satisfactorily fitted by a bi-exponential equation with decay time values in the range of 4.6 ± 0.2 and 8.5 ± 0.3 ns. Furthermore, in agreement with the spectral behaviour, the relative weight of the two components changed with the emission wavelength. In particular, the contribution of the long lived component became

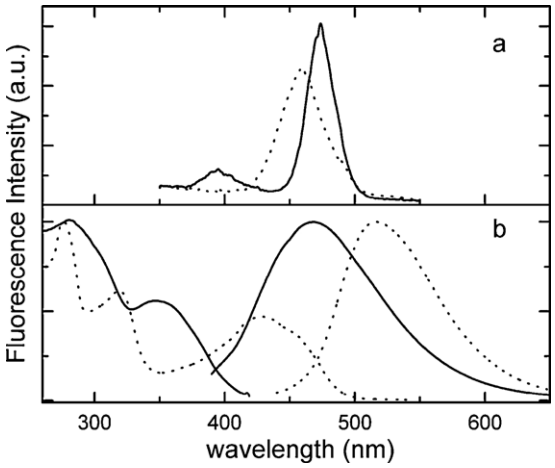

Fig. 5. Excitation and emission spectra of **1b** in butanol (λ_{exc} = 350 nm, solid line; λ_{exc} = 425 nm, dashed line; λ_{em} = 430 nm, solid line and λ_{em} = 560 nm, dashed line); upper panel synchronous scan **1b** in butanol (solid line) and in water (dashed line).

Table 1
Absorption and emission maxima (cm^{-1}) of **1a**, **1b** and **1c** as function of Kamlet–Taft parameters.

Solvent	α[a]	β[a]	π[a]	**1a** H5-I^-		**1b** H5-NO_3^-		**1c** H5-$COOCF_3^-$	
				ν_{abs}	ν_{em}	ν_{abs}	ν_{em}	ν_{abs}	ν_{em}
Water	1.17	0.47	1.09	26,178	19,762	26,110	19,608	26,246	19,646
Etanol	0.83	0.75	0.54	27,300	21,141	26,315	20,618	26,385	20,703
Butanol	0.79	0.84	0.47	26,385	19,881	26,246	20,283	26,300	21,140
Octanol	0.77	0.81	0.40	27,100	21,326	26,737	21,276		
Acetonitrile	0.19	0.31	0.66	26,455	20,080	26,246	20,074	26,246	20,533
Chloroform	0.20	0.10	0.58			26,501	20,408		
Toluene	0	0.11	0.49	27,777	22,675	27,500	22,205		
Heptane	0	0	−0.08	28,500	22,883				

[a] From Refs. [15–17].

higher with increasing the emission wavelength (Table 2). These observations suggest that in the alcoholic media a new emitting species is detected in agreement with fluorescence synchronous scan; the comparison of the decay time values indicates that the decay of new species is longer than the one of the monomeric chromophore. Furthermore, the constancy, within the experimental error, of the short lived decay time (about 4.6 ± 0.2 ns) upon changing solvent (with the exception of toluene) and counter ion suggests that no evident quenching processes or complex formation involving the counterions are taking place, differently from what reported for acridinium salts [19] unless their efficiency is below the instrumental sensitivity. Only when toluene was used as solvent an evident shortening of the decay times was detected, likely because in that conditions the ion couple (H5-I) is strongly associated.

These findings indicated that emission properties of the helicenium derivatives depend on the solvent. The data obtained in alcoholic media are in agreement with the presence of different emitting species, which cannot be explained by solvatochromism of the ion-couples, as shown by time resolved data. The dual fluorescence behaviour observed in neat alcoholic solvents is likely due to interactions occurring in the ground state, such as aggregation phenomena. As already reported for similar compounds [20–22], the solvent properties (polarizability, hydrogen bonding capacities, etc.) are able to modulate solvation of the ionic couples and hence the self-assembled process of helicenium derivatives, thus allowing to tune the fluorescence properties of the system. The nature of the species formed in alcoholic solutions and the role of the counterions are currently under investigation.

3.2. Cell growth experiments

In order to test the cell labelling capacities of helicenium salts, their effect on cell growth was monitored through the MTT assay.

Table 2
Fluorescence decay parameters for the three compounds under investigation.

Compound	Solvent	λ_{em} (nm)	τ_{F1} (ns)	A_1 (%)	τ_{F2} (ns)
1a	Water	505	4.7	100	–
	Acetonitrile	500	4.4	100	–
	Butanol	440	4.6	90	8.3
		540	4.6	60	9.0
	Toluene	440	2.6	100	
		500	2.0	87	5.0
1b	Water	505	4.8	100	–
	Acetonitrile	500	4.6	100	–
	Butanol	440	4.4	80	8.0
		540	4.6	55	8.8
1c	Water	505	4.8	100	–
	Acetonitrile	500	4.6	100	–

Increasing concentrations of **1b** (from a stock solution in PBS) were added to cell cultures and then incubated for 48 h; afterwards, MTT was added and the formation of the reduced product, formazan, was determined by measuring the absorbance at 570 nm. Fig. 6 shows that cell growth is inhibited in the presence of increasing **1b** concentrations. In particular, in the presence of 10 μM of **1b,** cell proliferation is reduced to a half compared to the number counted without the organic compound, suggesting that this concentration is enough to modify cell natural processes. However, cells did not apparently suffer any toxicity effect, from a morphological point of view, thus suggesting an arrest of cell proliferation and not an activation of cell death mechanisms. Similar results were obtained when cells were counted with a hemocytometer and the viability of the cells was estimated by examining their ability to exclude trypan blue (data not shown). The data indicate also that the helicenium concentration could be modulated to reach inhibition of cell growth.

However, the results of these tests were useful to choose a concentration below 5 μM for labelling experiments.

3.3. Cells labelling

Since it is well established that subcellular compartments are characterized by different medium properties (polarity, ionic strength, pH, density), the emission properties of **1a–c** suggested to verify the ability of helicenium to chromatically distinguish different subcellular compartments and to optically follow the cell uptake process. Incubation experiments were carried out by use of compound **1b**.

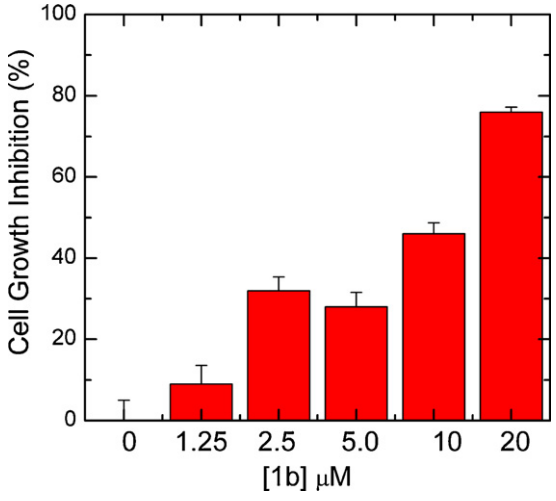

Fig. 6. Growth inhibition of Hude-cells as function of **1b** concentration.

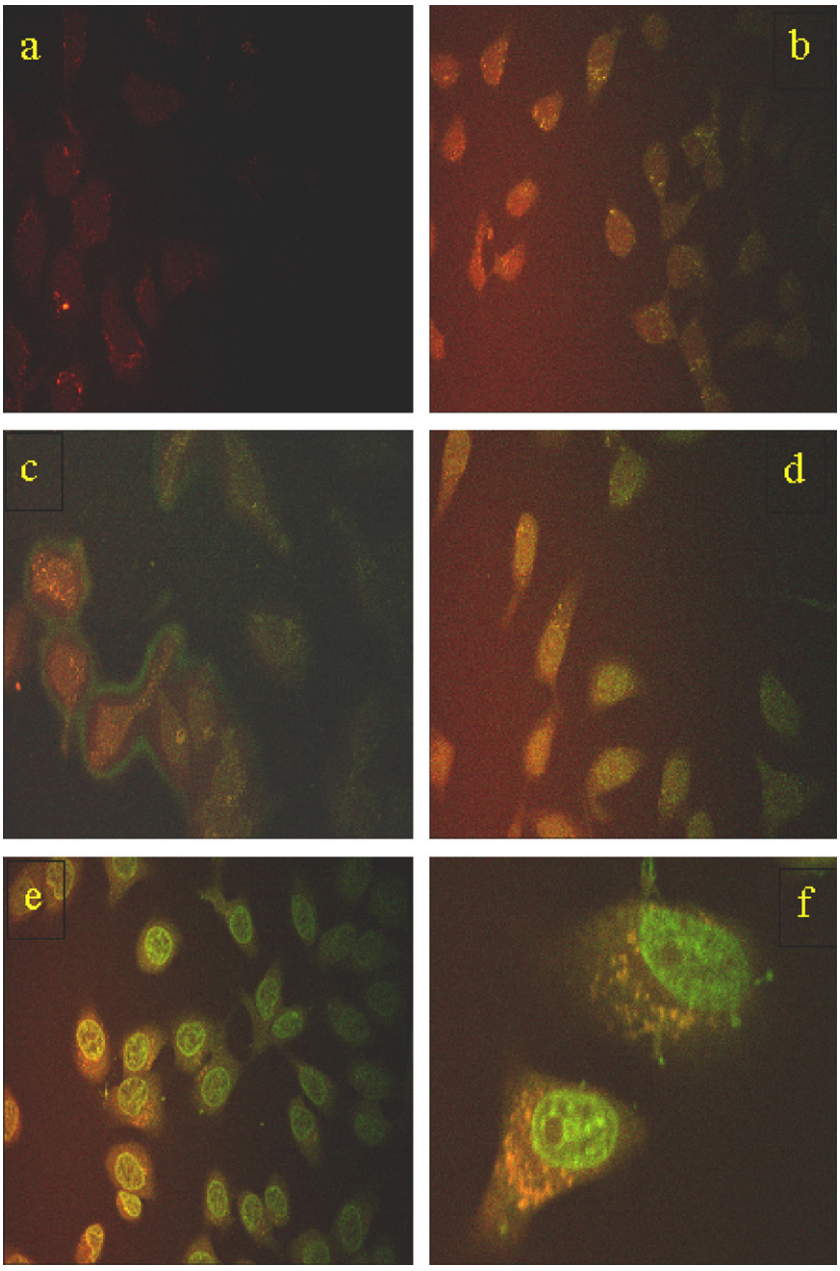

Fig. 7. Confocal fluorescence images of Hude-cells (a, control) incubated with 1**b** for 1 (b) 5 (c), 20 (d) and 60 (e and f) min.

Hude fibroblast cells were incubated in the presence of **1b** for different times in order to follow the uptake process. Confocal fluorescence imaging revealed that **1b** is able to penetrate inside the cells (Fig. 7). The cell samples prepared after 5 min contact display a pale fluorescence indicating accumulation of the dye at the cell rim. After 20 min incubation, the samples show fluorescence coming from the cell interior and after 60 min incubation, the uptake was completed since no further localization changes were observed for longer incubation times. Interestingly, the images recorded at the end of the uptake process (Fig. 7e–f) display dual colour fluorescence observable in different cell regions.

Indeed, the images evidenced a specific labelling of the cell interior by green colour, due to light emitted in the 500–530 nm region (see Section 2). The high affinity of helicenium salts already observed for DNA [10] might suggest that **1b** is localized in the nuclear region of the cells. The more external parts of the cells appeared as red coloured areas indicating that the dye molecules localized in these regions emits in the 580–620 nm range. These findings were evaluated on the basis of the spectral features of the dye molecules allow to suggest that the shorter wavelength emission observed in the cell interior is due to the helicenium units in monomeric form while the fluorescence in the external area of the cells is assigned to the emission from helicenium aggregate species which can be formed in the cytoplasm due to the reduces solubilization of the salt.

Furthermore, clearance experiments by confocal imaging (Fig. 8) were able to show that the labelling process is completely reversible. In fact, the dye was completely released in about 5 h without apparent alteration of the cell structure (within the experimental error due to the instrumental resolution) indicating that the labelling process is based on weak and reversible interactions. However, the dye degradation by cell metabolism during the clearence time cannot be excluded.

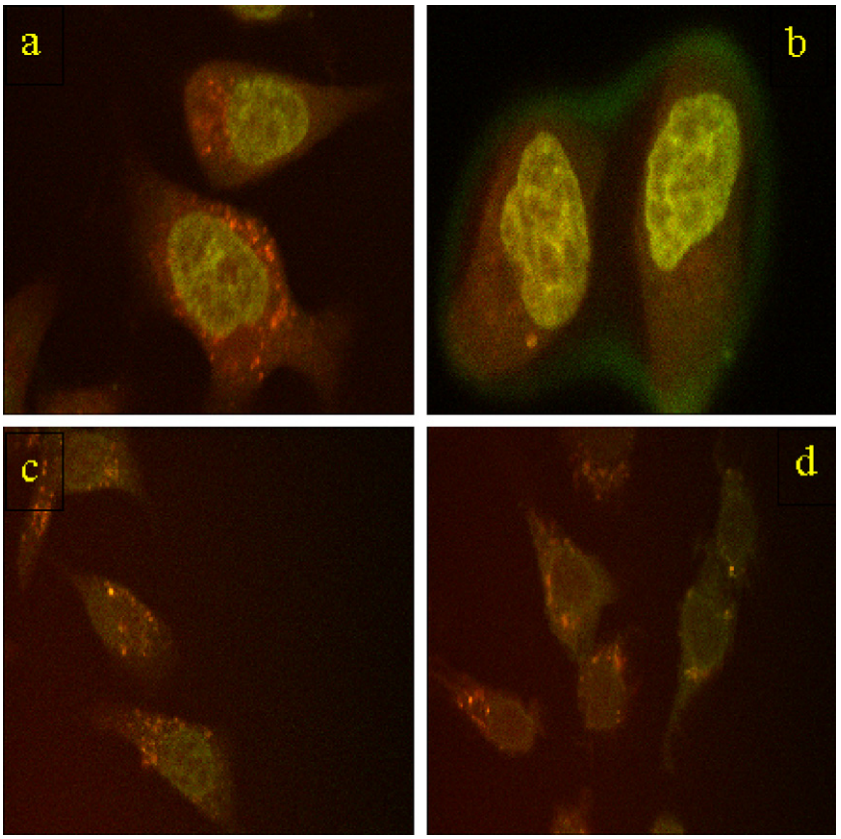

Fig. 8. Confocal fluorescence images of Hude-cells treated with 1**b** for 60′ and then washed and cultured for 0 (a), 60 (b) 180 (c) and 300 (d) min.

4. Conclusions

The optical properties of three N-methyl-aza-[5]-helicenium salts having the same organic moiety but different counterions (I^-, NO_3^- and $COOCF_3^-$) could be finely tuned upon changing the medium properties. The changes in the absorption and fluorescence spectra of the compounds were likely due to aggregation phenomena controlled by the solvent polarity. Indeed, time resolved fluorescence measurements and synchronous fluorescence scans indicated the presence of two emitting species in low polarity solvents. The emission properties of the compounds were tested for cell labelling aims using Hude cells as models. The occurrence of aggregation processes resulted in a dual colour labelling in different cell compartments. In particular, in the internal region the dye was present in monomeric form, originating a short wavelength emission, while in cytoplasm the helicenium units form aggregates giving rise to longer wavelength emission.

Acknowledgments

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