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Octa-BODIPY dye for monitoring live cell parameters using fluorescence lifetime imaging microscopy

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Monitoring of various parameters inside living cells allows to get deeper into understanding of mechanisms of their functioning in normal and pathological states. Currently, various fluorescent dyes are actively used for this purpose. Fluorophores sensitive to various intracellular analytes and cell parameters are of particular interest since they allow monitoring intracellular changes in real time. In the present work, we have evaluated the potential applicability of a multichromophore compound consisting of eight BODIPY chromophores linked via an aliphatic spacer to a siloxane core to monitor membrane parameters in living eukaryotic cells. This compound exhibits significant solvatochromism due to intramolecular chromophore interactions. Environmental parameters significantly affect the fluorescence properties of the dye and, in particular, the fluorescence lifetime. This allowed us to use it for monitoring the parameters of cell membrane structures by fluorescence lifetime imaging microscopy (FLIM).

Keywords: BODIPY, aggregation, cell imaging, microscopy, FLIM.

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Introduction

Fluorescent dyes are currently used widely in studies of processes in living systems [1]. In most cases, stable and bright fluorophores insensitive to environmental parameters are needed for visualization of individual cell compartments and localization of individual proteins within a cell. However, compounds with optical properties depending on external conditions are also in demand in cell biology, since they allow for monitoring of individual cell parameters. For example, numerous sensors of inorganic ions, organic low-molecular compounds, enzymes, etc., have been discussed in literature [2]. The fluorescence of such sensors changes with the analyte concentration. Compounds allowing one to measure viscosity in individual cell compartments and temperature-sensitive probes have also been developed. They are usually based on compounds with quantum yield and fluorescence lifetime varying with the mobility of individual elements of the chromophore. Styryl derivatives [3–5] and hemicyanines [6,7], which have aromatic rings separated by double bonds, and molecular rotors based on 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene (BODIPY) [8–10] are striking examples of such compounds (Fig. 1). With an increase in viscosity of the medium or a reduction in temperature, the quantum yield of fluorescence of these compounds grows due to a reduction in vibrational mobility of the chromophore. The interest in materials with aggregation-induced emission (AIE) has been on the rise lately. The aggregation of dyes normally leads to quenching of fluorescence (ACQ, aggregation-caused quenching), but the quantum yield of fluorescence of compounds with AIE increases due to, on the one hand, steric hindrance on the interactions of π-systems of chromophores with each other and, on the other hand, the restriction of vibrations in the condensed state [11,12]. We have recently synthesized a series of compounds containing several (2–8) BODIPY fluorophores linked via an aliphatic spacer to a siloxane core [13]. They are highly soluble in many organic solvents, but chromophores aggregate due to their close proximity. This resulted on the one hand, in the effects similar to ACQ: the quantum yield of fluorescence decreased with an increase in the number of chromophores in the molecule. On the other hand, the appearance of new fluorescence bands, which are most likely associated with the formation of excimers, was observed; this effect can be attributed to AIE. The fluorescent properties of these oligomeric dyes depended strongly on the parameters of the ambient medium (solvent), which determined the balance of ACQ–AIE effects in the molecule. These effects were most pronounced in the dye with eight BODIPY chromophores (**octa-BDP**; see Fig. 2, *a*). Owing to its high hydrophobicity, this compound should accumulate well in membrane structures of cells. In the present study, we assess the applicability of octa-BDP in monitoring of membrane parameters in living cells.

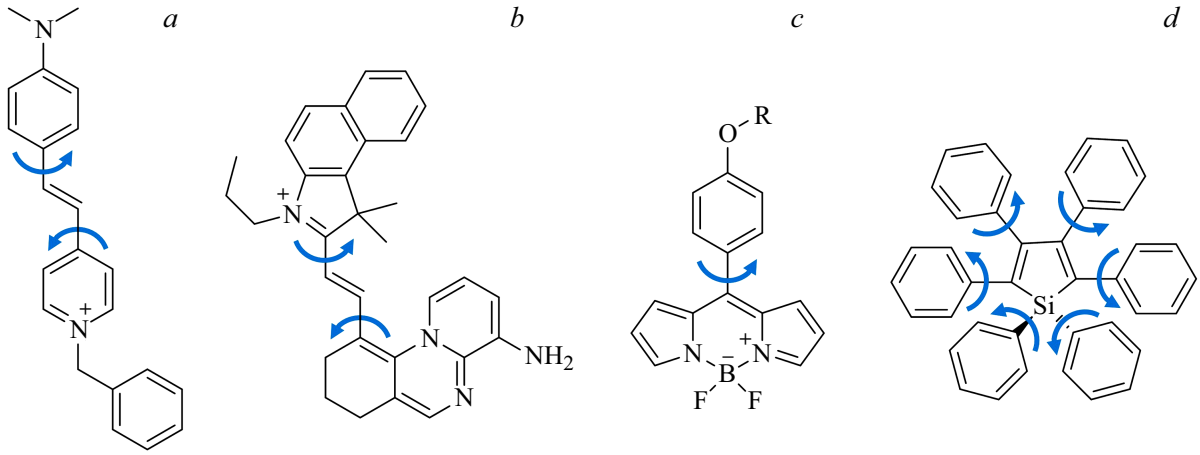

Figure 1. Examples of viscosity and temperature sensitive compounds based on molecular rotors: styryls [3] (*a*), hemicyanines [6] (*b*), BODIPY [8] (*c*), and hexaphenylsiol, which demonstrates AIE [12] (*d*). The arrow indicates rotational freedom that enable nonradiative relaxation from the excited state.

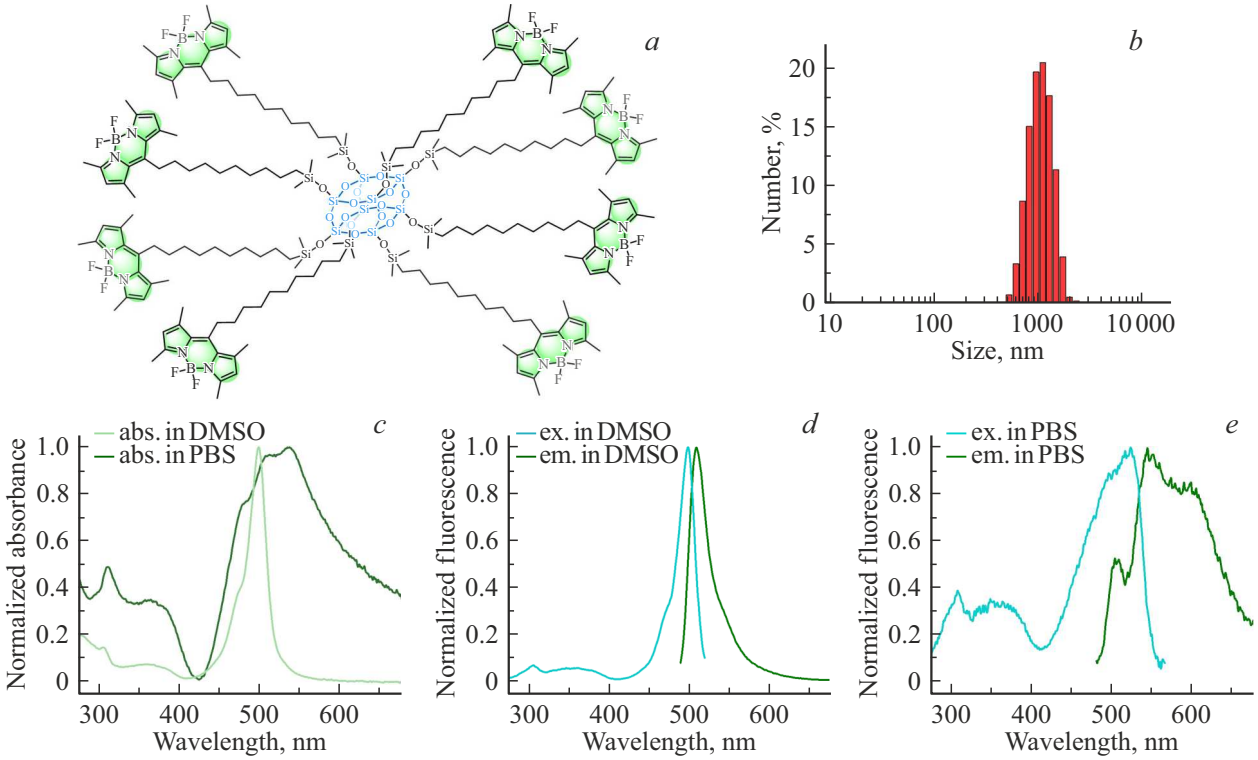

Figure 2. Structure of octa-BDP (*a*) and its properties in solution: size of octa-BDP particles in an aqueous buffer according to dynamic light scattering data (*b*) and absorption, fluorescence excitation and emission spectra in DMSO and an aqueous phosphate buffer (*c*–*e*).

Materials and methods

The synthesis procedure of octa-BDP was described earlier [13]. Absorption spectra were measured using a Cary50 Bio spectrophotometer (Varian). Fluorescence excitation and emission spectra were recorded with a Cary Eclipse spectrofluorimeter (Varian). A ZetaSizer Nano ZS nanosizer (Malvern) was used to determine the size of octa-BDP nanoparticles in water.

Human cervical carcinoma (HeLa) and human ovarian carcinoma (SKOV3) cells were grown in the RPMI 1640 medium (Thermo Fisher Scientific, Inc.) supplemented with fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.) to 10%, L-glutamine to 2 mM (PanEco), 100 u/ml of penicillin, and 100 μg/ml of streptomycin (Thermo Fisher Scientific, Inc.). Cells were cultured in a humidified atmosphere at 37°C and 5% CO_2. The medium was renewed every 2–3 days. In experiments on the analysis

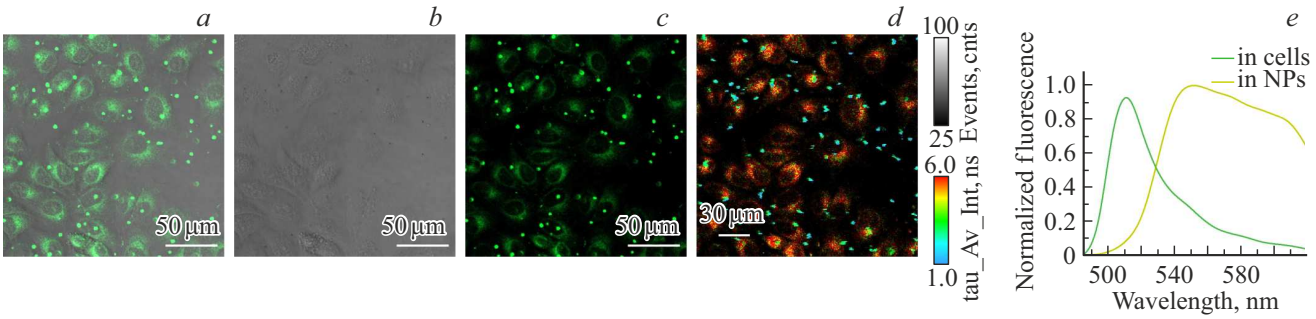

Figure 3. Fluorescence microscopy of HeLa cells incubated with octa-BDP NPs: *a* — overlay of the green channel and transmitted light, *b* — cells imaged in transmitted light, *c* — cells in the green detection channel (laser excitation at 488 nm; emission detection in the 490–675 nm range), *d* — FLIM with laser excitation at 485 nm and detection in the 505–545 nm range, and *e* — fluorescence emission spectra of the dye in cells and NPs recorded using the QUASAR detector of the Zeiss LSM 980 microscope in the 490–620 nm range under laser excitation at 488 nm.

of cell apoptosis under the influence of benzyl viologen and hydrogen peroxide, cells were cultured in the DMEM medium (Thermo Fisher Scientific, Inc.).

A Zeiss LSM 980 confocal laser scanning microscope was used for cell microscopy. Cells in a 96-well plate were imaged through a Plan-Apochromat 10×/0.3 objective with additional 4x magnification provided by the microscope scanner. A PicoQuant LSM Upgrade with a 485 nm pulsed diode laser (a frequency of 20 MHz was set) and a hybrid detector with a time resolution < 50 ps were used for fluorescence lifetime imaging microscopy. A 520/30 nm light filter was mounted in front of the detector to cut off excitation laser radiation. Fluorescence decay curves were analyzed in SymPhoTime 64 software (PicoQuant). To determine the fluorescence lifetime of individual cells, the frame area occupied by a cell was selected and the fluorescence decay kinetics was approximated. Despite the complex nature of the octa-BDP [13] fluorescence decay kinetics, the data were satisfactorily fitted by a monoexponential function (χ^2 remained within the 0.9–1.3 range), since the number of detected photons was about 100000 ($\sim 10^2$ at the kinetics maximum). Data for several (from 9 to 40) cells in a frame were processed statistically in Origin 2021 software.

In order to prepare octa-BDP nanoparticles (NPs), a small amount of this dye was first dissolved in 20 μl of dichloromethane; 50 μl of DMSO were then quickly added, and dichloromethane was evaporated under vacuum for half an hour. Following that, 5 μl of the resulting dye in DMSO were mixed quickly with 95 μl of phosphate buffered saline (PBS), which was then diluted to 1 ml either with PBS (for cuvette experiments) or with a growth medium (for experiments with cells). The final dye concentration was $\sim 5\,\mu$M. Rapid dilution of a small aliquot of the dye in an organic solvent with an aqueous buffer exactly in this sequence, not the reverse, as it is common when working with small volumes was critical to obtain NPs that remained stable over a long period of time.

Results and discussion

The solvatochromic properties of octa-BDP in organic solvents have been analyzed thoroughly in recent study [13]. However, since organic solvents are incompatible with living cells, we studied the behavior of octa-BDP in an aqueous buffer. With this aim in view, octa-BDP was first dissolved in DMSO and then diluted with phosphate buffered saline. Owing to its low polarity, the dye was not freely soluble in DMSO, but aggregated after dilution with PBS. This was evidenced by a change in its optical properties: a number of new bands with both bathochromic and hypsochromic shifts emerged in the absorption and fluorescence spectra (Figs. 2, *c*–*e*), and significant fluorescence quenching caused by aggregation was observed. Since octa-BDP should not dissolve in water due to its high hydrophobicity, one might expect it to precipitate after the addition of water. Although the solution after the addition of water was fairly transparent and no characteristic light scattering was observed in the absorption spectra, octa-BDP did indeed precipitate, but in the form of NPs. This was evidenced by microscopy data (see below) and dynamic light scattering (DLS; Fig. 2, *b*) results. According to DLS data, the average size of NPs was 1080 nm with a standard deviation of 280 nm.

We have tested whether octa-BDP would accumulate in cells after incubation with the obtained NPs. Human cervical cancer (HeLa) cells, which are utilized widely in routine cell experiments, were used. The cells were incubated with NPs in a growth medium solution for 30 min and imaged via fluorescence microscopy (Fig. 3). It can be seen that the green dye did indeed accumulate in cells, but numerous fluorescent particles in Brownian motion were also observed. The obtained microscopic images of HeLa cells are consistent with the accumulation of octa-BDP in membrane structures of a cell. It is known that lipophilic uncharged dyes (BODIPY included) get localized inside a cell in such membrane structures as the endoplasmic reticulum, the Golgi apparatus, and lipid droplets [2]. In

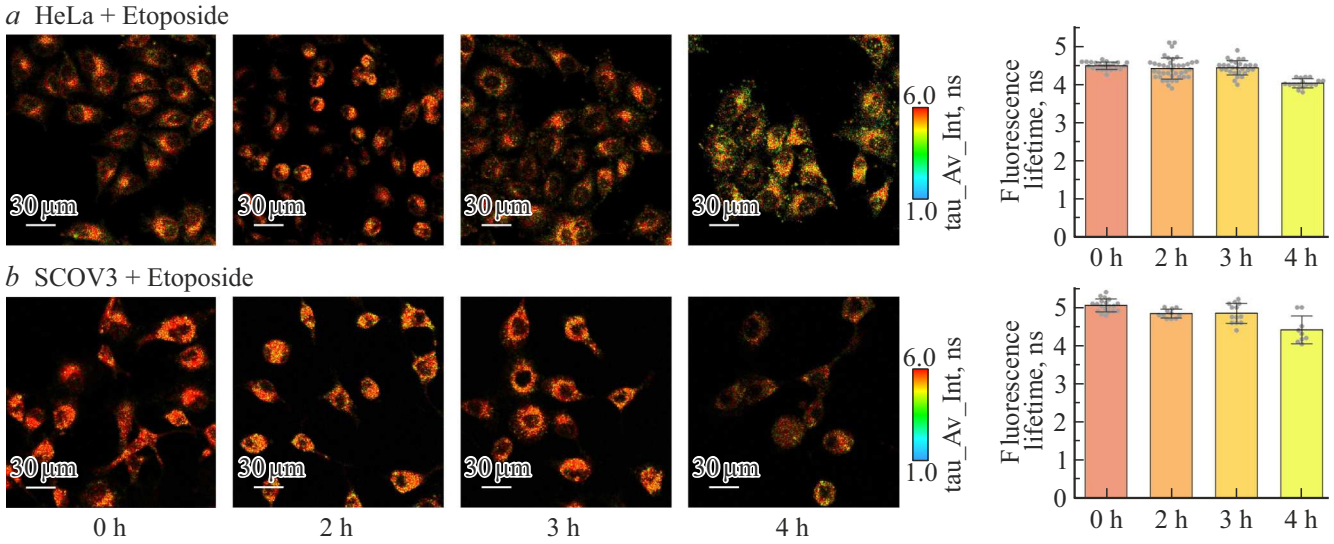

Figure 4. Changes in the fluorescence lifetime of octa-BDP in HeLa (*a*) and SCOV3 (*b*) cells occurring 0, 2, 3, and 4 h after the addition of etoposide. Histograms of the fluorescence lifetime of cells at different incubation times are shown on the right. Mean fluorescence lifetimes of cell populations and standard deviations are indicated; dots represent the data for individual cells.

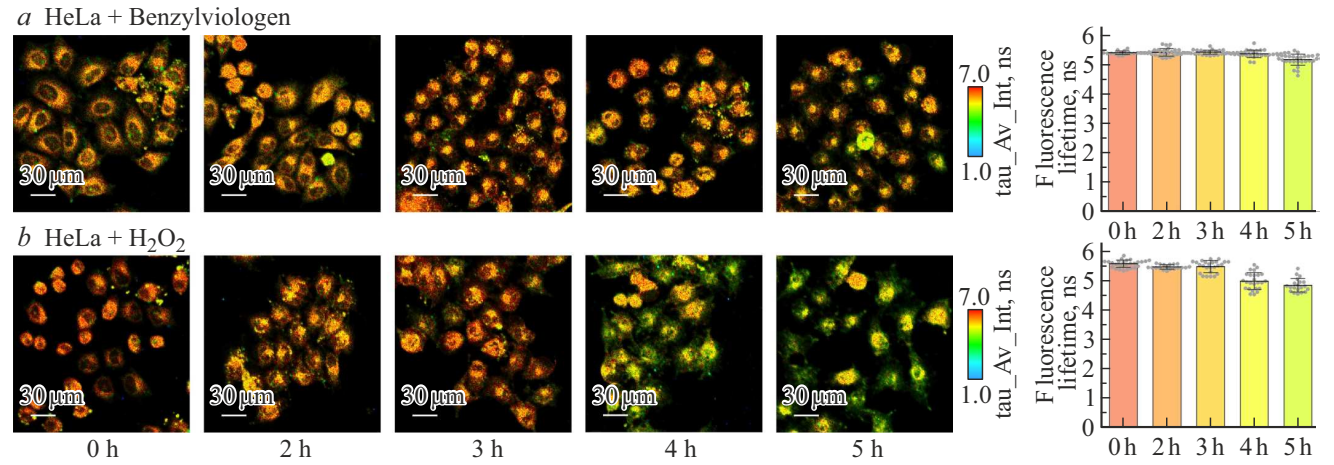

Figure 5. Changes in the fluorescence lifetime of octa-BDP in HeLa cells occurring in the process of apoptosis induced by benzyl viologen (*a*) and hydrogen peroxide (*b*) 0, 2, 3, 4, and 5 h after reagent addition. Mean fluorescence lifetimes of cell populations and standard deviations are indicated in the histograms; dots represent the data for individual cells.

general, the fluorescent images of cells were similar to those obtained earlier in experiments with homologous BODIPY derivatives [14]. Fluorescence lifetime imaging microscopy (FLIM; see Fig. 3, *d*) data revealed that the dye has different fluorescence lifetime in cells and NPs (5.1 and 1.6 ns, respectively). This is consistent with a decrease in aggregation upon dissolution of the dye in cells. The fluorescence emission spectra of cells and NPs obtained using the microscope detector (Fig. 3, *e*) also confirm this. It is clearly seen that aggregate bands in the 550–600 nm region disappear in cells.

To evaluate the applicability of octa-BDP in monitoring of cellular parameters, we treated HeLa and SCOV3 cells with etoposide. This agent is an apoptosis inducer and causes an increase in the viscosity of cell membrane structures; therefore, it is often used to test viscometers based on fluorescent dyes [3]. Following 1, 2, and 3 h of incubation of cells with etoposide, a solution of octa-BDP NPs (the dye concentration was $\sim 5\,\mu$M) was added, and incubation proceeded for another hour. After washing the unbound dye off, microscopic images of cells were obtained (Fig. 4). The fluorescence lifetimes of the dye in HeLa and SCOV3 cells were different at the start of the experiment: 4.5 and 5.1 ns, respectively. This is probably indicates the differences in the lipid composition of these cells and is consistent with other experiments demonstrating that the composition and viscosity of the lipid bilayer may differ from one cell culture to the other [15,16]. In fact, the fluorescence lifetime of octa-BDP in HeLa cells in this experiment differed from the one determined in the experiment described above (Fig. 3),

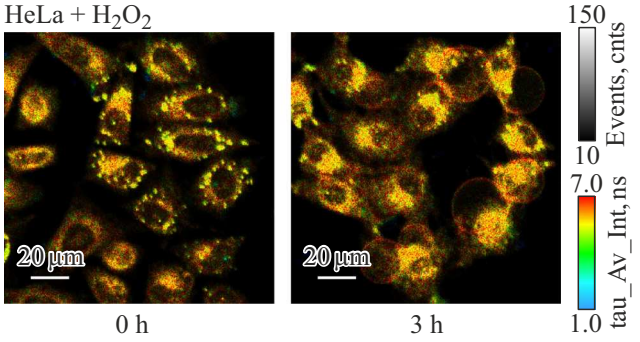

Figure 6. FLIM of octa-BDP-labeled HeLa cells before and after incubation with hydrogen peroxide for 3 h. Cells were not rinsed with PBS before imaging.

indicating that the lipid bilayers of examined cells had different compositions. The addition of etoposide (250 μM) to HeLa and SCOV3 cells resulted in a reduction in the dye fluorescence lifetime, which dropped to 4.0 and 4.4 ns, respectively, in 4 h (Fig. 4).

Other apoptosis-inducing reagents were also tested: benzyl viologen (5 μM) and the more traditional hydrogen peroxide (400 μM). Experiments were performed on HeLa cells cultured in DMEM instead of RPMI. Prior to apoptosis induction, the fluorescence lifetime of octa-BDP in cells was 5.4–5.5 ns (Fig. 5). These values differ from the ones obtained in previous experiments, confirming once again that the lipid composition of cells depends on the culture conditions. Under 60× magnification, individual compartments with different fluorescence lifetimes (approximately 5.1 ns in punctate structures and 5.6 ns in the main part of a cell; see Fig. 6) could be distinguished in octa-BDP-labeled HeLa cells. The dye fluorescence lifetime did also increase to 6.0 ns in plasma membrane protrusions (blebs) characteristic of apoptosis. As in the case of etoposide, the fluorescence lifetime in cells decreased under the influence of benzyl viologen and hydrogen peroxide (to 5.2 and 4.8 ns, respectively, in 5 h; see Fig. 5).

This behavior of octa-BDP drastically distinguishes it from molecular rotors, since an increase in their membrane viscosity induced by etoposide and other cytotoxins leads to an increase in the quantum yield and the lifetime of fluorescence due to the restriction of vibrational freedom [3,17,18]. The octa-BDP compound operates differently: its fluorescence lifetime is governed by interchromophore aggregation that depends on parameters of the medium in which it is located [13]. Notably, the formation of intramolecular aggregates of chromophores in octa-BDP was largely determined not by viscosity, but by polarity. The reduction in fluorescence lifetime of octa-BDP indicates an increase in the number of interchromophore aggregates due to deterioration of their solubility in the medium. This may be associated with a change in the composition of cell membrane structures (specifically, the amount of polar/nonpolar components and, in a lesser extent, the amount of saturated/unsaturated fatty acid residues that determine viscosity). A substantially more pronounced reduction in fluorescence lifetime of octa-BDP in experiments with hydrogen peroxide (compared to benzyl viologen) is consistent with this statement. Benzyl viologen induces apoptosis through oxidative stress and does not interact directly with lipids, whereas hydrogen peroxide, in addition to inducing oxidative stress in a cell, may be involved in peroxidation of lipids, which increases their polarity significantly. This leads to increased BODIPY aggregation and a reduction in fluorescence lifetime of octa-BDP.

It is worth noting in conclusion that the fluorescence lifetime of certain molecular rotors has recently been found to depend not only on viscosity, but also on polarity of the medium [19].

Conclusion

Thus, it was demonstrated that octa-BDP may indeed be used to monitor parameters of membrane structures in living cells. Unlike rotor-based fluorescent dyes that are currently used widely for this purpose, the fluorescence lifetime of octa-BDP is governed by chromophore aggregation and the resulting ACQ–AIE effects within the molecule. Since these effects depend largely on polarity of the medium, octa-BDP may serve as a complement to existing molecular rotor viscometers.

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Conflict of interest

The authors declare that they have no conflict of interest.

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